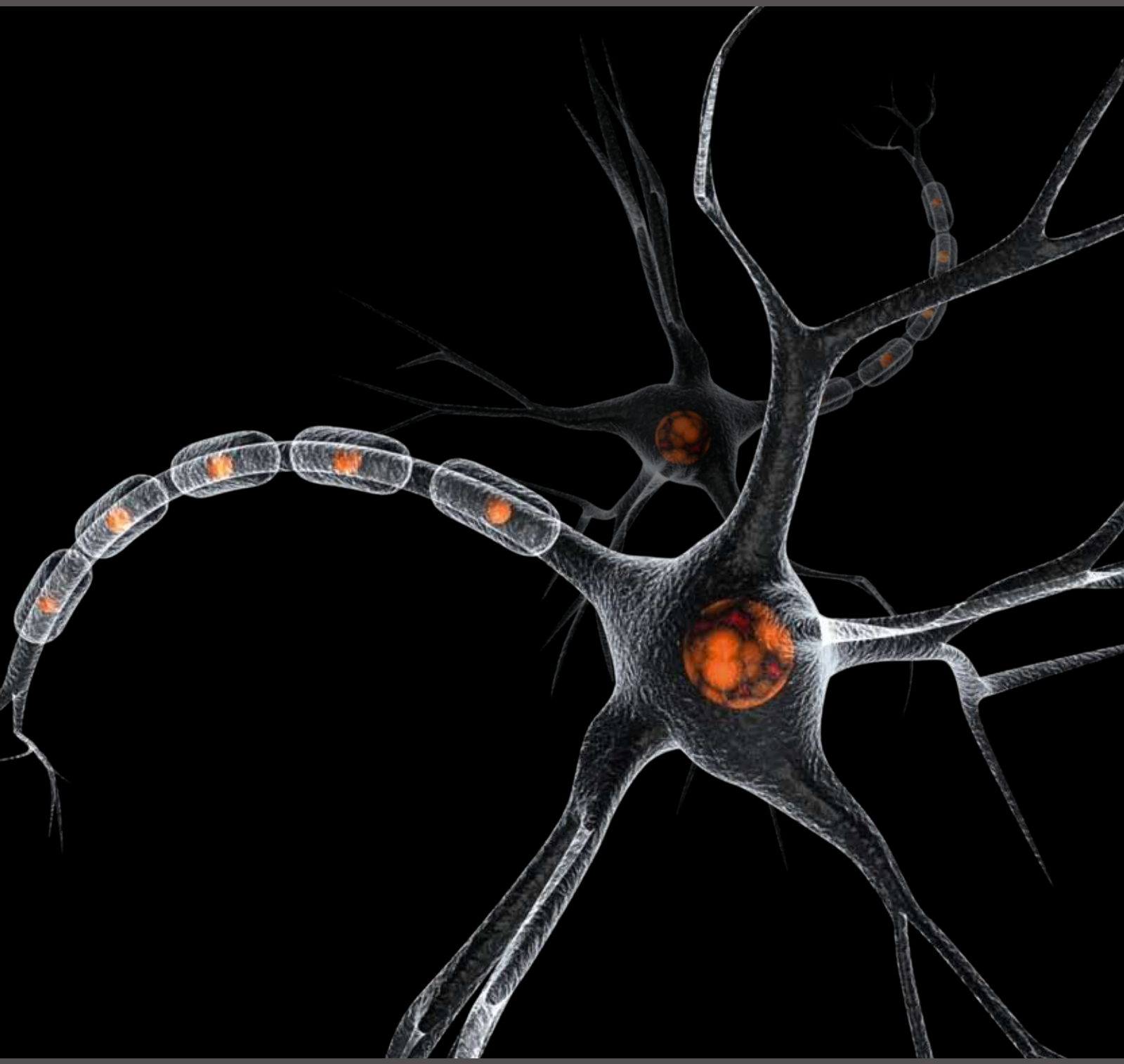
Neural Plasticity

Plasticity and Anxiety

Guest Editors: Patrice Venault and Georges Chapouthier



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Editorial

Plasticity and Anxiety

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Anxiety is a very broad behavioural trait, helping animals to cope with dangerous environmental situations. As anxiety is linked to other emotional processes and to cognitive functions such as learning and memory, it involves a number of cerebral structures and brain transmitter systems, thereby giving rise to a high degree of plasticity. Within the limited space of a single issue, it is obviously impossible to cover all aspects of anxiety processes occurring in the brain. By including both extensive reviews and articles reporting on experiments, the present issue wishes to present clearly different approaches to this ubiquitous behaviour trait.

The reviews start with the extensive review by D. M. Diamond et al. covering the effects of stress on LTP in the hippocampus, amygdala, and prefrontal cortex. Here the authors are presenting challenging new hypotheses on putative "temporal dynamics" of plasticity, involving an "activational" period, followed by a consolidation process and making it possible to recall traumatic memories. Another approach, which is complementary to this model, focuses on the relationship between stress and memory processes and is reviewed by C. Sandi and M. T. Pinelo-Nava who made a classification according to five factors: (1) the source of the stress, (2) the duration, (3) the intensity, (4) the timing, that is, in relation to the memory phase, and (5) the type of learning experience; a clear analysis has been made of the effects of stress on memory and of the main neurobiological mechanisms involved. I. Akirav and M. Maroun have investigated the role of the medial prefrontal cortex-amygdala circuit in the effects of stress on fear and the extinction of fear, and the key role played by the GABA transmitter system.

A. V. Kalueff has conducted an extensive review covering neurogenetic, neurochemical, and behavioural aspects, and the essential relationship between anxiety and memory, that is, between emotional and cognitive factors in the functioning of the brain. A strong relationship found in both human and animals was investigated by A.V. Kalueff and D. L. Murphy: the relationship between anxiety, depression, and stress-related disorders; such stress-related disorders can also lead to cognitive dysfunctions affecting learning and memory. The importance of hippocampal neurogenesis in these phenomena and their link with antidepressant treatment were studied by E. Paizanis et al. The involvement of the endocannabinoid system in the regulation of anxiety and in brain plasticity of emotional states is the subject of the analysis by M.-P. Viveros et al. In patients with early onset Alzheimer's disease (under the age of 65), apolipoprotein E4 is a risk factor. In rodents, this same protein plays a role in the regulation of anxiety; this is reviewed by J. Raber. C. Belzung and P. Philippot chose a phylogenetic approach ranging from specific reactions to danger in simple organisms to more elaborate physiological and behavioural responses in "higher" animals, and culminating with autonoetic consciousness of anxiety in great apes and humans.

Experimental data provide evidence for key arguments in the reviews. G. Legradi et al. studied rats and highlighted the action of pituitary adenylate cyclase activating polypeptide (PACAP) when administered in the central nucleus of the amygdala; they have shown that PACAP induces reorganization of stress-coping behaviour, with a shift from an active mode (burying) to a passive mode (withdrawal or immobility). V. Brinks et al. used mice to study the involvement of high affinity mineralocorticoid receptors and low affinity glucocorticoid receptors in the regulation of emotion and cognition in mice. Increased corticosterone concentrations and the gradual switch from mineralocorticoid to glucocorticoid receptors produced a "strong emotional arousal at the expense of cognitive performance." By studying two strains of mice in tests classically used to assess anxiety and comparing the results through a principal component analysis study, Y. Clement et al. identified four essential behaviour patterns: novelty-induced anxiety, general activity, exploratory behaviour, and decision making. In clinical

practice with humans, L. Carmilo-Granado et al. found evidence for a new treatment of anxiety in cases of arachnophobia. Patients were shown computer images, not of spiders but of objects with spider-like features (e.g. the Atomium in Brussels), and the authors managed to induce a sharp reduction in the symptoms of arachnophobia.

As can only be expected, and as can be seen with all the data and arguments presented here, this issue presenting a variety of approaches will not give a clear-cut answer to the question of the plasticity of anxiety, but instead will open a number of new paths for future research and discovery.

Patrice Venault
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Review Article

The Temporal Dynamics Model of Emotional Memory Processing: A Synthesis on the Neurobiological Basis of Stress-Induced Amnesia, Flashbulb and Traumatic Memories, and the Yerkes-Dodson Law

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Recommended by Georges Chapouthier

We have reviewed research on the effects of stress on LTP in the hippocampus, amygdala and prefrontal cortex (PFC) and present new findings which provide insight into how the attention and memory-related functions of these structures are influenced by strong emotionality. We have incorporated the stress-LTP findings into our "temporal dynamics" model, which provides a framework for understanding the neurobiological basis of flashbulb and traumatic memories, as well as stress-induced amnesia. An important feature of the model is the idea that endogenous mechanisms of plasticity in the hippocampus and amygdala are rapidly activated for a relatively short period of time by a strong emotional learning experience. Following this activational period, both structures undergo a state in which the induction of new plasticity is suppressed, which facilitates the memory consolidation process. We further propose that with the onset of strong emotionality, the hippocampus rapidly shifts from a "configural/cognitive map" mode to a "flashbulb memory" mode, which underlies the long-lasting, but fragmented, nature of traumatic memories. Finally, we have speculated on the significance of stress-LTP interactions in the context of the Yerkes-Dodson Law, a well-cited, but misunderstood, century-old principle which states that the relationship between arousal and behavioral performance can be linear or curvilinear, depending on the difficulty of the task.

1. INTRODUCTION

Numerous reviews in recent years have advanced our understanding of the interactions among long-term potentiation and depression (LTP/LTD), stress, and memory. These reviews have focused on specific topics, such as the cognitive implications of stress-LTP-LTD interactions (Kim and Diamond [1]; Diamond et al. [2]; Diamond et al. [3]; Kim et al. [4]), stress, LTP, and psychopathology (Post et al. [5]; McEwen and Magarinos [6]; Elzinga and Bremner [7]; Vermetten and Bremner [8]; Jay et al. [9]; Diamond et al. [10]; Buwalda et al. [11]), stress and metaplasticity (Abraham and Tate [12]; Kim and Yoon [13]), the effects of glucocorticoids on LTP (McEwen [14]; Garcia [15]; Joëls [16]), a comparison of stress effects on LTP in different brain regions (Diamond et al. [17]; Abe [18]; Richter-Levin and Akirav [19]; Richter-Levin [20]; Kim and Jung [21]; Akirav and Richter-Levin [22]), and a molecular analysis of stress-LTP interactions (Cremer et al. [23]; Popoli et al. [24]; Huang et al. [25]). Here, we have provided a different perspective on stress and LTP than has been considered previously. We have speculated on the functional significance of the finding that stress has different effects on LTP in different brain structures. Thus, stress has been shown to block the induction of LTP in the prefrontal cortex (PFC), and to enhance, as well as to impair, LTP in the hippocampus and amygdala. This review explores the idea that understanding the differential effects of stress on LTP in the PFC, hippocampus, and amygdala provides a framework towards understanding the neurobiological basis of flashbulb and traumatic memories, stress-induced amnesia, and the Yerkes-Dodson Law.

2. FLASHBULB MEMORIES AND VICISSITUDES OF THE WELL-CITED, BUT MISUNDERSTOOD, YERKES-DODSON LAW

One of the earliest researchers to comment on how emotion affects memory was James [26], who stated that "*an impression may be so exciting emotionally as almost to leave a scar upon the cerebral tissues*." This early observation that strong emotionality can generate a long-lasting memory of the arousing event was also studied by Colgrove [27] in his descriptions of the recollections people had of emotionally charged events. Colgrove noted that most adults could describe, in great detail, events that had transpired on the day when they had learned, over three decades before, that President Lincoln had been assassinated. Other rapidly formed, vivid, and durable memories have been described by people who experienced events of great importance, such as assassinations of international leaders and the terrorist attacks on America on September 11, 2001 (Somer and Saadon [28]; Christianson [29]; Wright and Gaskell [30]; Terr et al. [31]; Kvavilashvili et al. [32]; van Giezen et al. [33]; Berntsen and Thomsen [34]; Curci and Luminet [35]). The powerful strengthening of memories of events occurring in times of strong emotionality was referred to as "hypermnesia" by Stratton [36] and then as "flashbulb memories" by Brown and Kulik [37].

A decade after Colgrove's description of the influence of emotion on memory, Yerkes and Dodson [38] studied the effects of different shock intensities on the rate of learning by mice in a discrimination avoidance task. These investigators showed that when mice were trained in a simple, that is, black/white, visual discrimination task to avoid shock, their rate of learning improved linearly with an increase in the intensity of the shock. When mice were trained in a more difficult, that is, black/gray, visual discrimination task, their rate of learning was more efficient with an intermediate intensity of shock than with the highest intensity of shock. Their findings, which were then replicated separately by Yerkes [39] and later by Dodson [40], became known as the Yerkes-Dodson Law, which essentially stated that a high level of motivation can enhance learning on an easy task and impair learning on a difficult task (see also Yerkes [39]). Figure 1 provides a subset of the data from the Yerkes and Dodson [38] study, which illustrates the finding that the relationship between shock intensity and performance on the task was linear (increased shock intensity produced increased performance) for the simple discrimination and nonlinear (an intermediate intensity of shock produced optimal performance) for the complex discrimination.

With rare exceptions (Ni [41]; Young [42]; Postman [43]), the work of Yerkes and Dodson and the law it spawned were largely ignored in the first half of the twentieth century. Five decades passed from the formation of the Yerkes-Dodson law before it was first tested by Broadhurst [44] with modern techniques and statistical data analyses. In Broadhurst's work, rats were trained to escape from submersion in water in a task with different levels of difficulty and motivation. Broadhurst showed that rats tested on an easy visual discrimination task learned rapidly when they were trained with the highest level of motivation (stress). He also showed that an intermediate degree of stress produced the best performance in rats trained on a more difficult version of the task. Thus, Yerkes and Dodson [38] and then Broadhurst [44] demonstrated that high levels of stress impaired performance on a difficult, but not on an easy, task. Other studies on people and rodents have reinforced the notion of the importance of taking into account the difficulty of the task as an intervening variable in arousal effects on performance (e.g., Dickman [45]; Hammes [46]; Denenberg and Karas [47]; Telegdy and Cohen [48]; Bregman and McAllister [49]; Anderson [50]; Mesches et al. [51]; Diamond et al. [52]).

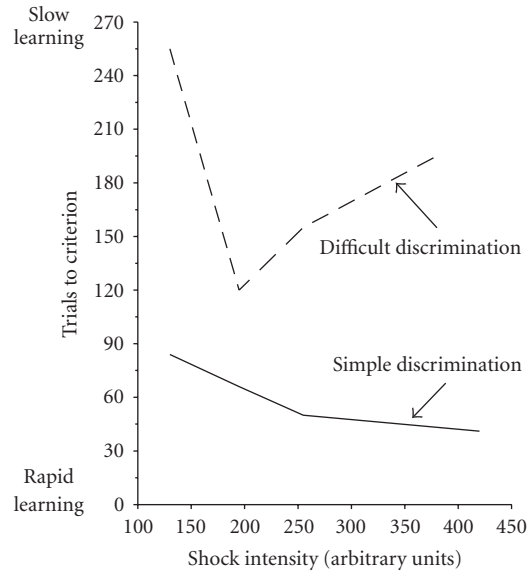

Figure 1: A subset of data from Yerkes and Dodson [38]. Mice were trained to avoid shock in a simple versus difficult visual discrimination task. The simple task involved a dark versus bright discrimination and the more difficult task involved a discrimination in which the two sides of the escape box were at similar levels of illumination. Behavioral performance increased linearly with increased levels of shock in the simple task, but performance was maximal at an intermediate level of shock for mice trained in the difficult discrimination.

In the 1950s, major figures in the field of cognitive psychology appear to have been unaware of, or ignored, the findings of Yerkes and Dodson when they stated that the relationship between arousal and performance was exclusively curvilinear. Thus, Schlosberg [56], Hebb [53], and Duffy [57] all asserted, without reference to Yerkes and Dodson, that there is a curvilinear relationship between arousal and performance. For example, Hebb's [53] view was that "there seems no doubt: the *(right side of the arousal-performance curve)* must come down to a low level" (page 251). Similarly, Duffy [57] stated that "the optimal degree of activation appears to be a moderate one, the curve which expresses the

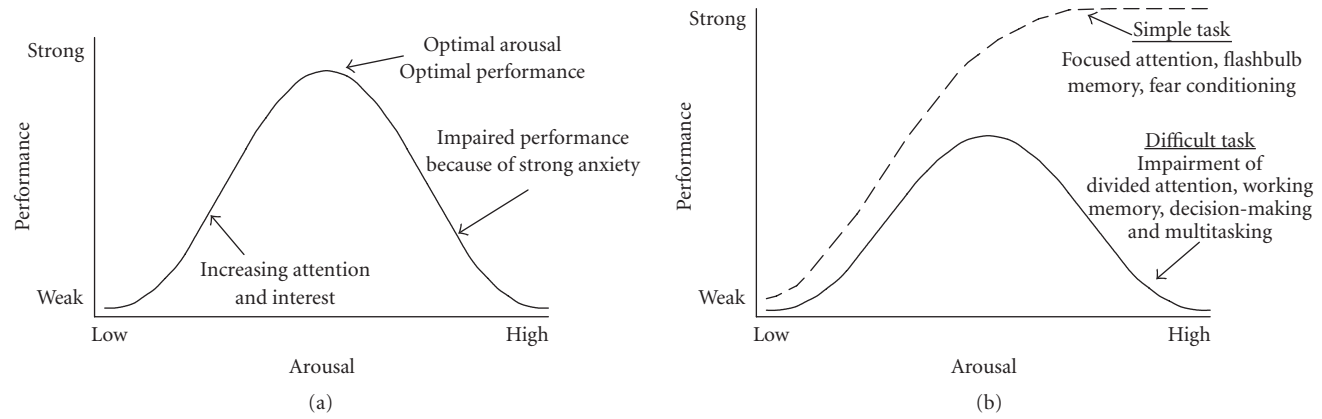

Figure 2: A comparison of the Hebbian version of the Yerkes-Dodson law, as it has been commonly represented for the past 50 years (a), and the original version, based on the actual findings and theorizing of Yerkes and Dodson ([38]; (b)). The Hebbian version incorrectly states that high levels of stress, anxiety, or motivation produce a monolithic impairment of performance. The original version based on the actual [38] Yerkes-Dodson findings takes into account the finding that strong emotionality can enhance performance under "simple" learning conditions, such as when learning involves focused attention on a restricted range of cues, and impairs performance under more complex or challenging learning situations, such as in divided attention, multitasking, and working memory tasks. Graph (a) is adapted from 5 decades of publications and books, for example, Hebb [53], Loftus [54], and Radvansky [55].

relationship between activation and quality of performance taking the form of an inverted U" (page 268).

The exclusion of the findings of Yerkes and Dodson in these reviews cannot be explained by a complete loss of interest in the Yerkes-Dodson law by the mid-twentieth century. At about this time, Postman [43] provided an exhaustive review of animal and human research conducted in the first half of the twentieth century on emotion and learning. He cited the findings of Yerkes and Dodson when he stated that "relatively severe punishment (intensive shock) is most effective in learning simple habits such as black-white discrimination ... and relatively mild punishment is optimal in the case of difficult tasks, such as complex types of discrimination" (page 507). Similarly, Harlow ([58, page 27]) noted, in his application of the Yerkes Dodson law to primate learning, that the "intensity of nociceptive stimulation may be positively related to the speed of formation of conditioned avoidance responses ... but the use of intense nociceptive stimulation prevents the monkey from solving any problem of moderate complexity." Thus, the idea that arousal interacts with task difficulty to positively or negatively influence performance was well established in cognitive psychology in the first half of the twentieth century.

It is ironic that not only were the original findings of Yerkes and Dodson ignored in major reviews on emotion and learning in the 1950s, but Hebb's incomplete illustration of the arousal-performance relationship as exclusively curvilinear (Figure 2 in Hebb [53]) incorrectly came to be known as the Yerkes-Dodson law by later researchers. Beginning in the 1960s (e.g., Broadbent [59]), the Yerkes-Dodson law devolved into a single inverted U-shaped curve, which has been promulgated, to this day, by introductory psychology textbooks (e.g., Radvansky [55]). Even contemporary scholars in the field of emotion, brain, and memory have relegated the linear component of the original Yerkes-Dodson law to the status of a mere footnote (Christianson [29]) or they have disregarded it entirely, focusing solely on the Hebbian view that a single inverted-U shaped curve represents how arousal interacts with cognition (e.g., Loftus [54]; Neiss [60]; Metcalfe and Jacobs [61]; Aston-Jones et al. [62]; Mendl [63]; Aston-Jones et al. [64]; Morris [65]).

As one of us noted recently (Diamond [66]), debates have raged for the past 5 decades regarding the validity of the Yerkes-Dodson law, but it is primarily the incomplete (Hebbian) version of the Yerkes-Dodson law that has become one of the most debated and even vilified doctrines in cognitive psychology (Deffenbacher [67]; Neiss [60]; Christianson [29]; Baumler [68]; Teigen [69]; Watters et al. [70]; Dutton and Carroll [71]; Hanoch and Vitouch [72]). Thus, the Yerkes-Dodson law suffered the indignity to be largely ignored during the first half of the twentieth century, and once it was revived, to be misrepresented to the present day. This five-decade-long misrepresentation of Yerkes and Dodson's findings has occurred despite the unambiguous statement by these authors that "an easily acquired habit may be readily formed under strong stimulation, whereas a difficult habit may be acquired only under relatively weak stimulation. That this fact is of great importance to students of animal behavior and animal psychology is obvious" (Yerkes and Dodson [38, pages 481-482]). With its thousands of reference citations in the past century, Yerkes and Dodson [38] may have the dubious distinction to be the most highly cited, but largely unread, paper in the history of science.

In a rare example of a scholarly analysis of the Yerkes-Dodson law, Hanoch and Vitouch [72] assessed a half century of misdirection by stating that "what Yerkes and Dodson had in mind was more sophistication than what their U-entranced successors made of it ... later generations let the law collapse into one single curve with its idealized and highly abstract, quasiunidimensional axes" (see also Teigen

[69, pages 430-431] for related discussion). As we approach the 100th anniversary of the publication of their study, we honor Yerkes and Dodson with a representation of a subset of the data from their [38] paper in Figure 1, which illustrates the linear and curvilinear (task difficulty-dependent) aspects of their findings. In addition, we have provided our version of the original (dual linear/curvilinear) and near-ubiquitous, Hebbian (curvilinear), version of the Yerkes-Dodson law in Figure 2.

Whereas leaders in the field of cognitive psychology have fiercely debated the heuristic value of the Hebbian version of the Yerkes-Dodson law, behavioral neuroscientists, by contrast, have universally accepted and incorporated the Hebbian version of the Yerkes-Dodson law into their theorizing on brain-emotion interactions (e.g., Foy et al. [73]; Diamond et al. [74]; LeDoux [75]; Metcalfe and Jacobs [61]; Aston-Jones et al. [62]; Mendl [63]; Aston-Jones et al. [64]; Richter-Levin [20]; Elzinga et al. [76]; Andreano and Cahill [77]; Morris [65], but see Schulteis and Martinez [78]). A recent study provides an example of the application of the Hebbian version of the Yerkes-Dodson law to behavioral neuroscience research. Andreano and Cahill [77] found an inverted-U-shaped relationship between cortisol levels and memory consolidation in people, that is, an intermediate level of cortisol correlated with peak memory performance. These investigators stated that their findings were consistent with the Yerkes-Dodson law, which, according to them, would predict that there should be a curvilinear relationship between cortisol levels and memory performance (pages 467–469). Actually, the Yerkes-Dodson law does not make this prediction. The Yerkes-Dodson law, in its original form, would predict that on simple tasks, stress levels of cortisol should enhance memory, and on more complex tasks, stress levels of cortisol should impair memory. Consequently, Andreano and Cahill's findings are relevant, specifically, towards enhancing our understanding of the stress side of the curvilinear component of the Yerkes-Dodson law. A more thorough understanding of how cortisol interacts with memory would need to address how high levels of cortisol (or corticosterone, the rodent form of cortisol) and drugs that activate cortisol receptors interact with task difficulty to impair, as well as enhance, memory consolidation (Sandi et al. [79]; Sandi [80]; Cordero and Sandi [81]; Buchanan and Lovallo [82]; Cordero et al. [83]; Rimmele et al. [84]; Hui et al. [85]; Het et al. [86]).

We introduced this section by mentioning "flashbulb memories," which are durable memories formed in response to strong emotional experiences. Had Schlosberg, Duffy, and Hebb been correct in their assertion that strong emotion reliably impairs cognition, then flashbulb memories should not exist. That is, if the right (high stress) side of the stress-performance curve always declines to produce poor performance, as it does in the Hebbian version of the Yerkes-Dodson law (Figure 2(a)), then strong emotionality should universally impair all forms of cognition. On the contrary, extensive research has shown that strong emotionality can, under some conditions, enhance memory (Ni [41]; Cahill et al. [87]; McGaugh [88]; Sharot et al. [89]; Nielson et al. [90]). The well-described flashbulb memory phenomenon is just one example of how arousing experiences can strengthen memories. Although emotional memories may not be flawless representations of the original experiences (Tekcan et al. [91]; Schmidt [92]; Laney and Loftus [93]; Loftus [94]), their general accuracy and durability which can span decades are remarkable (Tekcan and Peynircioğlu [95]; Berntsen and Thomsen [34]).

In summary, we have asserted that the Hebbian version of the Yerkes-Dodson law (Figure 2(a)) is an incomplete representation of the effects of emotionality on cognition because it does not address how memories can be strengthened by strong emotional experiences. Traumatic experiences place a subject at the highest right side of the arousal-performance curves depicted in Figure 2, and they can generate intrusive memories so powerful and durable that they can have long-lasting pathological consequences which underlie anxiety and mood disorders, including depression and post-traumatic stress disorder (PTSD) (Ehlers and Clark [96]; Layton and Krikorian [97]; Rubin et al. [98]; Ehlers et al. [99]; Bremner [100]; Michael et al. [101]; Nemeroff et al. [102]). Only the original version of the Yerkes-Dodson law (Figure 2(b)) can incorporate the finding that emotional trauma can produce an enhancement of memory. Hence, the original version of the Yerkes-Dodson law is of greater value to behavioral and psychiatric research than the Hebbian version because it incorporates the enhancement, as well as impairment, of memory in times of strong emotionality.

3. EASTERBROOK'S "CUE UTILIZATION" HYPOTHESIS: A CUE-BASED DISTINCTION BETWEEN SIMPLE AND COMPLEX TASKS

A problem with "task difficulty" as a critical factor in understanding emotion-memory interactions is that it is a subjective measure. It is therefore difficult, if not impossible, to operationally define the term "task difficulty" with objective criteria. Nevertheless, investigators over the past 5 decades have taken on this task. One of the earliest attempts to understand how task variables interact with performance was accomplished in a landmark paper by Easterbrook [103], in one of the most comprehensive and insightful analyses of how emotion affects cognition. Easterbrook assessed the influence of emotionality on cue utilization and the organization of behavior. He noted that strong emotionality "acts consistently to reduce the range of cues that an organism uses, and that the reduction in the range of cue utilization influences action in ways that are either organizing or disorganizing" (page 183). "On some tasks, reduction in the range of cue utilization *under high stress conditions* improves performance. *In these tasks*, irrelevant cues are excluded and *strong emotionality* is motivating. In other tasks, proficiency demands the use of a wider range of cues, and *strong emotionality* is disorganizing. There seems to be an optimal range of cue utilization for each task" (pages 197-198). Importantly, Easterbrook interpreted these observations as an indication that "the effect *of emotionality* on proficiency would depend

on the complexity of the *task* studied" (page 187). Easterbrook emphasized that performance on only the most demanding and complex tasks would suffer a "disintegration" *(i.e., severe impairment)* as a result of strong emotionality (page 187; text in italics are paraphrased). He noted that there was an impairment in behavioral performance in complex tasks in response to strong emotionality because "the range of cue utilization is reduced in response to strong emotion" (page 197), and that "tasks requiring the use of smaller numbers of cues were facilitated by drive increments" (page 192).

Easterbrook's cue utilization hypothesis stated that with increased emotionality, there is a reduction in the range of cues that an individual can process. According to Easterbrook, if a task is complex, that is, involves attention to multiple cues, then performance will deteriorate under conditions of high stress. If, on the other hand, a task is simple, that is, involves focused attention to a single cue, as occurs, for example, with the "weapon focus" phenomenon (Christianson [29]; Safer et al. [104]; Pickel [105]), then performance will improve under high-stress conditions.

Easterbrook's approach towards identifying systematic relationships between cue utilization and emotionality has been fruitful in understanding how emotionality affects behavioral performance in people and rodents (Telegdy and Cohen [48]; Geen [106]; Cohen et al. [107]; Christianson [29]; Hanoch and Vitouch [72]). Thus, Easterbrook's cue utilization hypothesis and the original version of the Yerkes-Dodson law are complementary explanations for the finding that strong emotionality can enhance performance on a simple task and impair performance on a complex task.

We will return to the challenge of how to distinguish simple versus complex levels of task difficulty and how they relate to performance in a later section addressing the cognitive functions of the prefrontal cortex. First, we will review literature on the effects of stress on synaptic plasticity in different forebrain structures, and then we will present a physiological model which may prove to be of value in explaining how stress can impair memory and can also generate flashbulb memories.

4. EFFECTS OF STRESS ON LTP IN THE HIPPOCAMPUS, AMYGDALA, AND PREFRONTAL CORTEX

Most of the research on stress and LTP has focused on the CA1 and dentate gyrus regions of the hippocampus, with a lesser volume of work on the PFC and amygdala. In addition, most of the stress-LTP studies have been conducted on male rats. This is an important issue because female rats and women appear to respond differently to acute stress than do the males of each species, a finding which was first suggested by Stratton [36] and then substantiated in contemporary research (Shors [108]; McEwen [109]; Beiko et al. [110]; Conrad et al. [111]; Kudo et al. [112]; Shansky et al. [113]; Cahill [114]). Therefore, we acknowledge that our speculation here is based primarily on research conducted on the hippocampus of male rats. The extension of this synthesis to females, in general, and to amygdala and PFC processing, in particular, needs to be substantiated with additional research.

Another issue worth mentioning is the potential role of long-term depression (LTD) in stress and memory processing. Elsewhere, Diamond et al. [2, 3] and others (Xu et al. [115]; Abraham and Tate [12]; Kim and Yoon [13]; Braunewell and Manahan-Vaughan [116]; Kemp and Manahan-Vaughan [117]; Sajikumar and Frey [118]; Huang et al. [25]) have speculated on the potential significance of stress-LTD interactions in hippocampal functioning. However, as the hypothesis we have presented here is at an early stage of development, we have restricted our speculation to the potential link between LTP and emotional memory processing.

Finally, we have arrived at the critical question that underlies the basis of our theorizing: what does it mean, from physiological and cognitive perspectives, for stress to affect the induction of LTP? Our approach to addressing this question is different from the conventional view that LTP can be understood exclusively as a physiological model of memory. We suggest here, as in previous theorizing (Diamond et al. [17]), that the successful versus unsuccessful induction of LTP can serve as a "diagnostic" measure with which to assess the functional state of a brain structure. If, for example, the induction of hippocampal LTP is enhanced 2 minutes after a rat is placed in a novel environment, then we would interpret this finding as evidence that hippocampal information processing has been enhanced by novelty, but the interpretation applies only to the influence of novelty on the hippocampus at the 2-minute time point. If, on the other hand, the induction of hippocampal LTP is blocked 30 minutes after a rat is placed in a novel environment, then we would interpret that finding narrowly, as well, as evidence that hippocampal information processing is inhibited 30 minutes after exposure to novelty. In this example, exposure of a rat to a novel environment, per se, does not generate a global excitatory or inhibitory effect on hippocampal functioning. Rather, it produces both effects, with each effect occurring at different times after the onset of the arousing experience. Therefore, the successful versus unsuccessful induction of LTP can serve as a diagnostic test to reveal whether the hippocampus has been *transiently* shifted into an enhanced or impaired state of plasticity induction at different times after the onset of an emotional experience.

With this diagnostic perspective on LTP induction in mind, we can now address the functional significance of the finding that stress blocks the induction of hippocampal LTP. In 1990, our group suggested that the reason why stress blocks LTP was because stress, itself, activates endogenous mechanisms of plasticity in common with mechanisms that are also activated by exogenously induced LTP (Diamond et al. [119]). We hypothesized that the stress-induced saturation of endogenous mechanisms of plasticity would render plasticity mechanisms refractory in response to subsequent stimulation. The stress-induced activation, followed by inhibition, of hippocampal plasticity mechanisms would thereby explain why stress interferes with the induction of LTP. Our hypothesis was supported by later work which

revealed commonalities between the mechanisms underlying stress and tetanizing (LTP-inducing) effects on plasticity (discussed further by Shors and Dryver [120]; Diamond et al. [2]; Diamond et al. [3]; Huang et al. [25]). According to this view, stress blocks the induction of LTP because the tetanizing stimulation was delivered when the hippocampus was in a refractory phase for plasticity induction, which occurs following an initial stress-induced activation of NMDA-receptors. Support for this hypothesis is the finding that NMDA receptor blockade during stress blocks the stress-induced suppression of LTP (Kim et al. [121]).

In the following sections, we have extended our earlier speculation that stress activated endogenous mechanisms in common with LTP in the hippocampus with the hypothesis that the hippocampus and amygdala both undergo a stress-induced activation, followed by an inhibition, of mechanisms underlying synaptic plasticity. We suggest that the rapid activation of plasticity mechanisms in these two structures underlies the well-described, arousal-induced enhancement of memory, producing flashbulb and traumatic memories in people, and fear conditioning in rodents. We also hypothesize that the PFC does not undergo a stress-induced enhancement phase followed by an inhibitory phase. We interpret the stress-induced inhibition of LTP in the PFC as an indication that stress produces an immediate inhibition of the functioning of the PFC, which is revealed behaviorally as a narrowing of attention and impaired multitasking, or more globally, as an impairment of complex learning.

5. STRESS BLOCKS HIPPOCAMPAL LTP, THEREFORE STRESS IMPAIRS HIPPOCAMPAL FUNCTIONING

For almost four decades, Bruce McEwen's group has been studying how stress hormones affect the brain and behavior. He and his coworkers first showed that the hippocampus has the greatest density of glucocorticoid receptors of all brain structures (McEwen et al. [122]; McEwen et al. [123]), indicating that the hippocampus was highly responsive to stressful experiences. Subsequent glucocorticoid-related behavioral work from his group led to the conclusion that "hippocampal function may indeed be suppressed during periods of prolonged stress" (Micco et al. [124, page 328]). This view of stress interfering with hippocampal functioning was incorporated into theorizing by Jacobs and Nadel [125] as an explanation of how stress reactivates childhood fears and phobias. These authors speculated that phobias can develop during infancy, before the hippocampal "locale" system, with its context-specific learning system, develops. They suggested that "under severe stress, behavioral control devolves on the taxon *(nonhippocampal)* systems that are, in this state, unusually sensitive ..." (page 518, text in italics added). They further proposed that "stress disrupts the function of the hippocampally based locale system and its context-specific learning capacities while potentiating taxon systems and their context-free associations" (page 518), and that the stress-induced suppression of the hippocampus would enable phobias that had been formed in childhood to be expressed in adulthood.

The first electrophysiological evidence that stress inhibited hippocampal functioning was provided by Richard Thompson and his coworkers, with their finding that stress (restraint with or without tail shock) blocked the induction of LTP in CA1 in vitro (Foy et al. [73]). They interpreted their findings of a stress-induced blockade of hippocampal LTP within the context of the Hebbian version of the Yerkes-Dodson law by stating that "cognitive performance deteriorates at extreme levels of arousal (which was) recognized by Yerkes and Dodson" (page 145). Their discussion provided the first suggestion that the stress-induced suppression of LTP could be linked to the presumed stress-induced impairment of hippocampal functioning.

At about the time that Thompson's group was studying restraint stress and paw shock effects on LTP in vitro, our group was investigating how stress affected a low threshold form of LTP in CA1 of behaving rats. This form of plasticity, which is referred to as primed burst (PB) potentiation, can be induced by a total of only 5 physiologically patterned pulses delivered to CA1 (Rose and Dunwiddie [126]; Diamond et al. [127]; and see also Larson and Lynch [128]; Larson et al. [129]; Staubli and Lynch [130] for related work). We found that the induction of PB potentiation was blocked in rats that were exposed to an unfamiliar environment (Diamond et al. [119]; Diamond et al. [131]). We also showed that when rats were explicitly acclimated to the environment, as indicated by a significant reduction in their levels of serum corticosterone, the blockade of PB potentiation was no longer present (Diamond et al. [131]). Importantly, when these same rats were then exposed to a second, stress-provoking (corticosterone-elevating) environment, once again, PB potentiation was suppressed. These findings demonstrated that the capacity for the hippocampus to generate plasticity, and presumably its memory storage functioning, was continuously influenced by an animal's emotional state.

Thus, the nascent stress-LTP field in the 1980s and early 1990s, led by McEwen's early research on hippocampal sensitivity to glucocorticoids (in conjunction with his pioneering work with Robert Sapolsky on the stress- and glucocorticioid-induced increases in the susceptibility of the hippocampus to damage; Sapolsky et al. [132]), the electrophysiological studies on the stress-induced suppression of LTP and PB potentiation (reviewed in Diamond and Rose [133]), and the theorizing by Jacobs and Nadel [125] on the psychopathological effects of stress on the hippocampus, all fully supported the view that stress exerts a disruptive influence on hippocampal functioning.

The hypothesis that stress inhibited hippocampal functioning was supported by a large number of cognitive and electrophysiological studies conducted in the past decade. For example, we have found that stress, involving exposure of rats to either an unfamiliar environment or to a predator, impaired hippocampus-dependent memory (Diamond et al. [134]; Diamond et al. [52]; Woodson et al. [135]; Sandi et al. [136]; Diamond et al. [137]; Park et al. [138]) and blocked the induction of PB potentiation in vivo (Diamond et al. [139]; Vouimba et al. [140]) and in vitro

(Mesches et al. [51]). Our findings are consistent with recent work from other laboratories indicating that acute stress or corticosterone administration blocks hippocampal LTP (Shors et al. [141]; Shors and Thompson [142]; Pavlides et al. [143]; Pavlides et al. [144]; Pavlides et al. [145]; Garcia et al. [146]; Pavlides and McEwen [147]; Akirav and Richter-Levin [148]; Zhou et al. [149]; Wang et al. [150]; Garcia [15]; Kim et al. [151]; Alfarez et al. [152]; Xiong et al. [153]; Jay et al. [9]; Kim et al. [154]; Krugers et al. [155]; Wiegert et al. [156]) and can impair hippocampus-specific memory processing in rats (de Quervain et al. [157]; Conrad et al. [158]; Roozendaal et al. [159]) and people (Kirschbaum et al. [160]; de Quervain et al. [161]; Wolf et al. [162]; Payne et al. [163]; Buss et al. [164]; Wolf et al. [165]; Elzinga et al. [76]; Kuhlmann et al. [166]; Kuhlmann et al. [167]; Payne et al. [168]; Buchanan et al. [169]).

An illustration of the widespread acceptance of the idea that strong stress impairs hippocampal functioning was in statements by LeDoux [75] in his scholarly and widely read book on the brain and emotion. He commented that memory "may be interfered with if stress is sufficiently intense and prolonged to raise the level of adrenal steroids to the point where the hippocampus is adversely affected," and he further suggested that "if the hippocampus was completely shut down by the stress to the point where it had no capacity to form a memory during the event, then it will be impossible through any means to dredge up a conscious memory of the event" (pages 243-244). Similar views of how traumatic experiences affect the hippocampus were expressed by van der Kolk [170], who suggested that "extreme emotional arousal interferes with hippocampal memory functions" (page 282), and by Joseph [171, 172] who stated that "under conditions of overwhelming terror, the hippocampus becomes desynchronized . . . what is experienced may be forgotten or stored abnormally and independently of the hippocampus . . . emotional memory and recall are in part mediated by the amygdala" ([171, page 175]).

The pervasive view in the 1990s that stress impairs hippocampal functioning and enhances amygdala functioning led Metcalfe and Jacobs [61] to propose a novel hypothesis which addressed the neurobiological basis of traumatic memory formation. These investigators categorized brain memory systems in terms of whether brain structures were activated (hot) or impaired (cool) by strong emotionality. According to Metcalfe and Jacobs [61], the amygdala is a component of the "hot" memory system, because it functions optimally under emotionally intense conditions. The hippocampus, by contrast, is a component of the "cool" memory system because it functions optimally under emotionally neutral conditions and is impaired by traumatic stress. The theorizing by Metcalfe and Jacobs [61], as well as by Nadel and Jacobs [173], were consistent with LeDoux's [75] speculation that stress induces a "shutdown of the hippocampus" (page 246), and "may even enhance amygdala functions" (page 245).

Metcalfe and Jacobs [61] also noted that intermediate levels of stress appeared to have a facilitatory effect on hippocampal plasticity. This view was based, in part, on the finding of an inverted-U-shaped relationship between the level of serum corticosterone and the magnitude of hippocampal PB potentiation or LTP (Bennett et al. [174]; Diamond et al. [74]; Kerr et al. [175]). That is, the magnitude of hippocampal synaptic plasticity was maximal in animals with intermediate levels of corticosterone, and was the lowest in animals with either low or high (stress) levels of corticosterone. In addressing the significance of this finding, Diamond et al. [74] and Metcalfe and Jacobs [61] perpetuated the misrepresentation of the Yerkes-Dodson law by suggesting that the U-shaped relationship between PB potentiation and corticosterone was a physiological manifestation of the (Hebbian version of the) Yerkes-Dodson law (Figure 2(a)).

This overview of studies on stress and hippocampal plasticity summarizes the view of many researchers over the past two decades that strong stress inhibits hippocampal functioning (e.g., Jacobs and Nadel [125]; van der Kolk [176]; Diamond and Rose [133]; LeDoux [75]; van der Kolk [170]; Nadel and Jacobs [173]; Kim and Yoon [13]; Joseph [172]; Diamond and Park [177]; Garcia [15]; Layton and Krikorian [97]; Kim and Diamond [1]; Lynch [178]; Diamond et al. [2]; Diamond et al. [3]; Kim and Jung [21]; Akirav and Richter-Levin [22]). In the next section, we will present a new perspective on this issue by integrating a broader range of research on stress-hippocampus-LTP interactions than has been considered previously.

6. CRACKS IN THE EDIFICE OF THE HYPOTHESIS THAT STRONG EMOTIONALITY GLOBALLY SUPPRESSES HIPPOCAMPAL FUNCTIONING

As discussed above, research conducted over the past two decades has demonstrated conclusively that stress blocks the induction of hippocampal synaptic plasticity (LTP and PB potentiation) and impairs spatial and declarative memory. Based on these findings, major figures in the field have stated that stress adversely affects hippocampal functioning. For example, according to Nadel and Jacobs [173], "high levels of stress impair the functioning of the hippocampus, weakening or totally disrupting those aspects of spatial and explicit memory subserved by this structure. A number of studies, with both humans and animals, have demonstrated this now well-accepted fact" (page 155). This perspective was discussed further by Metcalfe and Jacobs [61], who stated that memory processing was accomplished by the amygdala, and not by the hippocampus, during times of stress. These authors speculated that during traumatic stress, the hippocampus "becomes dysfunctional" (page 205). Similarly, Diamond et al. [17] and Layton and Krikorian [97] hypothesized that the amygdala becomes activated and temporarily stores information as the hippocampus is rendered nonfunctional during a traumatic experience. More recently, Akirav and Richter-Levin [22] summarized the consensus viewpoint by stating that "under certain stressful conditions, emotional memory storage in the amygdala will be facilitated at the expense of hippocampus-dependent spatiotemporal processing" (page 29).

Finally, perhaps the ultimate denial of a necessary role of the hippocampus in emotional memory processing was stated by Dalgleish [179], in his review of the history of research on affective neuroscience. Dalgleish discussed MacLean's [180] introduction of the term "limbic system," which is still currently in use to describe the group of brain structures considered to be involved in emotion (but see commentary by LeDoux [75]). According to MacLean, the hippocampus was the core structure of the limbic system, responsible for integrating visceral with external information. Dalgleish, however, justified the expulsion of the hippocampus from the limbic system because it had only a relatively small role in emotionality, as it was "more involved in higher cognitive processes" (page 584).

We now suggest that the idea that hippocampal functioning is globally impaired by strong emotionality is incomplete and inaccurate. The following observations illustrate inconsistencies with the idea that strong stress impairs hippocampal functioning.

(1) The hippocampus is an important component of contextual fear conditioning (Phillips and LeDoux [181]; Maren [182]; Sanders et al. [183]; Rudy et al. [184]). Moreover, hippocampal cells exhibit plasticity of their place fields in response to contextual fear conditioning (Moita et al. [185]; Moita et al. [186]), leading these authors to conclude that hippocampal "place cell remapping was related to the rat's learned fear of the environment" (Moita et al. [186, page 7015]). Fear conditioning training has stress-provoking elements which have been shown to block LTP and PB potentiation, such as exposure of rats to a novel environment (the training context) and electric shock, and yet, the formation of the contextual component of the fear memory is dependent on the integrity of the hippocampus. How is it possible for the hippocampus to exhibit fear-induced place cell plasticity and to form a contextual memory of a fear-provoking experience when fear suppresses hippocampal functioning?

(2) Researchers outside of the stress-LTP field have long contended that activation of the amygdala exerts a facilitating effect on memory-related processing by other brain regions, including the hippocampus (McGaugh et al. [187]; Roozendaal et al. [188]; Nathan et al. [189]). In one example, Packard and Teather [190] demonstrated that the amphetamine-induced activation of the amygdala enhanced hippocampus-dependent spatial memory. In related work, neuroimaging studies have provided strong support for the idea that the conjoint activation of the hippocampus and amygdala under arousing counditions is a critical component of emotional memory storage and retrieval processes (Maratos et al. [191]; Dolcos et al. [192]; Dolcos et al. [193]). The finding that activation of both the amygdala and hippocampus is necessary for the formation of an emotional memory is incompatible with the view that stress "shuts down" the hippocampus.

(3) Flashbulb memories are highly durable, explicit recollections of the details of events that had transpired during emotional experiences (Brown and Kulik [37]; Schmidt [92]). A traumatic memory is a type of flashbulb memory which is generated in response to a horrific and possibly life-threatening event. According to van der Kolk [170, 176], the suppression of hippocampal functioning and activation of the amygdala during horrific experiences underly the implicit, fragmented, and primarily sensory structure of traumatic memories. Traumatic memories certainly have a powerful implicit (nondeclarative) component, and PTSD patients commonly have amnesia, or "memory gaps," for events that occurred during their trauma (van der Kolk et al. [194]; van der Kolk [176]; van der Kolk [170]; Joseph [171]; Yovell et al. [195]; Michael et al. [196]; Ehlers et al. [197]). However, traumatized people commonly provide explicit (declarative) descriptions of the event(s) that precipitated their PTSD symptoms. For example, Ehlers et al. [198] noted that PTSD patients could describe sensory elements of their traumatic experiences, such as a victim of a motor vehicle accident described hearing the sound of crunching metal which occurred during the accident, and a rape victim described the feel of the rapist's hands over her eyes. The ability of PTSD patients to verbally describe features, albeit only fragments, of their traumatic experiences suggests that their memories of trauma are not entirely implicitly based. If hippocampal functioning actually was shut down during emotional experiences, then emotional memories would be similar to those observed in amnesics with temporal lobe damage. That is, an individual with a complete loss of the hippocampal functioning, such as HM, can acquire implicit information, such as perceptual and motor skills, but completely lacks an explicit memory of the learning experience (Scoville and Milner [199]; Squire [200]). It is evident from the descriptions of PTSD patients' recollections of their traumatic experiences that traumatic memories are not equivalent to the complete loss of declarative memory processing that occurs in patients with temporal lobe damage. The combination of intense implicit components interwoven with fragmented declarative recollections of isolated sensory elements of the experience in traumatic memories is perhaps a unique category of memory. Nevertheless, since PTSD patients can consciously recall details of aspects of their traumatic experiences, it would appear that the hippocampus is involved, perhaps in an abnormal manner, in the formation of traumatic memories.

These three points illustrate inconsistencies in the literature as to how stress affects the hippocampus. On the one hand, a large body of research unequivocally indicates that stress interferes with cognitive and electrophysiological measures of hippocampal functioning. On the other hand, however, emotional memories, including flashbulb and traumatic memories, can have a hippocampal (conscious/declarative) component. In the next section, we present a model of stress-hippocampus interactions which addresses how hippocampal functioning can be impaired by stress, and can also be involved in the formation of emotional memories.

7. TEMPORAL DYNAMICS MODEL OF STRESS-HIPPOCAMPUS INTERACTIONS

We suggest that the discrepancies between theory and research on emotion, memory, and hippocampal functioning

discussed in the previous section may be resolved with a thorough assessment of the literature on the influence of emotion on LTP. A critical finding in this area of research is that manipulations that produce strong emotionality in rats can actually *enhance* hippocampal LTP. This finding was first described by Seidenbecher et al. [201], who showed that water-deprived rats given access to water around the time of tetanizing stimulation exhibited an *increase* in the duration of LTP recorded in the dentate gyrus (DG). Numerous other studies have replicated and extended this finding to show that a variety of arousing experiences, such as water immersion, exposure to novel places and objects, and spatial learning occurring around the time of the delivery of tetanizing stimulation, all increased the duration of LTP in CA1 and DG (e.g., Seidenbecher et al. [202]; Frey [203]; Li et al. [204]; Straube et al. [205]; Davis et al. [206]; Almaguer-Melian et al. [207]; Uzakov et al. [208]; Ahmed et al. [209]).

A critical component of the emotion-induced enhancement of LTP involves the activation of the hippocampus by the amygdala. Electrical stimulation of the amygdala can mimic the emotion-induced enhancement of hippocampal LTP (Ikegaya et al. [210]; Akirav and Richter-Levin [148]; Akirav and Richter-Levin [211]; Frey et al. [212]; Akirav and Richter-Levin [213]), and damage to, or inactivation of, the amygdala blocks stress effects on hippocampal LTP and spatial memory (Almaguer-Melian et al. [214]; Kim et al. [154]; Korz and Frey [215]; Kim and Jung [21]). In addition, input from the hypothalamus (Nakanishi et al. [216]) and the locus coeruleus (Harley and Sara [217]; Sara et al. [218]; Kitchigina et al. [219]; Bouret and Sara [220]), via activation of β-adrenergic receptors (Ikegaya et al. [221]; Vermetten and Bremner [8]; Strange and Dolan [222]; Nathan et al. [189]; Hurlemann et al. [223]), as well as the dopaminergic innervation of the hippocampus from the ventral tegmental area (VTA) (Li et al. [204]; Lisman and Grace [224]) and local release of corticotropin releasing hormone (CRH; Adamec et al. [225]; Wang et al. [226]; Wang et al. [227]; Blank et al. [228]; Chen et al. [229]), all appear to contribute to the rapid stress-induced enhancement of hippocampal LTP. *These studies indicate that hippocampal mechanisms of memory storage are rapidly engaged, rather than suppressed, by an arousing and stressful experience*.

Recent work has implicated corticosterone in the stress-induced enhancement, as well as the impairment, of hippocampal synaptic plasticity. Joëls et al. have shown that brief application of corticosterone around the time of tetanizing stimulation enhanced LTP in CA1 in vitro via nongenomic activation of mineralocorticoid receptors (Karst et al. [230]; Wiegert et al. [231]). Complementary work by Ahmed et al. [209] demonstrated that brief stress transforms protein synthesis-independent LTP into a long-lasting protein synthesis-dependent form of LTP, via activation of mineralocorticoid (MR) receptors. This group also showed that stress rapidly initiated dynamic changes in gene expression (Morsink et al. [232]), and levels of cellular signaling molecules in the hippocampus, including phosphorylated mitogen-activated protein kinase 2 (pMAPK2) and calcium/calmodulin-dependent protein kinase II (pCaMKII). Conversely, stress levels of corticosterone applied for a longer period of time (>20 minutes) increased the magnitude of inhibitory components of electrophysiological activity, such as the afterhyperpolarization (Joëls and de Kloet [233]; Kerr et al. [234]; Joëls and de Kloet [235]; Karst and Joëls [236]), and suppressed the induction of LTP (Pavlides et al. [237]; Rey et al. [238]; Kerr et al. [175]; Pavlides et al. [143]; Pavlides et al. [144]; Pavlides et al. [145]; Zhou et al. [149]; Alfarez et al. [152]; Krugers et al. [155]).

Extensive research indicates, therefore, that one cannot conclude that strong emotionality or corticosterone globally enhances or impairs hippocampal functioning; work discussed above indicates that stress or corticosterone can have both effects on the hippocampus. We propose that the manner in which emotionality affects the hippocampus follows a consistent pattern: an arousing experience must occur in close temporal proximity to the delivery of tetanizing stimulation to enhance LTP. Studies in which stress blocked LTP consistently involved a substantial (>20 minutes) delay from the initiation of the stress experience before tetanizing stimulation was delivered.

The time dependency of stress or amygdala activation effects on LTP was demonstrated directly in a series of studies by Akirav and Richter-Levin [148, 211, 213]. These investigators showed that stimulation of the amygdala 30 seconds, but not 1 hour, prior to perforant path stimulation of the hippocampus enhanced LTP in the DG. Similar findings were reported by Abe's group (Ikegaya et al. [239]; Ikegaya et al. [240]). In our studies in which stress blocked the induction of PB potentiation in vivo and in vitro (discussed above), tetanizing stimulation was always delivered at least 1, and as many as 4, hour after the stress manipulation began. Overall, these findings indicate that for a relatively brief period of time, stress or amygdala activation enhances the induction of hippocampal LTP, followed by a later developing phase when the induction of LTP is suppressed.

Figure 3 represents the temporal dynamics model, which illustrates our hypothesis that stress initiates dynamic time-restricted shifts in the efficacy of hippocampal functioning (as well as the amygdala and PFC, which are discussed in subsequent sections). This model is consistent with and extends recent theorizing by Joëls et al. [241] on the time-dependent effects of stress and corticosterone on memory and LTP, and the "emotional tagging" hypothesis of Richter-Levin and Akirav [19, 20], which states that there is a time-dependent activation, followed by inhibition, of neuroplasticity in the hippocampus in response to stimulation of the amygdala. Our model is also an extension of findings which have shown that strong emotionality briefly activates hippocampal mechanisms of synaptic plasticity, thereby increasing the duration of LTP when emotionality and tetanizing stimulation coincide in time (Ahmed et al. [209]; Reymann and Frey [242]). We emphasize more broadly in our model that stress, or any sufficiently arousing experience, briefly enhances the memory processing features of hippocampal functioning. We further speculate that this relatively brief stress-induced enhancement of hippocampal functioning underlies the declarative component of flashbulb and traumatic memories in

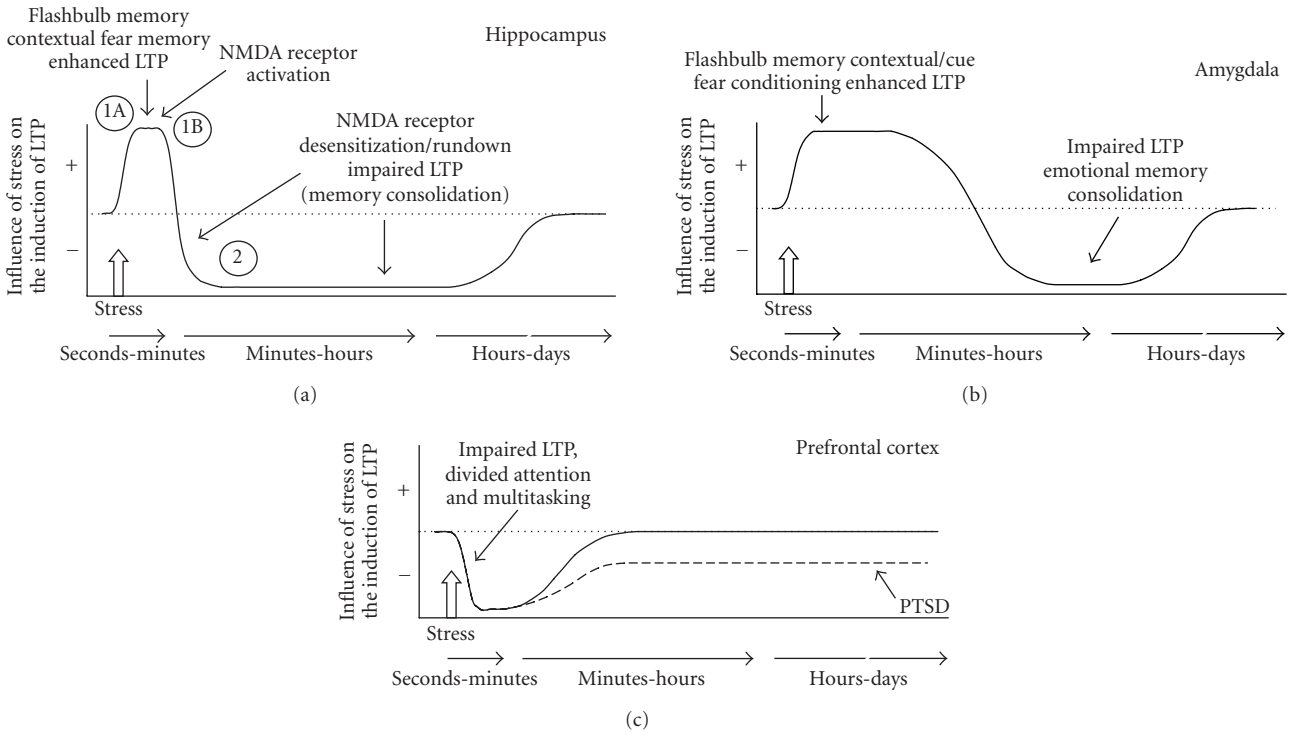

Figure 3: Temporal dynamics model of how stress affects memory-related processing in the hippocampus, amygdale, and prefrontal cortex. The initiation of a strong emotional experience activates memory-related neuroplasticity in the hippocampus and amygdala, and suppresses PFC functioning (phase 1). The most rapid actions would involve increases in ACTH, CRF, NE, acetylcholine, dopamine, and changes in GABA receptor binding (phase 1A), followed within minutes by elevated levels of glucocorticoids (phase 1B). The combination of the activation of the hippocampus by these neuromodulators with coincident tetanizing stimulation produces a great enhancement of LTP. Within minutes of the initiation of phase 1, the hippocampus undergoes a reversal of its plasticity state, based, in part, on the reduction in the sensitivity of NMDA receptors (phase 2). Tetanizing stimulation delivered to the hippocampus during phase 2 will thereby result in an impairment of the induction of LTP. The amygdala continues in its form of phase 1 longer than the hippocampus, but eventually, the amygdala, as well, exhibits an inhibitory phase, perhaps as it is involved in the consolidation of the emotional memory. The PFC is only inhibited by stress; the recovery from its suppression of functioning would depend on the nature and intensity of the stressor, interacting with the ability of the individual to cope with the experience. In the case of trauma-induced PTSD, the PFC may not recover to its original state of efficiency in suppressing the activity of lower brain areas, such as the amygdala and brain stem nuclei.

people, and contextual fear conditioning in rodents. Following the brief period in which hippocampal plasticity is activated is a refractory period, in which there is an increase in the threshold for the induction of new plasticity. Therefore, tetanizing stimulation delivered during the poststress refractory period is less effective at inducing LTP than if it is delivered at the onset of a stress experience.

According to the temporal dynamics model, the onset of an emotional experience activates endogenous forms of neuroplasticity in the hippocampus for a period of seconds to minutes, which is revealed as an enhancement of LTP when tetanizing stimulation occurs in this narrow-time window (Ahmed et al. [209]; Reymann and Frey [242]). The activational period, identified by the "1A" and "1B" in Figure 3, involves a stress-induced increase in glutamatergic transmission and activation of AMPA and NMDA receptors (Bagley and Moghaddam [243]; Venero and Borrell [244]; McEwen et al. [245]; Kole et al. [246]). The initial component (1A) would involve the rapid activation of the hippocampus by the amygdala, in conjunction with local increases in levels of neuromodulators, such as corticotrophin-releasing hormone (CRH) (Adamec et al. [225]; Wang et al. [226]; Wang et al. [227]; Blank et al. [228]; Chen et al. [229]), acetylcholine (Ye et al. [247]; Ovsepian et al. [248]), dopamine (Li et al. [204]; Lisman and Grace [224]; Ahmed et al. [209]; Lemon and Manahan-Vaughan [249]), and norepinephrine (Gray and Johnston [250]; Hopkins and Johnston [251]; Katsuki et al. [252]; Izumi and Zorumski [253]), all of which have been shown to enhance hippocampal LTP. Rapid alterations in GABA receptor binding dynamics (Trullas et al. [254]), as well, would contribute to the almost immediate activation of the hippocampus in response to the onset of a strong emotional learning experience.

It is noteworthy that the initial component of the stress-induced activation of the hippocampus would *not* include a corticosteroid influence. The substantial delay after the onset of stress before corticosteroids would be released into the bloodstream and then reach the brain (Cook [255]) would make the steroidal modulation of hippocampal plasticity a delayed component of phase 1, identified by the

"1B" in Figure 3. Thus, no sooner than several minutes after the onset of a stress experience, corticosterone would begin to activate mechanisms involved in hippocampal plasticity, thereby producing an enhancement of LTP (and memory) via nongenomic activation of mineralocorticoid receptors (Karst et al. [230]; Wiegert et al. [231]).

Ultimately, the rapid stress-induced activation of the hippocampus by steroidal and nonsteroidal neuromodulators would produce a dramatic increase in intracellular calcium levels (Kole et al. [256]; Joëls [257]; Joëls et al. [258]). This rapid influx of calcium would trigger the initiation of a cascade in the phosphorylation of molecules involved in synaptic plasticity and in the formation of memories of the events that had occurred in phase 1 (Blair et al. [259]; Poser and Storm [260]; Lisman et al. [261]; Rongo [262]; Suenaga et al. [263]).

The next phase, identified by the "2" in Figure 3, is a prolonged period of time in which the threshold for the induction of LTP is increased. When the hippocampus is in phase 2, its capacity to generate new plasticity, and therefore to form new memories, would be impaired. In theory, phase 2 can develop within minutes of the onset of a strong emotional experience, and may last from hours to days (Garcia et al. [146]; Shors et al. [264]). The initiation of phase 2 would involve the desensitization (Zorumski and Thio [265]; Rosenmund et al. [266]; Swope et al. [267]; Nakamichi and Yoneda [268]) or rundown (Rosenmund and Westbrook [269]; Alford et al. [270]; Price et al. [271]) of NMDA receptors, which occurs in response to a dramatic increase in postsynaptic calcium concentation.

The magnitudes and durations of phases 1 and 2 are variable, and would depend on the intensity and duration of the emotional experience. A weak stimulus that produces a negligible phase 1 response, as well as a weak hormonal response, would produce minimal activation of endogenous hippocampal plasticity, and thereby result in poor memory (Sandi et al. [79]). By contrast, activation of the hippocampus in phase 1 in conjunction with elevated levels of adrenal hormones (e.g., epinephrine and corticosterone) during phase 2 would facilitate the consolidation of the emotional memory. This component of the temporal dynamics hypothesis is consistent with a vast literature which has demonstrated that epinephrine- or corticosteroids- (Gold and Van Buskirk [272]; Sandi et al. [79]; McGaugh and Roozendaal [273]; Cahill and Alkire [274]; Sandi [275]; McGaugh [88]; Akirav et al. [276]; Hui et al. [85]; Roozendaal et al. [277]) administered posttraining under weak learning conditions can strengthen the consolidation of a memory that might otherwise not have been stored. Therefore, during phase 2, adrenal hormones, as well as other neuromodulators, are involved in the consolidation of information that was acquired during phase 1.

The idea that the threshold for LTP induction is raised in phase 2, rather than there being a complete suppression of hippocampal plasticity, has important functional considerations. We have commented previously that stress appears to reduce the efficiency of hippocampal processing, but does not produce the equivalent of a hippocampal lesion (Diamond et al. [52]; Diamond and Park [177]). Empirical support for this idea is the finding that, unlike stress, hippocampal lesion or inactivation produces a general impairment of spatial learning and memory in rats (O'Keefe and Nadel [278]; Olton et al. [279]; Steele and Morris [280]; Diamond et al. [52]; Morris et al. [281]; Nakazawa et al. [282]). For example, we showed that stress impaired memory in a task that placed a great demand on spatial working memory capacity, but stress had no effect on a less demanding, but still hippocampus-dependent, version of the same task (Diamond et al. [52]). Moreover, in electrophysiological studies, stress or stress-related neuromodulators have been shown to block LTP produced by relatively weak (primed burst or theta burst) tetanizing stimulation, but stress has been shown to have no effect on LTP produced by stronger forms of tetanizing stimulation (Corradetti et al. [283]; Mesches et al. [51]; Diamond et al. [139]; Alfarez et al. [152]; Vouimba et al. [140]). We interpret these findings to indicate that while the hippocampus is in the phase 2 state, it can process new information and generate plasticity, but it does so at a reduced level of efficiency. Additional support for this speculation is the finding that when the hippocampus is in a phase 2 state, it shifts to non-NMDA receptor-, rather than NMDA-receptor-, dependent LTP (Krugers et al. [155]; Wiegert et al. [156]).

The temporal dynamics model is consistent with the strong evidence, reviewed in the previous sections, that led researchers to conclude that the hippocampus is rendered "dysfunctional" or "shut down" by stress. We suggest that the idea that the hippocampus is impaired by stress was based entirely on research in which tetanizing stimulation or learning occurred while the hippocampus was in the poststress refractory period (phase 2).

In summary, we have reviewed literature which indicates that the onset of stress activates the hippocampus, thereby producing a rapid and dramatic increase in levels of intracellular calcium. The increased calcium serves as the trigger stimulus to briefly produce an enhancement (phase 1), followed by an impairment (phase 2), of the induction of endogenous synaptic plasticity in the hippocampus. Although the initiation of phase 2 is theorized to involve a calcium-triggered reduction in the sensitivity of NMDA receptors, its maintenance over hours to days may involve depotentiating mechanisms as well (Xu et al. [284]; Rowan et al. [285]; Zhuo et al. [286]; Ghetti and Heinemann [287]; Adamec et al. [288]; Lin et al. [289]; Manahan-Vaughan and Kulla [290]; Kemp and Manahan-Vaughan [117]; Gerges et al. [291]; Xia and Storm [292]; Diamond et al. [3]; Aleisa et al. [293]).

8. EMPIRICAL SUPPORT FOR THE MODEL

The temporal dynamics model of hippocampal functioning leads to specific predictions. First, hippocampus-dependent learning occurring coincident with the onset of an emotional experience (phase 1, Figure 3) should produce intact memory. Emotionality should rapidly activate, that is, prime, mechanisms involved in hippocampal plasticity, thereby enabling memory formation occurring while the hippocampus

is in phase 1 to be intact or enhanced. Second, hippocampus-dependent memory formation should be impaired if new learning occurs during phase 2 (Figure 3).

We have begun to test aspects of the temporal dynamics hypothesis with two different, but well-established, tests of hippocampus-dependent memory. In the first test, adult male rats were trained in the radial arm water maze according to methods we have described in recent publications (Sandi et al. [136]; Diamond et al. [137]). In brief, rats were handled for three days and then they were given a single session of water maze training to find a hidden platform located in 1 of 6 swim arms. The rats were given only 4 sequential training trials to learn the location of the hidden platform (1 minute maximum swim time/trial, followed by 15 seconds on the platform). After completion of the four learning trials, all rats were given memory test trials 1 and 24 hours later. Results from the control (no stress) group showed that 4 learning trials were a sufficient amount of training to produce good performance on the 1-hour memory test, but was insufficient to produce good performance on the 24-hour memory test (Figure 4(a)).

According to the temporal dynamics model, the weak memory at 24 hours produced by minimal water maze training should be strengthened if training were to occur during phase 1, but not if training was to occur during phase 2. To evaluate this possibility, rats were placed for 2 minutes near a cat within the cat's housing room, as described previously (Mesches et al. [51]; Diamond et al. [52]; Woodson et al. [135]; Vouimba et al. [140]; Diamond et al. [137]; Park et al. [138]). The rats were then brought to the main laboratory, where they were given minimal water maze training, either immediately or 30 minutes later. In theory, the brief exposure of the rat to a cat should rapidly initiate an activational (phase 1) response in the rat's hippocampus. This activational phase should be followed a sufficient time later (e.g., 30 minutes) by an inhibitory (phase 2) response. Therefore, rats given water maze training immediately, but not 30 minutes, after brief exposure to a cat, should exhibit enhanced long-term spatial memory.

We have found that rats given 2 minutes of cat exposure immediately before minimal water maze training demonstrated strong spatial memory 24 hours later (Figure 4(a)). This observation of a predator stress-induced enhancement of memory is in complete contradistinction to our prior findings that exposing rats to a cat impaired their consolidation, as well as retrieval, of spatial memory (Diamond et al. [52]; Woodson et al. [135]; Sandi et al. [136]; Diamond et al. [137]; Park et al. [138]). The critical differences between the methodology of our prior studies and the current one are that here, predator stress was brief (2 minutes versus 30–60 minutes) and, more importantly, the brief stress occurred immediately before the learning phase. Therefore, 2 minutes of predator stress enhanced 24-hour memory only when it occurred immediately, but not 30 minutes, before training (Figure 4(a)).

It is important to point out that brief cat exposure enhanced the rat's memory for the location of the hidden platform, despite the fact that predator stress occurred in a completely different context from where spatial learning occurred. That is, predator stress occurred in the cat housing room and water maze training occurred in a different room. This finding does not support the theorizing of Joëls et al. [241], who stated that memory will be facilitated only for cues occurring in both the time and space in which stress occurs. The predator stress-induced enhancement of water maze memory indicates that time, but not space, is the critical element in determining which features of the stress experience will be remembered. Cues that are the focus of attention while the hippocampus is in phase 1, independent of whether they are in or out of the stress context, will be given priority for access to long-term memory storage.

This experiment leads to one other prediction. Since we hypothesized that exposure of the rats to the cat should drive the hippocampus into a phase 1 state of enhanced plasticity, then the rats also should have a strong memory of their cat exposure experience. In the water maze-cat exposure experiment (described above), the memory of the rats' exposure to the cat was not measured, but in other work, we have found that rats develop a strong, extinction-resistant, fear of the context temporally associated with their exposure to the cat (Halonen et al. [294]). This preliminary finding provides further support for the idea that the hippocampus is powerfully activated by traumatic stress to form a durable memory of the arousing experience, as well as other, temporally contiguous, experiences.

In theory, once the phase 1 activational "window" closes, and phase 2 begins, the hippocampus becomes less efficient at processing new information. Therefore, 30 minutes after cat exposure occurred, the hippocampus would have been less efficient at storing the memory of the platform location, which explains why rats given minimal water maze training 30 minutes after cat exposure had poor memory for the platform location 24 hours later.

We have conducted a second test of the temporal dynamics hypothesis by examining the influence of pretraining stress on new learning occurring when the hippocampus presumably was in phase 2, which is a time when we would expect that memory formation (for phase 2 events) should be impaired. It is well known that hippocampal damage or inactivation can interfere with contextual, but not cued, fear conditioning (Phillips and LeDoux [181]; Maren [182]; Sanders et al. [183]; Rudy et al. [184]). Therefore, we hypothesized that an impairment of contextual (hippocampus-specific) memory should occur if fear conditioning were to occur when the hippocampus was driven into the phase 2 state.

Adult male Sprague-Dawley rats (n = 8/group) were given 1 (brief stress) or 10 (prolonged stress) inescapable immersions in a tank of water (1.7 m diameter, 30 cm depth, 23-24°C). Two groups of rats were given a single water stress (1 minute of water immersion) and then they were given fear conditioning training either immediately (brief stress-no delay) or 8 minutes later (brief stress-delay). The group of rats given prolonged water stress swam for an average of 35 seconds per immersion, followed by a 15-second period out of the water, which was repeated 10 times in an

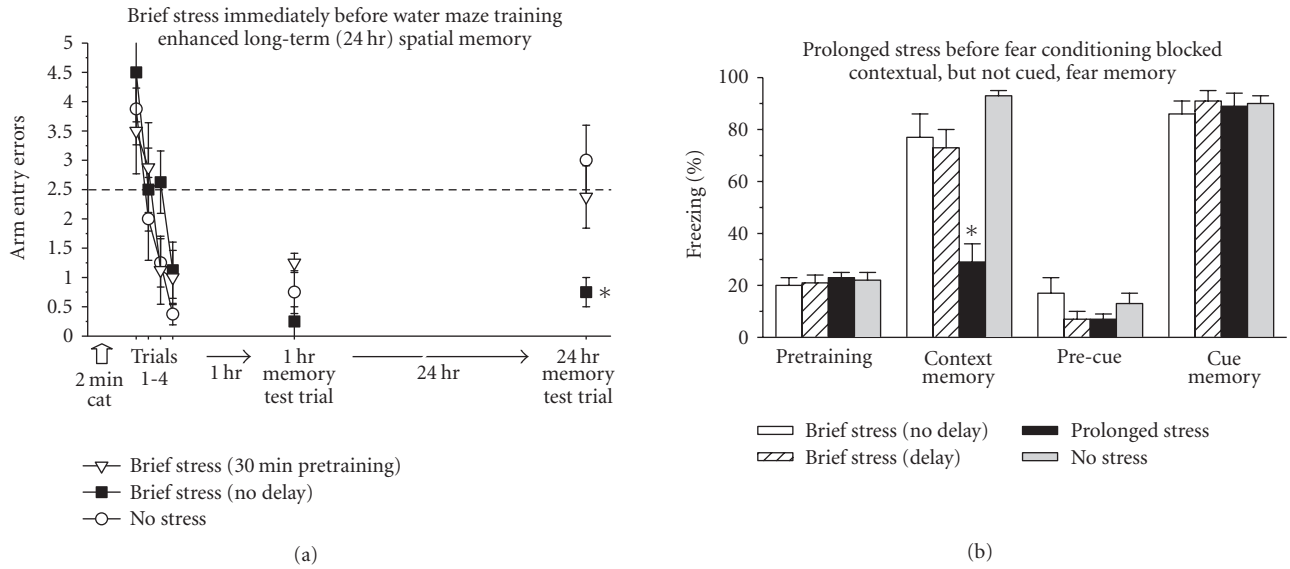

Figure 4: Brief stress immediately before training enhances, and prolonged stress impairs, hippocampus-dependent memory. (a) illustrates the influence of 2 minutes of predator exposure on spatial memory. Rats were exposed to a cat for 2 minutes and then they were given minimal radial arm water maze training (4 trials to find the hidden platform) either immediately or 30 minutes later. Rats trained under nonstress conditions or with cat exposure 30 minutes before training showed no evidence of memory for the platform location 24 hours later (open circle and open triangle). In contrast, rats trained immediately after brief exposure to a cat showed strong 24-hour memory (filled square). The dashed line at 2.5 errors indicates chance level of performance. (b) illustrates the effects of brief versus prolonged water immersion on contextual and cued fear conditioning. Rats given brief water stress either immediately (open bar) or 8 minutes (diagonal lines) before fear conditioning exhibited intact contextual and cued fear memory which was equivalent to that found in the no-stress group (gray bar). Rats given repeated pretraining water immersions (solid bar), by contrast, exhibited intact cued fear memory, but had a complete absence of contextual fear memory. "Precue" indicates baseline freezing in the nonshock context (3-minute duration) prior to the delivery of the tone (3-minute duration). Prolonged pretraining stress, therefore, completely suppressed contextual (hippocampus-dependent) fear conditioning without having any effect on cued (amygdala-dependent) fear conditioning. In both graphs, $* = P < .05$ (ANOVA and Holm-Sidak post-hoc test) compared to the no-stress group.

8-minute period. After the tenth immersion in water, the rats in this group were immediately given fear conditioning training (prolonged stress).

Fear conditioning training was designed in order to produce strong contextual and cued fear memory. Rats were placed into a conventional shock box for 2 minutes, followed by the delivery of 10 shocks (1 mA for 2 seconds) pseudorandomly delivered over 30 minutes (the range of time between shocks was 2–4.5 minutes, with an average delay of 3 minutes). Before each of the 10 shocks, a tone was delivered for 10 seconds, with the last 2 seconds of the tone coincident with the delivery of shock. Twenty four hours after training, all rats were reexposed to the shock environment for 5 minutes for the contextual fear memory test and then they were placed in a different environment where the auditory cue was delivered for 3 minutes. Conditioning was measured as the percent of time that the rats exhibited immobility (freezing) to the context or cue, as determined by automated detection of their movement (Coulbourne instruments).

The rats that were given a single 1-minute immersion in the water immediately before fear conditioning was expected to exhibit intact contextual fear memory because brief water exposure would be expected to drive the hippocampus into the phase 1 state. In contrast, the rats that experienced repeated immersions in the water were expected to exhibit impaired contextual fear memory because more prolonged stress would be expected to drive the hippocampus into the phase 2 state (Figure 3).

We have found that rats given brief pretraining water stress immediately before fear conditioning exhibited contextual and cued fear conditioning which was equivalent to the degree of conditioning observed in the nonstressed group (Figure 4(b)). Therefore, brief stress occurring immediately before fear conditioning did not adversely affect hippocampus-dependent memory processing (the fear memory under control training conditions was so strong that it was not possible to observe a brief stress-induced enhancement of the fear memory).

The memory performance of rats given prolonged water stress prior to fear conditioning training was quite different from the memory performance of rats given brief water stress. Rats given 8 minutes of pretraining stress exhibited intact cued (amygdala-dependent) fear memory, but they exhibited a complete absence of contextual (hippocampus-dependent) fear memory (Figure 4(b)). Thus, the performance of rats given prolonged pretraining stress was equivalent to the severe contextual memory impairment which has been reported in rats with an inactivated or damaged

hippocampus (Phillips and LeDoux [181]; Maren [182]; Sanders et al. [183]; Rudy et al. [184]).

It is important to point out that the inhibitory effect of water stress on contextual fear conditioning was produced by the repeated immersions of the rats in the water, and not only because the water stress began 8 minutes before fear conditioning training. Rats that were given only a single immersion in the water 8 minutes before fear conditioning developed intact contextual and cued fear memory (brief stress—delay group, Figure 4(b)). This finding indicates that there is an interaction between the strength and duration of the stress experience which is necessary to drive the hippocampus into a phase 2 state.

Taken together, our findings in which brief stress enhanced water maze memory (Figure 4(a)) and prolonged stress impaired hippocampus-specific (contextual) fear memory (Figure 4(b)) support our hypothesis that stress rapidly initiates dynamic shifts (enhancement followed by inhibition) in the efficiency of hippocampal memory processing. Moreover, the fear conditioning experiment suggests that phase 2 can be initiated within 8 minutes of the onset of a stressful experience if the stress is sufficiently strong and persistent. The basis of the delayed stress-induced suppression of hippocampal processing may involve a stress-induced increase in GABAergic transmission in the hippocampus (Trullas et al. [254]; Amitani et al. [295]), in addition to an activity-induced desensitization of NMDA receptors (discussed above).

The water maze and fear conditioning findings described here are potentially relevant towards understanding the physiological basis of flashbulb memories. The relatively brief period in which the hippocampus would be activated by stress would be a sufficient time to initiate NMDA, and perhaps non-NMDA, receptor-mediated plasticity (Joëls et al. [258]; Krugers et al. [155]; Wiegert et al. [231]; Morsink et al. [232]), which would induce the hippocampus to store information about the arousing experience. However, when the hippocampus is briefly in this global activational state, its mechanisms involved in memory storage are promiscuous, storing information not only about the arousing stimulus (the "to-be-remembered" (TBR) event; Christianson [29]), but also about temporally contiguous information unrelated to the TBR event. The end product would be an emotional memory which would be a montage of significant and insignificant events that co-occurred in time. In terms of flashbulb memory processing, the activation of the hippocampus by an arousing event would initiate the storage of the memory of a TBR event, such as the televised images of planes crashing into the World Trade Center on September 11, 2001, as well as coincident information, such as where people were and what they were doing, as they learned of the crisis.

Additional empirical support for the temporal dynamics model is derived from the "warning signal" hypothesis by Ehlers et al. [198]. These investigators noted that intrusive memories in PTSD patients were typically composed of the remembrance of stimuli that were present immediately before the traumatic event happened or shortly before the moments that had the largest emotional impact. They suggested that intrusive memories are not random sensory fragments of the traumatic experience. Instead, they noted that intrusive memories "can be understood as stimuli that—through temporal association with the traumatic event—acquired the status of warning signals; stimuli that if encountered again would indicate impending danger" (page 999). Our temporal dynamics model extends their "warning signal" hypothesis to a physiological level, as we propose that it is the abnormally intense and time-restricted activation of the hippocampus in phase 1 that can produce a powerful association between coincident neutral and traumatic stimuli which is commonly described as "burnt into memory" (Elbert and Schauer [296]).

Other findings from our group are consistent with the idea that within 30 minutes after the onset of phase 1, the hippocampus undergoes a prolonged period in which the induction of new plasticity or the formation of new memories is impaired. First, we have shown that 30 minutes of cat exposure not only impaired spatial memory (Kim and Diamond [1]; Diamond et al. [10]; Diamond et al. [2]; Diamond et al. [3]), it also suppressed molecular (Sandi et al. [136]) and structural (Diamond et al. [137]) measures of plasticity in the hippocampus. Specifically, 30 minutes of cat exposure impaired spatial memory and dramatically reduced hippocampal levels of neural cell adhesion molecules (NCAMs) (Sandi et al. [136]), which are important structural components of long-term memory storage (Sandi [297]). Second, we have found that 30 minutes of pretraining cat exposure suppressed the learning-induced increase in dendritic spine density in CA1 (Diamond et al. [137]). Overall, these findings, in conjunction with related work by Kim et al. [154] support our hypothesis that a strong stressor generates a powerful inhibitory influence on hippocampal memory processing for events occurring 30–60 minutes after the onset of a stressful experience.

9. WHAT IS THE BENEFIT OF SUPPRESSING THE INDUCTION OF HIPPOCAMPAL PLASTICITY IN PHASE 2?

Why does the hippocampus undergo a prolonged phase of inhibition of the induction of synaptic plasticity following the activational phase? We can suggest three benefits of the phase 2 state of inhibition. First, if a stress-induced increase in hippocampal activation, with its increase in glutamate levels and enhanced calcium influx, were to continue unabated, hippocampal neurons would be at an increased risk for glutamate-induced neurotoxicity (Sapolsky [298]; Slemmer et al. [299]; Petrović et al. [300]). The decrease in the sensitivity of NMDA receptors during phase 2 would reduce calcium influx, thereby protecting hippocampal neurons from developing excitotoxicity in times of strong and persistent stress (Moudy et al. [301]; Moulder et al. [302]).

A second explanation for why the desensitization and rundown of NMDA receptors occur during phase 2 is that it serves a "memory protective" function. In theory, the activation (phase 1) followed by inhibition (phase 2) of hippocampal plasticity would produce a relatively brief period,

an isolated fragment of time, when the formation of the memories of events occurring at the onset of an emotional experience would be optimized, thereby enhancing the association between otherwise neutral cues with the onset of a traumatic experience (Ehlers et al. [198]). Thus, a primary component of the neurobiology of flashbulb memories is the brief activation of neuroplasticity in the hippocampus while it is in the phase 1 state. The subsequent suppression of the induction of new plasticity from being generated in phase 2 would reduce, but perhaps not completely block, the corruption of the memory of phase 1 events by later occurring events (Laney and Loftus [93]; Loftus [303]).

Third, processes initiated during phase 1 and then active in phase 2, such as the corticosterone-mediated activation of the GR receptor, genomically mediated events, and protein synthesis, would underlie the first phase of the consolidation of the emotional memory. As hippocampal neurons proceed through the molecular sequence of events leading to structural plasticity underlying the storage of the memory of the emotional event, it would be prudent for the storage process to occur without being contaminated by the processing of new information. Therefore, as the hippocampus descends into phase 2, it goes partially "offline" for a period of hours as the hippocampus begins to consolidate information acquired during phase 1.

10. A PLACE FOR THE TEMPORAL DYNAMICS MODEL IN THEORIES OF HIPPOCAMPAL FUNCTIONING

Our temporal dynamics model suggests that qualitative features of hippocampal memory processing in response to stress should be different from the type of memory processing which is normally attributed to the hippocampus. That is, over the past few decades, investigators have developed the view that the hippocampus plays a role in binding together the elements of an experience to generate a "cognitive map" (O'Keefe and Nadel [278]), or a "conjunctive" (Sutherland et al. [304]; Rudy and O'Reilly [305]; O'Reilly and Rudy [306]) and flexible (Cohen and Eichenbaum [307]) representation of a learning experience. Extensive research supports these theories, indicating that the hippocampus enables the formation of "complex, bound representations of episodes replete with spatiotemporal and contextual details" (Metcalfe and Jacobs [61, page 187]). Thus, the different theories on the role of the hippocampus in memory processing have in common the idea that the hippocampus generates a higher-order representation of the contextual components of a learning experience (Teyler and DiScenna [308]; Eichenbaum [309]; O'Reilly and Rudy [306]; Brassen et al. [310]).

The extensive evidence of a stress-induced impairment of LTP and spatial memory provided strong support for the view that stress suppresses hippocampal functioning. But we suggest that another reason why the hippocampus was considered to be dysfunctional in times of emotional trauma is not only because of the stress-LTP work, but because the characteristics of traumatic memories did not conform to the well-accepted view that the hippocampus generates memories which contain a higher-order (cognitive map/conjunctive) representation of the learning context. Traumatic memories have been described as disembodied fragments of the original experience only weakly connected with contextual details (van der Kolk [176]; van der Kolk and Fisler [311]; van der Kolk [170]; van der Kolk [312]; Ehlers et al. [198]; Hackmann et al. [313]; van der Kolk [314]), which is inconsistent with the cognitive map/conjunctive view of the hippocampal representation of a learning experience. This perspective is illustrated by the following perspective by van der Kolk [170] on why the hippocampus is impaired in times of trauma:

"very high levels of emotional arousal may prevent the proper evaluation and categorization of experience by interfering with hippocampal function. One can hypothesize that when this occurs, sensory inprints of experience are stored in memory; however, because the hippocampus is prevented from fulfilling its integrative function, these various inprints of experience are not organized into a unified whole. The experience is laid down, and later retrieved, as isolated images, bodily sensations, smells, and sounds that feel alien and separate from other life experiences. Because the hippocampus has not played its usual role in helping to localize the incoming information in time and space, these fragments continue to lead an isolated existence" (page 295).

Our temporal dynamics model provides a different perspective from van der Kolk's on the possible involvement of the hippocampus in emotional and traumatic memory processing. The model proposes that in times of emotional trauma, the memory storage reportoire of the hippocampus rapidly shifts from its normative cognitive map mode to a flashbulb memory mode, which processes time-restricted, contextually disembodied, fragments of the details of emotional experiences. We hypothesize that the great enhancement and durability of memory for the details of arousing experiences is produced in part by the rapid induction of neuroplasticity in the hippocampus in phase 1 (Figure 3), mediated by arousal-related afferents, including the amygdala (Abe [18]; Roozendaal et al. [315]; Abe et al. [316]; Richter-Levin [20]; McGaugh [88]; Akirav and Richter-Levin [22]), hypothalamus (Nakanishi et al. [216]), ventral tegmental area (Ovsepian et al. [248]; Lisman and Grace [224]), and locus coeruleus (Sara and Devauges [317]; Harley and Sara [217]; Kitchigina et al. [219]).

We would also speculate that in the days, weeks, and even years after a traumatic event occurs, with repeated rehearsals of the experience, a person's hippocampus may attempt to reconstruct a more contextually rich representation of the original emotional experience (Foa et al. [318]; Diamond et al. [17]). The reconstructed memory would therefore be a hybrid representation of information processed by the hippocampus (and amygdala) in a fragmented manner at the time of the experience, in conjunction with postevent reconstructions of the memory. The repeated reconstruction, as well as reconsolidation (Przybyslawski and Sara [319]; Nader et al. [320]; Duvarci and Nader [321]), of the representation of the original experience by the hippocampus could produce a hypermnesic (strengthening) of the memory of

the traumatic experience (Scrivner and Safer [322]; Klein et al. [323]; Bornstein et al. [324]; Kern et al. [325]). However, repeatedly reconsolidating the memory could render it susceptible to modification, and potentially reduce its veracity (Foa et al. [318]; Garry et al. [326]; Christianson and Lindholm [327]; Wright and Loftus [328]; Loftus [303]).

Despite the well-described evidence of the modifiability of flashbulb memories, it appears that information acquired during phase 1, which is when there would be the most intense activation of hippocampal and amygdala neuroplasticity, is highly resistant to develop reconstructive errors over time (van der Kolk et al. [329]; van der Kolk [176]; Koss et al. [330]). As noted by van der Kolk [170], "aspects of traumatic events appear to become fixed in the mind, unaltered by the passage of time or by the intervention of subsequent experience" (page 282). Thus, the "warning signal" hypothesis of Ehlers et al. [198], which emphasizes that traumatic memories commonly include events that had occurred at the onset of the traumatic experience, and the resistance of traumatic memories to corruption by later occurring events, both indicate that phase 1 of our temporal dynamics model is a period of highly efficient hippocampal processing. When the hippocampus is driven into phase 1 by strong emotionality, its focusing on events associated with emotional experiences, referred to as "tunnel memory" by Safer et al. [104] results in powerful memories of isolated sensory experiences which are extremely resistant to degradation over time. We would suggest that it is the memory for events occurring during phase 2 (Figure 3) and for events occurring outside of the focus of attention during the emotional experience that are more susceptible to corruption over time than events which were the focus of attention during phase 1 (Christianson [29]).

In summary, we have proposed that the initiation of a stressful experience produces an intense, but brief, activation of memory-encoding plasticity within the hippocampus. This process would involve a shift by the hippocampus from its normative cognitive mapping mode to a "print-now" (Brown and Kulik [37]) flashbulb memory mode. Within minutes after being activated by the emotional experience, the hippocampus would descend into the phase 2 state, which would involve an increase in the threshold for the induction of new plasticity. It is during the phase 2 state that the hippocampus would exhibit an impairment in the induction of LTP, and therefore, be impaired at storing the memory of events that occur during phase 2. Long after the termination of the emotional experience, the hippocampus would slowly return to its cognitive mapping mode and it would attempt to generate a contextually rich representation of the experience. With the hippocampus in this reconstructive phase, post-trauma experiences and ideations may become "spliced" into memories of the original events. In this manner, information stored around the time of the emotional experience may become incorporated into a more complete, but possibly corrupted, representation of the original experience (Neisser and Harsch [331]; Neisser [332]).

11. FLASHBULB MEMORIES AND THE STRESS-INDUCED MODULATION OF LTP IN THE AMYGDALA

It is well known that the amygdala is a critical component of emotional learning and memory. This topic has been reviewed extensively by others (LeDoux [333]; McIntyre et al. [334]; Fanselow and Gale [335]; McGaugh [88]; Dityatev and Bolshakov [336]; Maren [337]; Kim and Jung [21]; Sigurdsson et al. [338]) and will not be discussed at length here. The primary issue we are concerned with is how an emotional experience affects endogenous mechanisms of plasticity, as well as electrical stimulation-induced LTP, in the amygdala. An early study that addressed this issue was the work by Rogan et al. [339]. These investigators demonstrated that fear conditioning produced an enhancement of CS-evoked activity in the amygdala. Comparable results were reported by McKernan and Shinnick-Gallagher [340], who showed that fear conditioning produced a presynaptic facilitation of AMPA-receptor-mediated transmission, in vitro. In both studies, the increases in intrinsic excitability in the amygdala produced by fear conditioning were specific to associative processes, as shock, alone, did not produce a change in excitability. These studies, as well as subsequent work from this group (Schroeder and Shinnick-Gallagher [341]) and studies by Adamec et al. employing naturalistic (predator) stress (Adamec et al. [342]; Adamec et al. [288]; Rosen et al. [343]), all indicate that fear conditioning produces long-lasting increases in excitability in the amygdala.

As we noted in an earlier section, whether or not tetanizing stimulation induces LTP can be viewed as a "diagnostic" measure of the functioning of a brain structure. How does stress or fear conditioning affect exogenously induced LTP in the amygdala? Our group, in conjunction with Richter-Levin's group, examined this issue in recordings from the basal amygdala of behaving rats (Vouimba et al. [344]). We showed that stress exerted different effects on LTP in the DG versus the basal amygdala in response to stimulation of the entorhinal cortex. In general, stress either had no effect or suppressed LTP in the DG, and enhanced LTP in the basal amygdala. In more recent work, our group has shown that predator stress blocked PB potentiation in CA1 and enhanced LTP in the basolateral nucleus of the amygdala (Vouimba et al. [140]). These studies suggest that when the hippocampus passes into the phase 2 (inhibitory) period, the amygdala continues to exhibit a stress-induced enhancement of plasticity (Figure 3).

The finding of an enhancement of LTP in the amygdala under stress conditions is consistent with the well-established role this structure serves in emotional memory. There are, however, accounts in which amygdala LTP has been suppressed in response to emotional learning conditions. For example, Tsvetkov et al. [345] found that 3 days of fear conditioning resulted in a profound suppression of LTP in the cortico-amygdala circuit, and Schroeder and Shinnick-Gallagher [341] found a suppression of amygdala LTP 10 days after fear conditioning. Comparable findings were reported recently by Kavushanky et al. [346], who showed

that rats given water maze training exhibited a reduction in the magnitude of LTP in the basal amygdala in response to tetanizing stimulation of the EC. The findings of an emotional learning-induced suppression of LTP in the amygdala suggest that this structure, as with the hippocampus, has an initial activational phase of processing, followed by a slowly developing inhibitory phase. The amygdala appears to remain in phase 1 longer than the hippocampus, but eventually, the phase 2 (inhibitory) period develops, perhaps while the amygdala is involved in the consolidation of the emotional memory (Izquierdo and Medina [347]; Pelletier and Paré [348]; McGaugh [88]).

We should emphasize that the amygdala excitability curve in Figure 3 serves only to illustrate our idea that the amygdala, as with the hippocampus, appears to undergo activational and inhibitory phases which may be involved in the consolidation of emotional memories. The actual shapes of perhaps multiple plasticity-shift curves in different amygdala nuclei would reflect interactions between activational and inhibitory influences in response to an emotional experience. Despite these caveats, our model is potentially useful in providing insight into the neurobiology of emotional, in particular flashbulb and traumatic, memories. For example, because the model indicates that the amygdala and hippocampus each develops endogenous plasticity independently with the onset of a stressful learning experience, there should be distinguishable hippocampal versus amygdaloid components of flashbulb memories. This feature of the model is consistent with almost a century of observations of people with organic, as well as emotion-induced, memory disorders. One example is a well-known case study of an amnesic patient, presumably with hippocampal damage, studied by Claparède [349]. He conducted an experiment in which he shook the patient's hand, and at the same time, stuck her with a pin which was hidden between his fingers. The patient, some time later, exhibited a reluctance to shake his hand, but she did not have a specific recollection of the handshake/pin prick incident (translated to English in Claparède [349]). Similarly, Bechara et al. [350] reported that a patient with bilateral damage to the hippocampus failed to make a CS-US association at a cognitive (explicit) level, but did develop a subconscious CS-US association. Conversely, another patient with damage to the amygdala given fear conditioning failed to develop a conditioned emotional response, but did learn the factual (explicit) information about the CS-US contingency. Finally, a patient with bilateral damage to the hippocampus and amygdala failed to acquire either the explicit details or a conditioned emotional response. These cases are only a subset of a substantial literature consistent with the idea that the hippocampus and amygdala process different features of emotional memories (Phillips and LeDoux [351]; LeDoux [352]; Bechara et al. [350]; Fanselow [353]; Sanders et al. [183]; Bechara et al. [354]).

One other case is particularly instructive towards understanding how the amygdala and hippocampus process different components of emotional (traumatic) memories, with potential relevance towards understanding the etiology of post-traumatic stress disorder (PTSD). Krikorian and Layton [355] reported on a case of a healthy adult man who was rendered anoxic for approximately 15 minutes when he was suddenly buried under 5.5 meters of sand. In the weeks following his recovery, he exhibited a change of personality, which was presented largely as persistent cognitive impairments and symptoms of PTSD. He spent his days with a near- constant fear of imminent death and intrusive thoughts that the earth would open up and swallow him, and his nights were consumed with nightmares about being buried alive. Despite these powerful PTSD-like symptoms which could be directly tied to his traumatic experience, he had no recollection of the actual event.

We suggest that the initiation of the burying incident triggered a powerful activation of neuroplasticity simultaneously in his hippocampus and amygdala. The independent induction of plasticity in each of these two structures would normally function to form a flashbulb memory which would contain two components: (1) the explicit, hippocampus-dependent, information about the specific details of the experience; (2) more global, conscious, and subconscious, amygdala-dependent components which would generate the fear-provoking features of the memory. However, because the man remained in an anoxic state for so long, it is likely that he developed damage to his hippocampus (Zola-Morgan et al. [356]; Squire and Zola [357]; Rempel-Clower et al. [358]), which interfered with the consolidation of the explicit component of the memory of his traumatic experience. The cognitive deficits this patient exhibited post-trauma are consistent with our assumption that he developed hippocampal damage as a result of his anoxia. We would speculate that global and fear-provoking information about the experience was stored primarily by amygdala-centered memory processing, thereby underlying his general fear of being buried and his PTSD symptomology. This postulated role of the amygdala in the gist, rather than the details, of an emotional experience is consistent with recent findings (Adolphs et al. [359]; Cahill and van Stegeren [360]) and discussion (Phelps [361]) of the differential roles of the hippocampal versus amygdala in emotional memory processing.

In summary, findings from amnesics, in conjunction with observations of people with emotional trauma-induced amnesia, support our hypothesis that the hippocampus and amygdala both develop neuroplasticity in the seconds to minutes after the initiation of a traumatic experience. The engram of the resultant flashbulb memory is therefore a montage of hippocampal and amygdala representations of the experience.

12. STRESS TAKES THE PREFRONTAL CORTEX "OFFLINE"

In 1898, Overton [362] proposed that "Thinking is done by the cells of the brain behind the forehead ... if the forehead cells do not know how to think, the mind cannot make use of memories. We say that such a person is a fool, even though he has great knowledge."

A century later, Arnsten [363] stated that "stress impairs prefrontal cortex function through catecholamine receptor mechanisms … dopamine and norepinephrine synergize to take the prefrontal cortex "off-line" during stress."

The functioning of the PFC, and its susceptibility to be disrupted by stress, is aptly summarized by the two statements above by Overton [362] and Arnsten [363]. "Thinking," or higher-order cognitive functioning, is dependent to a great extent on the integrity of the PFC. Extensive research and recent imaging studies have shown that the PFC is critically involved in guiding behavior during divided attention (Nebel et al. [364]; Dannhauser et al. [365]) and working memory (Goldman-Rakic [366]; Adcock et al. [367]; Taylor et al. [368]; Marshuetz and Smith [369]; Müller and Knight [370]; Curtis [371]) tasks, as well as in planning (Rowe et al. [372]; Anderson et al. [373]) and decision making (Bechara [374]; Bechara [375]), which may be broadly referred to as "executive processes" (Baddeley and Della Sala[376]; McEwen [377]). In addition, the frontal cortex, in general, is an important component of brain circuitry involved in the extinction of conditioned responses (Maren and Quirk [378]; Likhtik et al. [379]; Milad et al. [380]; Milad et al. [381]), behavioral inhibition (Tillfors [382]; Levy [383]), and coping with controllable stressors (Ter Horst [384]; Gerrits et al. [385]; Rangel et al. [386]; Bland et al. [387]; Amat et al. [388]), as well as in interacting with the temporal lobe to faciltate memory formation and retrieval (Buckner and Wheeler [389]). Therefore, Overton's statement about cells at the front of the brain being involved in "thinking" is accurate in the sense that the PFC (and other frontal and parietal regions) is important for higher-order attentional and cognitive processes which enable an individual to use information and memory effectively. Foolish behavior, such as poor decision making, is well known to occur when frontal cortex functioning is impaired as a result of damage (Bechara et al. [390]; Bechara [374]; Bechara [375]) or acute stress (Arnsten and Goldman-Rakic [391]; Arnsten [392]; Arnsten [393]; Gray [394]; Morrow et al. [395]; Arnsten [396]; Moghaddam [397]; Birnbaum et al. [398]; Moghaddam and Jackson [399]; Goudriaan et al. [400]).

With regards to LTP work, we are aware of only two studies that have investigated how acute stress affects LTP in the PFC. Maroun and Richter-Levin [401] showed that electrical stimulation of the amygdala produced LTP in the PFC. These researchers demonstrated that the same stress that blocked LTP in CA1 (placement of rats on an elevated platform) also blocked LTP in the PFC. Similarly, Rocher et al. [402] demonstrated that LTP in the PFC produced by stimulation of the ventral hippocampus was blocked by elevated platform stress.

The inhibition of LTP in the PFC by stress, acting in large part, through excessive activation of dopamine (D1) receptors, supports the idea that PFC functioning, in general, including its capacity to maximize decision making, multitasking, and divided attention, is impaired by stress (discussed above). Therefore, we have illustrated a rapid and prolonged inhibitory shift in functional excitability in the PFC in our model of stress-LTP dynamics (Figure 3). This inhibitory phase of PFC functioning would be revealed electrophysiologically as a suppression of LTP, and behaviorally as an impairment of coping skills, executive functioning, multitasking, decision making, and a reduced ability to perform well in complex tasks.

The length of time it would take for the stress-induced inhibition of PFC functioning to recover fully to baseline would depend on the nature and intensity of the stressor, interacting with environmental and genetic factors, as well as with individual variability in coping effectively with the stressor (Yehuda [403]; Olff et al. [404]; Nemeroff et al. [102]). In extreme cases, individuals who develop PTSD in response to experiencing a traumatic event may be unable to recover fully to their original baseline (Figure 3). The ongoing impairment of PFC functioning would result in a chronic reduction in descending inhibitory influences from the PFC on brainstem nuclei and the amygdala (Williams et al. [405]), which could form the basis of certain symptoms of PTSD, such as chronic hypervigilance, attention deficits, and impaired executive functioning (Vermetten and Bremner [406]; Shin et al. [407]; Britton et al. [408]; Shin et al. [409]; Williams et al. [405]).

13. STRESS EFFECTS ON THE PFC, HIPPOCAMPUS, AMYGDALA, AND THE YERKES-DODSON LAW

The relationship between stress effects on the PFC, hippocampus, amygdale, and the Yerkes-Dodson law has been alluded to throughout this paper. For example, we have emphasized how the PFC (and related frontal areas) is involved in complex tasks that require working memory, executive processing, decision making, and divided attention. Therefore, the extent to which the PFC is involved in a task and the degree to which the PFC is suppressed by emotionality are primary determinants of whether a task's arousal-performance curve will be linear or curvilinear. That is, if the successful completion of a task requires PFC functioning, then performance on that task is likely to suffer under conditions of high arousal. One example of an application of this strategy is the finding that high states of anxiety have little to no effect on performance in simple, single-digit, mental calculations, which place minimal demands on PFC-based working memory capacity. Ashcraft [410] has shown that when people perform more complex mental calculations, such as double-digit calculations, which tax working memory and thereby increase PFC involvement in the task, they are more susceptible to be impaired by anxiety. It is notable that even single-digit calculations could be made susceptible to impairments by anxiety when a PFC-dependent component, decision-making, was included in the calculations (Ashcraft [410]). Therefore, one strategy with which to operationalize the distinction between "simple" and "complex" tasks is to determine whether the task involves a PFC-mediated component. We would suggest that, as a general rule, tasks that require the involvement of the PFC, which can be confirmed to some degree by neuroimaging techniques (Callicott et al. [411]; Ranganath et al. [412]; Taylor

et al. [368]; Ranganath and D'Esposito [413]; Curtis [371]), should all exhibit the curvilinear component of the Yerkes-Dodson law.

The mechanistic basis of the PFC-mediated curvilinear component of the Yerkes-Dodson law is well studied. A number of researchers have commented on the inverted-U-shaped relationship between dopamine receptor signaling in the prefrontal cortex and working memory performance (Arnsten et al. [414]; Murphy et al. [415]; Cai and Arnsten [416]; Arnsten [363]; Arnsten [417]; Brunel and Wang [418]; Dreher et al. [419]; Yamashita and Tanaka [420]; Williams and Castner [421]; Tanaka et al. [422]). The common finding among these studies is the importance of an intermediate, that is, optimal, level of dopaminergic (D1) receptor activation to enable working memory tasks to be accomplished. Stress, pharmacological treatments, or mental disease states (Russell [423]; Levy [383]; Jay et al. [9]; Anderson et al. [424]) that involve either an excessive increase or decrease in dopaminergic activity result in an impairment in working memory performance (Arnsten [363]; Williams and Castner [421]).

An inverted-U function has also been described for the relationship between locus coeruleus (LC) activity and performance in an attentional task (Aston-Jones et al. [62]; Aston-Jones et al. [64]). In the work by Aston-Jones' group, behavioral performance was impaired in animals with high levels of LC activity, perhaps because the task required sustained attention with distracting stimuli. Overall, there is strong support for the idea that intermediate levels of norepinephrine and dopamine in the PFC are an important component of efficient performance on complex tasks (Arnsten [363]; Williams and Castner [421]).

The second component of the Yerkes-Dodson law is the enhancement of performance under high levels of stress in relatively simple tasks (Figure 2(b)). If, for example, a task involves focused attention to an isolated cue with minimal cognitive (decision-making) demands, then performance may not only be unimpaired, it can even be enhanced, under conditions of high arousal. The well-described "weapon-focus" phenomenon, as well as fear conditioning in rats, illustrates a situation that involves an almost complete absence of decision making, multitasking, and peripheral attention (Christianson [29]; Conway et al. [425]; Safer et al. [104]; Pickel [105]). In threatening situations, there may be a great enhancement of memory for the sole focus of attention, such as the weapon that threatened someone's life, with perhaps impaired memory for other cues on the periphery of a person's attention (Christianson [29]; Safer et al. [104]; Pickel [105]). This shift in focus from thoughtful decision making to one of highly focused attention with rapid processing has clear adaptive value, enabling an individual to devote attentional resources (and maximal hippocampal and amygdaloid memory processing) to life-threatening stimuli in times of danger (Mineka and Öhman [426]; Flykt [427]).

As a first step in understanding how emotion enhances learning in simple tasks, consider the repercussions of the suppression of the PFC by strong emotionality. Descending projections from the PFC appear to provide an inhibitory influence over lower brain structures involved in emotionality, such as the amygdala, dorsal raphe and hypothalamus (Arnsten and Goldman-Rakic [428]; Sesack and Pickel [429]; Rempel-Clower and Barbas [430]; Hajós et al. [431]; Quirk and Gehlert [432]; Quirk et al. [433]; Milad et al. [434]; Likhtik et al. [379]; Amat et al. [388]). A consequence of the loss of PFC-mediated inhibition is that these structures will exhibit greater activation in times of strong emotionality, thereby enhancing their throughput. For example, the release of PFC-mediated inhibition over locus coeruleus cell activity will increase norepinephrine release throughout the forebrain, which would be manifested behaviorally as an enhancement of attention, and physiologically as enhanced memory-related neuroplasticity in the amygdala and hippocampus (Izquierdo and Medina [435]; Roozendaal [436]; McGaugh [437]; Strange and Dolan [222]; Hurlemann et al. [223]; Bremner [100]). Indeed, we would speculate that it is the release of PFC inhibition over brain stem and amygdala activity which would enable the great enhancement and focusing of attention towards threatening cues (Berridge et al. [438]).

Finally, errors in emotional memory processing are not attributable solely to an impairment of PFC function. Flaws in emotional memories have been a subject of extensive research, which has great relevance in clinical and legal settings, involving issues including, for example, the credibility of repressed memories (Loftus [439]; Loftus and Polage [440]) and eyewitness testimony (Loftus [441]; Sparr and Bremner [442]). Elsewhere, we have commented on the functional consequences of how acute stress appears to simultaneously enhance plasticity in the amygdala and impair plasticity in the hippocampus (Vouimba et al. [140]). One potential repercussion of the opposing effects of stress on these two structures is that in times of strong emotionality, amygdala plasticity is enhanced, thereby intensifying the emotional memory of an experience. However, if the enhancement of the amygdala processing occurs at a time when the hippocampus is in the stress-induced inhibitory period (Figure 3, phase 2), then the stress-induced impairment of hippocampal functioning could compromise the accuracy of the details of the emotional memory, despite an individual's great confidence in its veracity (Talarico and Rubin [443]; Wolters and Goudsmit [444]; Coluccia et al. [445]). Therefore, in addition to the reduced involvement of the PFC in controlling cognition in times of strong emotionality, reduced functioning of the hippocampus while it is in the phase 2 state, as well, contributes to the impairment of performance at the right side of the curvilinear component of the Yerkes-Dodson law.

In conclusion, a century after the passage of the Yerkes-Dodson law and almost 50 years after the publication of Easterbrook's cue utilization hypothesis, cognitive psychology and behavioral neuroscience research have provided an in-depth perspective on the neurobiological basis of how emotion interacts with memory formation. We have applied this research to develop a synthesis which addresses the linear and curvilinear components of the Yerkes-Dodson law. We have proposed that the enhancement of memory under

high stress conditions is subserved by the rapid and coordinated activation of hippocampal-amygdaloid circuitry, in conjunction with a suppression of the PFC. The emotional-induced enhancement of hippocampal and amygdaloid processing favors rapid processing of distinct cues with minimal demands on decision making, which is typified by phenomena such as weapon focus and flashbulb memories in people and fear conditioning in rats. We have also suggested that the high (declining) end of the curvilinear component of the Yerkes-Dodson law is generated largely by a stress-induced suppression of PFC functioning (see also Kensinger and Corkin [446] for related discussion). Our model predicts, therefore, that performance on all tasks that require the involvement of the PFC would suffer at times of strong emotionality. However, a complete understanding of the neurobiological basis of the curvilinear versus linear components of the Yerkes-Dodson law will require additional investigation of how stress rapidly enhances, and then suppresses, hippocampal functioning.

14. SUMMARY

In this synthesis, we have presented our perspective on the neurobiological basis of the stress-induced enhancement and impairment of memory. First, we have asserted that the view, developed in the 1950s, that imposed a monolithic curvilinear shape on all performance-emotion interactions led to decades of debates which inappropriately called for the repeal of the Yerkes-Dodson law. We have discussed how the original version of the Yerkes-Dodson law took into account the interaction of task difficulty with arousal level to address how strong motivation can either enhance or impair performance. We recognize, however, that one problem with the Yerkes-Dodson law is that it invokes an ill-defined distinction between "simple" versus "complex" tasks. We have suggested that identifying the involvement of the PFC in a task, which can be confirmed to some degree by neuroimaging analysis, may provide a general guideline for predicting whether performance on a task in times of strong emotionality will express a linear versus nonlinear shape.

Our neurobiological model of stress-memory interactions addresses the complex, and seemingly conflicting, findings of how stress affects hippocampal LTP, and therefore, how hippocampus-dependent memory is affected by strong emotionality. We have suggested that a rapprochement can be accomplished by examining the timing between an emotional experience and a test of hippocampal functioning, as measured by hippocampus-dependent learning or LTP induction. If the two coincide in time, then hippocampal functioning would be enhanced, but if there is a substantial delay between the stress onset and either hippocampus-dependent learning or tetanizing stimulation, then measures of hippocampal functioning (memory consolidation or LTP) would be impaired. We have substantiated this model with our finding that spatial memory was enhanced when stress and spatial learning occurred in close temporal proximity, but when there was a delay between stress and learning, memory consolidation was impaired. We have also suggested that strong emotionality changes the hippocampus from a "cognitive map" mode of memory processing to a "flashbulb memory" mode, which enables the hippocampus to store disembodied fragments of an experience which lack the depth of processing of context normally attributed to hippocampal memory encoding. Overall, our model of how the hippocampus, amygdala, and PFC are differentially affected by strong emotionality provides a framework for further advancements in our understanding of the neurobiology of traumatic memory processing.

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Review Article

Stress and Memory: Behavioral Effects and Neurobiological Mechanisms

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Stress is a potent modulator of learning and memory processes. Although there have been a few attempts in the literature to explain the diversity of effects (including facilitating, impairing, and lack of effects) described for the impact of stress on memory function according to single classification criterion, they have proved insufficient to explain the whole complexity of effects. Here, we review the literature in the field of stress and memory interactions according to five selected classifying factors (source of stress, stressor duration, stressor intensity, stressor timing with regard to memory phase, and learning type) in an attempt to develop an integrative model to understand how stress affects memory function. Summarizing on those conditions in which there was enough information, we conclude that high stress levels, whether intrinsic (triggered by the cognitive challenge) or extrinsic (induced by conditions completely unrelated to the cognitive task), tend to facilitate Pavlovian conditioning (in a linear-asymptotic manner), while being deleterious for spatial/explicit information processing (which with regard to intrinsic stress levels follows an inverted U-shape effect). Moreover, after reviewing the literature, we conclude that all selected factors are essential to develop an integrative model that defines the outcome of stress effects in memory processes. In parallel, we provide a brief review of the main neurobiological mechanisms proposed to account for the different effects of stress in memory function. Glucocorticoids were found as a common mediating mechanism for both the facilitating and impairing actions of stress in different memory processes and phases. Among the brain regions implicated, the hippocampus, amygdala, and prefrontal cortex were highlighted as critical for the mediation of stress effects.

1. INTRODUCTION

Nowadays, there is great consensus in the literature that stress is a potent modulator of cognitive function in general, and more precisely, of learning and memory processes McEwen and Sapolsky [1]; de Kloet et al. [2]; Lupien and Lepage [3]; Sandi [4, 5]; Diamond et al. [6]; Fuchs et al. [7]; Joëls et al. [8]; Shors [9]. Although stress effects are frequently regarded as deleterious to cognitive function, very intensive work during the past decade is delineating a great complexity, both in the nature of interactions between stress and memory functions and in their outcome. In addition to the overemphasized negative side of stress on brain and behavior, there are many instances in which neural function and cognition are either facilitated by stress (de Kloet et al. [2]; Joëls et al. [8]), or even not affected (Warren et al. [10]; Beylin and Shors [11]).

There have been several successful attempts to make sense of the confusion in the literature. By focusing on specific explanatory factors, different authors have successfully provided integrative and clarifying views of the impact of stress on memory function. For example, a great deal of the variability can be explained by the "intensity" of the stressor, either if the dosage reflects its physical characteristics (Cordero et al. [12]) or internal hormonal reactions (Baldi and Bucherelli [13]; Conrad [14]; Joëls [15]). The most general view is that stress—or stress hormones—levels induce inverted U-shaped dose effects in learning, memory, and plasticity (Baldi and Bucherelli [13]; Conrad [14]; Joëls [15]), although linear effects have also been proposed (Diamond

[16]). A second important factor that has been emphasized is stress "duration," with distinct effects frequently induced by *single* versus *repetitive*—or chronic- stress—or stress hormones activation-, and not only at the cognitive level, but also when evaluating brain structure and function (Sandi and Loscertales [17]; Pinnock and Herbert [18]; Pecoraro et al. [19]; Joëls et al. [8]). A third important factor that has been particularly highlighted by Roozendaal [20, 21] as relevant in this context is the memory phase at which stress acts. After reviewing the literature, Roozendaal [20, 21] has proposed opposing effects for stress—and stress hormones activation—during the phases of *consolidation* (generally facilitating) and *retrieval* (generally impairing) of information. A fourth factor that should be mentioned is psychological factors, notably stressor *controllability* and *predictability* that are well known to be key mediators of the psychophysiological impact of stress (Mineka and Hendersen [22]; Das et al. [23]). Convergent evidence indicates that experiencing uncontrollable—as opposed to controllable—stress has deleterious effects on further information processing (Maier and Watkins [24]). A fifth factor that seems to count for the outcome of stress in memory function is the importance of taking into account the existence of individual differences when trying to make sense of the literature on stress and memory, with *gender* appearing as a very highly important modulator of such interactions (Luine [25]; Bowman et al. [26]; Shors [27]). Finally, a sixth factor that has been identified as certainly relevant to understand how stress affects cognition is the relevance of the context in which stress—or stress hormones activation—is experienced, that is, whether stress is, or is not, *contingent* to the particular information processing under study (Sandi [28]; de Kloet et al. [2]; Joëls et al. [8]).

Despite the usefulness of the above-mentioned factors, a systematic view that integrates all the complexity (or at least much of it) of the apparently discrepant actions of stress in cognition is still lacking. Although not so ambitious as to try to develop a comprehensive model including all the factors highlighted above, our goal here is to come up with an integrative model that incorporates several of them along with new proposed factors. More specifically, our goal is to organize the literature among those selected factors to eventually provide integrative answers to the question: "what does it count for the outcome of stress interaction with memory function"? Finally, we will evaluate whether such integrative effort helps understanding better stress effects on memory function than other more reductionistic approaches already available in the literature. We should also state that the goal of this review is to discuss studies from the literature that help illustrating the mediating influence of the selected factors (see above) to understand the nature of stress actions on memory function. By no means, we attempt to include here an exhaustive account of a large number of studies that have proliferated in recent years. In addition, each subsection includes a brief account of the main neurobiological mechanisms proposed to account for the different effects of stress in memory function.

2. FACTORS SELECTED TO ANALYZE STRESS AND MEMORY INTERACTIONS

We should emphasize that the revision and potential final model will account for the impact of stress in adult male rodents according to the following factors.

(1) Source of stress: we will introduce a new factor, the source of stress, and emphasize its utility to understand the diversity of stress and memory interactions. It makes reference to the origin of stress with regard to the cognitive task. In a way, it is related to the above-mentioned factor *contingency to the contex* (de Kloet et al. [2]; Joëls et al. [8]), but it includes a more explicit nomenclature that hopefully will help clarifying the concept. More precisely, this factor classifies stress as either *intrinsic* (if stress is originated by elements related to the cognitive task) or *extrinsic* (if stress is originated by conditions completely unrelated to the cognitive task, i.e., in the outside world, and ideally occurring temporally dissociated from such task, i.e., either before or afterwards).

(2) Stressor duration: this factor makes reference to the length of stress. The differential effects of *acute* versus *chronic* (with some *subchronic* versions) stress have concentrated great interest in the field. In addition to the relevance to cognitive function, this factor is essential when evaluating the neural mechanisms whereby stress affects cognition.

(3) Stressor intensity: stressors can vary throughout a very wide range of intensities. Even though oversimplifications can have the drawback of being too superficial, for the sake of clarity, we will just use the categories of *low*, *medium*, *high* (and occasionally *very high*) intensities. Not surprisingly, very high (e.g., a clear life threat, such as a being in a combat) and mild (e.g., novelty exposure) stressors seem to have distinct effects on cognitive function (Cordero et al. [12]; Joëls et al. [8]). Importantly, since conspecifics frequently show marked individual differences in stress reactivity (Márquez et al. [29]), measuring individual behavioral and physiological responses to a particular stressor would be the ideal approach when trying to determine the actual stress magnitude experienced by each experimental subject. When such approach is not possible, it is important to be systematic in the gradation of the amount of stressor applied to the different animals, ideally including at least three different intensities.

(4) Stressor timing with regard to memory phase: this factor makes reference to the time when stress is experienced with regard to a particular memory phase. Memory phase stands for the type of the information process that is linked to stress. Generally, three phases are distinguished: acquisition (the learning process), consolidation (memory storage), and retrieval (access to stored information) of information (see Figure 1). As noted above, stress and stress mediators appear to exert opposing effects in consolidation and retrieval (Roozendaal [20, 21]; but see de Kloet et al. [2]; Joëls et al. [8]).

(5) Learning type: an additional key factor is the type of the learning process that is evaluated (i.e., implicit/nondeclarative learning, explicit/declarative learning,

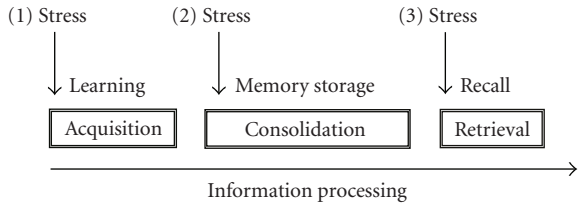


Figure 1: Diagram depicting the relevance of specifying timing of stress with regards to different memory phases. If stress (1) is given before learning (acquisition of information), it can potentially affect all cognitive phases involved in memory function; that is, acquisition, consolidation, and/or retrieval. However, if acquisition is already affected, that would be the main conclusion to extract from the particular experiment. If stress (2) is experienced after learning, any effect observed in retention could now be due to an impact of stress on either consolidation or retrieval, but any effects on acquisition can be discarded. However, effective treatments given at this time point normally disrupt the process of memory storage, instead of retrieval, which can be further tested by given the treatment at later time points (at a different—or outside the—consolidation phase) and assess whether recall is then also affected. If stress (3) is delivered before the recall test, it should just normally affect the retrieval processes. However, a note of caution should be mentioned depending on how close the retention test is applied with regards to training, since consolidation mechanisms are increasingly recognized to last longer than previously hypothesized and, therefore, this type of manipulation could influence both consolidation and retrieval processes. Research on this field should take into account this complexity and apply the necessary controls to ascertain which phase and mechanisms of the information processing is affected by the stress procedure under study.

nonassociative learning, etc.). Although there are different typologies of memory involving a variety of subtypes (Nelson et al. [30]; Squire and Zola [31]; Verfaellie and Keane [32]; Eichenbaum [33]; Moscovitch et al. [34]), this review will focus on a main dichotomy between a type of *implicit* memory processes, Pavlovian conditioning, and spatial types of learning (when reviewing the animal literature) as models for *explicit* memory processes.

Even though we will occasionally mention relevant studies in other species (notably, in humans), this is a review about the rodent literature. Importantly, we will not include as analytic factors two of the probably most important ones among the large list proposed above: (i) psychological factors, like controllability and predictability; (ii) individual differences in the vulnerability and response to stress. Whenever the effect of stress "from outside the context" is applied, we review studies that applied "uncontrollable" stressors and deliberately excluded the few studies that examined the role of "controllable" ones. Concerning the issue of individual differences, we concentrate on the studies performed in adult (but not old) male rodents. We have decided not to tackle here the role of gender, since there are still not enough studies performed in female rodents for each of the factor conditions included in the study. Moreover, we should clarify that we will not deal here with studies in which the impact of stress was evaluated from a developmental point of view, such as for example how pre- or postnatal stress affects cognition in adulthood. Typically, the type of stress whose effects we will examine is stress closely associated with the cognitive challenge under study/discussion, and therefore normally experienced from a few minutes to normally 1-2 days either before or after a particular memory phase.

We have selected the factor "source of stress" as the guiding line to structure this review. We hypothesize that intrinsic stress facilitates learning and memory processes, whereas "extrinsic" stress will normally have the opposite impairing effects. Although differing in some ways, this hypothesis shares some commonalities with the proposal formulated by Joëls et al. [8] stating (page 154):

> *"... that stress will only facilitate learning and memory processes: (i) when stress is experienced in the context and around the time of the event that needs to be remembered, and (ii) when the hormone and transmitters released in response to stress exert their actions on the same circuits as those activated by the situation, that is, when convergence in time and space takes place..."*

In the following pages, relevant studies from the literature will be first classified depending on whether the source of stress is intrinsic or extrinsic to the memory task, and then will be analyzed according to each of the other four factors selected for the analysis (stressor duration, stressor intensity, timing with regard to memory phase, and learning type).

3. THE IMPACT OF ACUTE INTRINSIC STRESS ON MEMORY FUNCTION

As stated above, intrinsic stress makes reference to those situations in which stress is either elicited by, or directly associated with, the cognitive experience. Let us first consider how the factors highlighted above account for intrinsic stress conditions in order to define the whole extent of settings that will be discussed here.

(a) Stressor duration: although intrinsic stress (or stress linked to a cognitive experience) can be experienced both acutely and chronically, to our knowledge, no study to date has systematically studied how chronic activation of stress systems during learning experiences contributes to the different phases involved in memory processes (from learning acquisition to memory consolidation, relearning, reconsolidation, retrieval of information, etc.). Therefore, the evaluation resulting from this review for intrinsic stress will only account for *acute* (not chronic) situations in which a memory is formed from a stressful learning experience.

(b) Stressor intensity: whenever possible, we will consider the whole range of stress intensities: *low*, *medium*, *high*, and occasionally *very high*.

(c) Stressor timing with regard to memory phase: as noted above, to be considered within the category of intrinsic

stress, stress should be linked to a particular cognitive challenge. This could be either a learning challenge or a retrieval challenge. Although several studies have focused on the role of intrinsic stress linked to the learning phase, to our knowledge, no study has systematically studied how stress elicited by the retrieval experience accounts for the effectiveness of the retrieval process. Therefore, the evaluation resulting from this review for intrinsic stress will only account for *learning* (not retrieval) processes. Importantly, stressful learning experiences might affect potentially the *acquisition* and/or *consolidation* of information. We will examine separately both memory phases.

(d) Learning type: as mentioned above, this review focuses in *Pavlovian conditioning* (as representative of implicit learning) and *spatial learning* (as representative of explicit learning). Since there are examples in the literature for both learning types, the discussion here will include and compare the impact of intrinsic stress upon both learning types.

Summarizing, in this subsection, we will evaluate how stress (in a dose-response fashion) triggered by a learning challenge (therefore, an acute condition) affects memory (both implicit and explicit types of memory) function.

Emotionally arousing experiences are better remembered than more neutral ones (Cahill and McGaugh [35]; Sandi [28]; McGaugh [36]). The emotional reaction can range from a mild activation to a strong stress response, and therefore, stress can be regarded as a critical component within the framework of the emotional modulation of memory. The evolutionary advantage of ensuring the future recalling of specific aversive stimuli and/or the successful strategies developed once by the individual to cope with such aversive stimuli is clear. The rapid identification of already experienced dangers, as well as the ability to enhance the speed and accuracy of behavioral reactions to threats, provides the individual with better survival possibilities if faced with similar dangerous circumstances in the future. Predictably, this will, in turn, revert on enhanced reproductive success.

Classically, research attempts addressed to characterize the facilitating effects of stressful learning on memory function have emphasized the role of stress-induced mechanisms on the *consolidation* of the information acquired during such stressful event (Roozendaal [20, 21]). However, enhanced memories resulting from stressful learning situations can also be due, on a first instance, to an effect of stress on the *acquisition* of information. This can be achieved by altering a variety of psychobiological functions (such as attention, motivation, sensory processing and integration, and motor function) that are known to be both sensitive to stress and able to modulate learning processes. Although these latter processes have been less explored in research programs, we will review here the contribution of stress to the spectrum of information encoding including both the storage—consolidation—and acquisition of information.

3.1. Effects of intrinsic stress on the consolidation of information

The effects of arousing or stressful experiences on memory consolidation—as well as the potential mediating mechanisms—have received much attention over the past decades (Sandi [28], Roozendaal [20, 21]; Conrad [14]; McGaugh and Roozendaal [37]; Richter-Levin and Akirav [38]; McGaugh [36]; de Kloet et al. [2]; Joëls et al. [8]).

Different approaches have been successfully undertaken to assess whether the degree of stress experienced during learning might be related to the strength of the memory that is formed. One of those approaches (reviewed below) is based on the manipulation of the intensity of the stressor used as the unconditioned stimulus (US) in a particular task, to subsequently evaluate whether any correlation can be observed between posttraining levels of stress hormones and the degree of memory displayed by the animals.

3.1.1. Pavlovian conditioning

Typical examples of this type of studies are those involving different shock intensities in fear conditioning tasks. Experiments performed in rats with the contextual fear conditioning task, involving groups that received different shock intensities (0.2, 0.4, and 1 mA), observed a direct relationship between the stressor intensity experienced at training and the level of freezing displayed by animals at the testing session (Cordero et al. [12, 39]; Merino et al. [40]). Similar shock-dependent effects on auditory fear conditioning have also been described for mice (Laxmi et al. [41]; Anagnostaras et al. [42]). Therefore, these data support the existence of a linear relationship between stressor intensity and the strength of fear conditioning memory formed (see Figure 2(a)). Although difficult to study for obvious ethical reasons restricting the magnitude of stress that can be delivered to animals, one would expect that the dose-dependent linear relationship would achieve an asymptotic, or ceiling effect, after certain stressor intensity is achieved (see Figure 2(a)). To our knowledge, no study has found evidence for impaired memory consolidation for fear conditioning at very high stress conditions. If we consider the normal range of experiences to which experimental animals are submitted in the laboratories worldwide, a stressor intensity-dependent linear relationship seems to account for the effects of stress in the formation of fear memories (Rau et al. [43]).

Conclusion

A linear relationship is proposed for the impact of different stress intensities on the consolidation of fear conditioning, with an asymptotic wave form for high-to-very-high stress intensities (Figure 2).

Neurobiological mechanisms

Interestingly, posttraining corticosterone levels showed a positive correlation with the strength at which fear conditioning is established into a long-term memory (Cordero

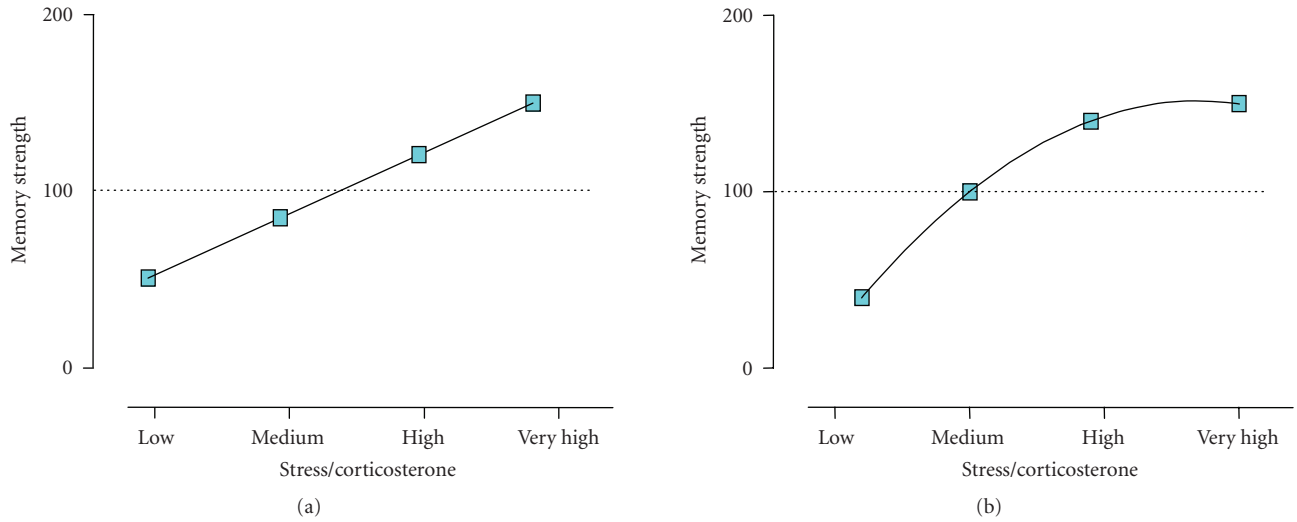


Figure 2: Impact of "intrinsic" stress on memory consolidation. Figures representing the linear (a) and linear-asymptotic (b) relationship between stress intensity (either defined by the stressor or by the physiological response indexed by the glucocorticoid corticosterone) experienced during the consolidation period (provided learning has taken place during the previous learning phase) and the strength of the memory formed.

et al. [12, 39]; Merino et al. [40]) (see Figure 2(a)). A causal role for a central action of corticosterone through glucocorticoid receptors has been supported by two complementary types of studies. First, posttraining administration of corticosterone (either peripherally or centrally) facilitates memory consolidation for both contextual (Pugh et al. [44]; Cordero and Sandi [45]; Revest et al. [46]) and auditory fear conditioning—an effect that was dose-dependent and specific for the conditioned tone (Hui et al. [47]). Second, inhibition of either training-induced corticosterone release (Cordero et al. [39]; Fleshner et al. [48]) or central antagonism of the glucocorticoid, but not mineralocorticoid, receptors (Cordero and Sandi [45]) inhibited the strength of the fear memory formed. Microinfusion of a glucocorticoid receptor antagonist in the basolateral nucleus of the amygdala (BLA) and ventral hippocampus was also found to interfere with long-term memory of contextual fear (Donley et al. [49]).

Recent evidence (Revest et al. [46]) has implicated the MAPK pathway within the hippocampus in the increase in contextual fear conditioning induced by glucocorticoids. Another research line has implicated the neural cell adhesion molecule (NCAM) in the stressor intensity-dependent effects on fear memory formation (Merino et al. [40]). Moreover, the enhancing effect of corticosterone on memory consolidation of auditory-cue fear conditioning requires posttraining noradrenergic activity within the BLA (Roozendaal et al. [50]) and is associated with increased expression of CRH mRNA in the amygdala (Thompson et al. [51]).

3.1.2. Spatial learning

In the spatial learning water-maze task, a similar dose-dependent phenomenon for stress regulation of memory consolidation has been described. In this case, stress intensity was varied by manipulating the temperature of the pool water during the acquisition phase (Sandi et al. [52]). Rats learning the task at a water temperature of 19°C showed a greater retention of the platform location on the second day of training than rats trained at 25°C. Again, a relationship was found between the strength of memory and corticosterone levels displayed by rats after the first training session, with rats trained on the experimental conditions that led to a stronger and longer-lasting memory (i.e., at 19°C) showing the highest circulating hormone levels. These hormonal data indicated that training at 19°C is more stressful than training at 25°C. Moreover, performance of rats trained at 25°C, but not at 19°C, was improved by peripheral injections of corticosterone given immediately after each training session. Therefore, these results further support the existence of a linear facilitating effect of stress on memory consolidation, with increasing glucocorticoid levels during the posttraining period reinforcing the strength of memory up to an asymptotic or ceiling effect (Figure 3.1.1).

Conclusion

A linear asymptotic relationship is also proposed for the impact of different stress intensities on the consolidation of spatial learning, with ceiling performance already achieved for high stressor intensities (Figure 2).

Neurobiological mechanisms

Several examples in the literature support a wider range for the dose-response relationship between glucocorticoid levels and consolidation of spatial learning. Detrimental effects of low glucocorticoid levels in learning and plasticity processes have been largely documented in different tasks. For

example, either metyrapone (an inhibitor of glucocorticoid synthesis and release) administration or adrenalectomy-inhibited special memory in a variety of mazes, including the water maze (Oitzl and de Kloet [53]; Roozendaal et al. [54]), radial arm maze (Vaher et al. [55]), and Y-maze (Conrad et al. [56]). In addition, blocking the activation of glucocorticoid receptors with the GR antagonist RU-38486 impaired spatial memory in the water maze (Oitzl and de Kloet [53]; Roozendaal and McGaugh [57]). Interestingly, similar results have also been obtained in humans; with metyrapone administration enhancing the rate of forgetting on a declarative memory task (Lupien et al. [58]). Glucocorticoid receptors can affect transcription both through DNA binding-dependent and independent mechanisms. Using male mutant mice in which homodimerization and DNA binding of the glucocorticoid receptor is largely prevented (GR(dim/dim)) while protein-protein interactions still can take place (Oitzl et al. [59]), the facilitating effects of corticosterone on spatial memory were shown to depend on DNA binding of the glucocorticoid receptor.

Interestingly, the activation of ERK2 in the hippocampus and the amygdala differs in animals trained at 19°C and 25°C. In the dorsal CA1, training induced an increased phosphorylation of ERK2 only in animals that had learned the task (irrespective of the level of stress). In contrast, in the amygdala, activation of ERK2 was found only in animals that learned the task well under high levels of stress (19°C) (Akirav et al. [60]).

Adrenergic mechanisms have also been implicated in the consolidation of spatial memories. Water-maze learning also triggers the release of adrenergic (adrenaline and noradrenaline) hormones. Mabry et al. [61] showed that plasma adrenaline and noradrenaline levels in young adult rats submitted to water swimming are correlated with water temperature, with 20°C inducing higher glucocorticoid hormonal levels than 25°C. Interestingly, good and bad learners in the water maze at 25°C have been suggested to differ in their task-induced endogenous activation of adrenergic hormone release (Cahill et al. [62]), since posttraining administration of the beta-adrenergic antagonist propranolol specifically impaired the good retention levels showed 24 hours after training by "good learners," without affecting performance in "poor learners." These findings were interpreted as the possible involvement of posttraining adrenergic activation in modulating memory consolidation processes after emotionally stressful events. Interestingly, direct injections of propranolol into the BLA cause retrograde amnesia in the same water-maze task (Hatfield and McGaugh [63]). Several findings in humans have provided support for the hypothesis that enhanced memory for emotionally arousing events depends critically on posttraining adrenergic modulation (Cahill et al. [64]; Southwick et al. [65]). The fact that the degree of activation of the noradrenergic system following training predicts retention performance supports the view that the noradrenergic system within the amygdala plays a central role in memory consolidation. In fact, this phenomenon is circumscribed within more general evidence that the modulation of long-term storage of an emotionally arousing event involves an important activation of the noradrenergic system within the amygdala (McGaugh [36]). Moreover, the dopaminergic system in the BLA has been suggested to be critically involved in memory modulation induced by the noradrenergic system (Lalumiere and McGaugh [66]).

3.2. *Effects of intrinsic stress on the acquisition of information*

Although the facilitating role of stress on consolidation has been emphasized for many years, less attention has been paid to the effects of intrinsic stress on acquisition of information. One of the main reasons for this reduced attention is the variability in the length and characteristics of learning protocols, some including one-trial training procedures and others involving multiple learning trials and even sessions. Such diversity makes it difficult to reach conclusions as to whether it is the acquisition of information that is affected by prior stress, working memory processes, or other types of mechanisms. Anyhow, more recent work raises the possibility that stress effects on acquisition might also underlie the potentation of long-term memory observed when learning under stress.

3.2.1. *Pavlovian conditioning*

Such possibility is quite clear for fear conditioning. When we talk of a linear relationship between shock intensity and long-term memory, we cannot neglect the fact that such linear relationship already exists during the conditioning phase between shock intensity and behavioral reactivity (Figure 3(a)). High shock intensities are typically followed by higher freezing responses than those displayed to lower shock intensities (Cordero et al. [12]; Merino et al. [40]; Laxmi et al. [41]).

However, and although in many occasions mechanisms operating during acquisition will already be key for the strength of the long-term memory formed, we cannot disregard the existence of an acquisition-independent dose-dependent effect for stress and consolidation. The fact that some of the treatments addressed to interfere with the cognitive actions of stress systems (such as, e.g., glucocorticoid administration, or interference experiments based on either corticosterone synthesis inhibition (Cordero et al. [39]) or antagonism of glucocorticoid receptors (Cordero and Sandi [45])) did not affect with the after-shock freezing response but did impair long-term memory reinforces the view that those physiological stress systems show a dose-dependent effect on memory consolidation. The possibility that initial encoding is also affected for such treatments should be more systematically addressed, and would require, for example, fine behavioral analyses during the conditioning processes as well as testing animals in the task at very short time intervals after conditioning.

Conclusion

A linear asymptotic relationship is observed for the impact of different stressor intensities in performance during the acquisition of fear conditioning (Figure 3(a)).

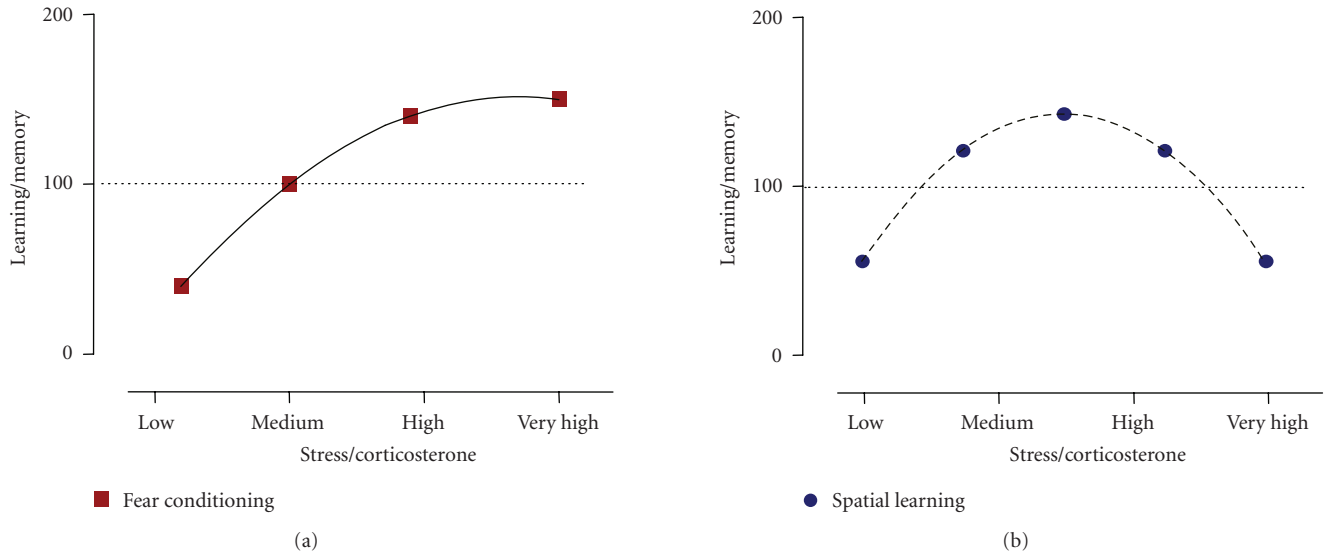


Figure 3: Impact of "intrinsic" stress on learning acquisition. Figures representing the linear-asymptotic—typical for fear conditioning—(a) and inverted U-shape—typical for spatial learning—(b) relationships between stress intensity (either defined by the stressor or by the physiological response indexed by the glucocorticoid corticosterone) experienced during the learning period and the degree of learning and memory acquired.

3.2.2. *Spatial learning*

The example given above for water-maze training at different water temperatures (Sandi et al. [52]) was a spaced learning protocol extended over a few consecutive days. It presented the advantage that by just giving a few training trials per day, groups of animals trained at either 19°C or 25°C water temperature did not differ in their performance on the first training session. However, clear differences were observed in their retention levels from the second training day on, with rats trained at 19°C showing better performance than animals that had been trained at 25°C. This effect was already on the first trial of the second training day; indicative of differences in the strength of memory raised during the consolidation period. The same effect was observed in animals that had been trained at 25°C followed by an injection of corticosterone. Altogether, those results reinforced the view of a facilitating action of stress and glucocorticoids (and note also that evidence is discussed above for adrenergic mechanisms) on consolidation mechanisms.

However, in spatial learning tasks, there are a few documented cases in which learning under different stress levels can have an immediate impact on the rate of learning. By using a modified version of the Morris water maze task that consists in a massed training protocol (1 hour of training in 1 day) that generates long-term spatial learning, Akirav et al. [60] showed that rats trained at 19°C and 25°C already differ in their acquisition rate during the training session. Rats trained at 19°C displayed shorter latencies to find the hidden platform than rats trained at 25°C. Interestingly, animals trained at 25°C could be split into two groups, one that performed as well as the 19°C trained animals and another that performed poorly (i.e., showed longer latency to reach the hidden platform in the water maze), with differences in performance at 25°C apparently being related to the anxiety trait of animals (Herrero et al. [67]).

Interestingly, Akirav et al. [60] also reported that differences in animals' learning curves correlated with corticosterone levels, with higher hormone levels observed in rats trained at 19°C. In a subsequent study, Akirav et al. [68] explored the role of glucocorticoids on learning and memory processes in the same training paradigm. Rats injected with the corticosterone synthesis inhibitor metyrapone (50 or 75 mg/kg, but not 25 mg/kg) showed an impaired learning rate at 19°C, as well as impaired spatial memory. Conversely, rats injected with corticosterone (10 mg/kg, but not 25 mg/kg) at 25°C showed both a better learning rate and better subsequent retention. Therefore, these data also strongly implicate corticosterone in the level of acquisition of spatial learning. They also indicate that there is a ceiling effect for the facilitating actions of corticosterone during acquisition of spatial information, since the dose of 10 mg/kg facilitated learning, whereas the higher dose of 25 mg/kg did not. This finding should be considered cautiously, since the dose of 25 mg/kg might, in fact, induce more pharmacological than physiological levels of the steroid, but it could also suggest the existence of biphasic effects of stress and glucocorticoids in learning acquisition. However, we should also note that rats trained at 25°C that showed a poor performance showed significantly enlarged corticosterone responses (Akirav et al. [60]). These results, together with the higher corticosterone levels displayed by poor performers trained 19°C

(see above), further suggest the existence of an inverted U-shaped relationship between corticosterone levels and performance at training (Figure 3(b)).

Such possibility (the existence of an inverted U-shape between stress levels and learning acquisition for spatial tasks) is reinforced by a previous study (Selden et al. [69]) that showed impaired spatial learning in animals trained at 12°C, a highly stressful condition for the animals. Such impairment was prevented by noradrenaline depletion in the dorsal noradrenergic bundle (ceruleocortical pathway), which only affected performance under such stressful condition, but not in animals trained at a higher temperature (26°C).

Conclusions

The reviewed data on spatial learning supports the view that the effectiveness of acquisition throughout a continuum of stress and/or corticosterone levels generally follows an inverted U-shaped function; the lower performance associated with very low and very high levels, and the optimal performance with intermediate stress levels (see Figure 3(b)).

Neurobiological mechanisms

How could stress systems activated by the training experience affect the learning rate? Whereas an immediate effect of noradrenergic systems in acquisition and performance can be explained by their well-known actions in modulating attention (Selden et al. [69]), explaining online actions of glucocorticoids might not be so straightforward. Typically, glucocorticoid actions were believed to be genomic, with activated corticosteroid receptors being able to modulate the transcription of a large number of genes (Beato and Sanchez-Pacheco [70]; Datson et al. [71]). Such effects are of slow appearance, and therefore cannot explain the described differences in performance throughout the massed spatial training protocol due to different stress conditions (water temperatures). However, increasing evidence supports the existence of rapid effects of glucocorticoid through nongenomic mechanism (Sandi et al. [72, 73]; Karst et al. [74]; for reviews see Makara and Haller [75]; Dallman [76]; Tasker et al. [77]). Glucocorticoids could rapidly modulate cognition through their ability to rapidly enhance extracellular glutamate levels, as shown in the hippocampus and prefrontal cortex, both during stress (Lowy et al. [78]; Moghaddam et al. [79]) and following a peripheral injection of corticosterone (Venero and Borrell [80]). In connection with these fast actions of corticosterone on glutamate release, Karst et al. [74] have recently reported that stress levels of corticosterone, by interacting with the mineralocorticoid receptor (MR), can rapidly enhance the frequency of miniature excitatory postsynaptic potentials in hippocampal CA1 pyramidal neurons and to reduced paired-pulse facilitation. Given that the MRs have been traditionally regarded as the mediators of tonic actions of glucocorticoids, it is important to mention recent evidence suggesting that MR protein expression in the brain can be rapidly regulated by changes in corticosteroid levels (Kalman and Spencer [81]). In addition, some of the rapid glucocorticoid actions can also be mediated through interactions of glucocorticoid metabolites on the gamma-aminobutyric acid (GABA) system (Strömberg et al. [82]).

In addition, the intriguing possibility that glucocorticoids could also rapidly affect the density and morphology of dendritic spines in CA1 pyramidal neurons within 1 hour has been recently put forward (Komatsuzaki et al. [83]). Dendritic spines are essential for information processing, and therefore for memory formation. Because the presence of the protein synthesis inhibitor cycloheximide did not block the effect of the synthetic glucocorticoid dexamethasone, the authors suggest that such rapid morphological changes are probably nongenomic. Moreover, this study presented evidence for the localization of the classical GR in synaptosomal fractions enriched in postsynaptic membranes, suggesting a possible action site of dexamethasone at spines. However, these findings were obtained in hippocampal slices, and therefore the validity for the in vivo situation still remains to be established.

4. THE IMPACT OF ACUTE EXTRINSIC STRESS ON MEMORY FUNCTION

We will deal here with those situations in which stress experienced by the individual is not related to the cognitive task, but is elicited by other circumstances happening either before or after the mnemonic experience (i.e., stress comes from "the outside world"). This condition, that we term extrinsic stress, resembles the concept of "out-of-the-learning context" proposed by other authors (de Kloet et al. [2]; Joëls et al. [8]). At difference to intrinsic stress for which there were not studies exploring the contribution of chronic conditions, there are many examples in the literature devoted to explore the effects of extrinsic stress, both for acute and chronic conditions. Therefore, we will deal with these two very different phenomena in separate subsections, starting here with those referring to acute extrinsic stress. As we did for intrinsic stress, we will first consider which of the factors selected for the current analysis (see above) account for acute extrinsic stress conditions.

(a) Stressor duration: as noted above, both *acute* and chronic situations are well documented in the literature. In this subsection, we deal with *acute* stress.

(b) Stressor intensity: although, hypothetically, the impact of a range of stressor intensities on cognitive performance could be studied, most reports that investigated extrinsic stress conditions generally just apply a single stressor intensity. Whenever possible, we will grade the stressor intensities delivered by the studies according to the same range as above: *low*, *medium*, *high*, and *very high*.

(c) Stressor timing with regard to memory phase: extrinsic stress can be delivered either before (acquisition) or after (consolidation) *learning*, or before *retrieval*. For Pavlovian conditioning, there are examples in the literature related to acquisition and consolidation, whereas for spatial learning the available examples are related

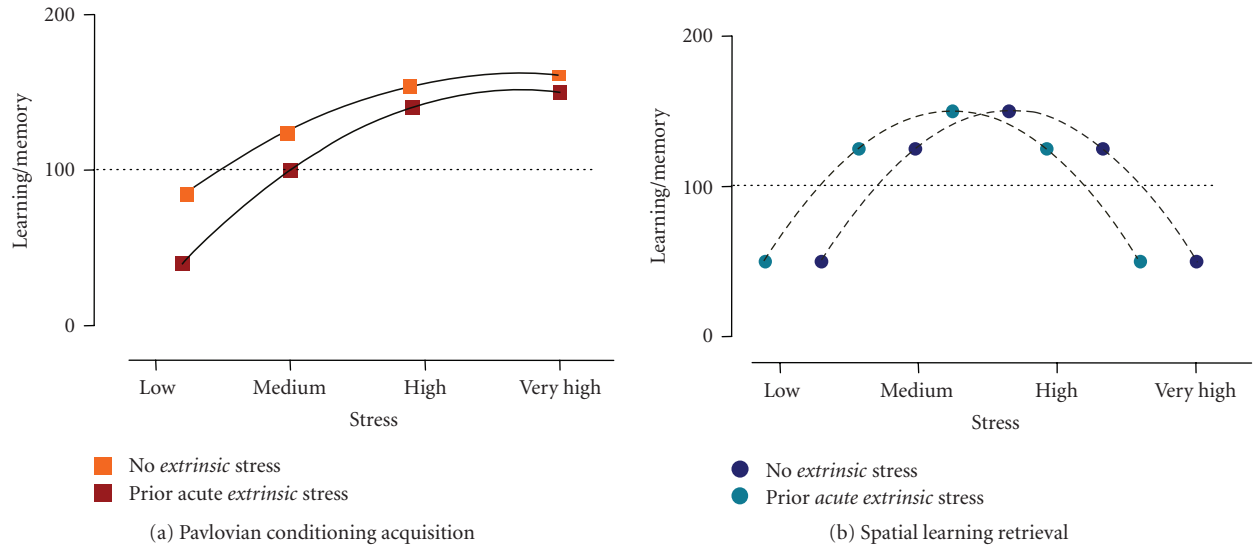


Figure 4: Impact of "acute extrinsic" stress on memory function. Figures representing how extrinsic stress can affect the linear-asymptotic (a) and inverted U-shape (b) relationships depending on the intrinsic stress of each of the learning tasks. Note that, according to the available knowledge in the literature, this model accounts for the "acquisition" of Pavlovian conditioning (a) and for the "retrieval" of spatial information (b). In both conditions, extrinsic stress is proposed to displace to the left the relationship between stressor-related relationship and performance (however, this displacement in the case of the inverted U-shape in (b) has only been described for the right part of the curve).

to acquisition and retrieval. We will review below each of these memory phases separately, as appropriate.

(d) Learning type: we will deal with examples for both *Pavlovian conditioning* and *spatial learning*.

Summarizing, in this subsection, we will evaluate how acute stress (at different intensities) experienced outside the learning challenge affects memory (both implicit and explicit types of memory) function.

4.1. Effects of acute extrinsic stress on the acquisition of information

4.1.1. Pavlovian conditioning

There are many examples in the literature in which prior exposure to acute stress affects subsequent learning in Pavlovian conditioning tasks. The topic has been addressed recently in several reviews (Shors [9, 27]).

Shors and collaborators have extensively illustrated that stress experienced before training consistently facilitates eyeblink conditioning in male rats of different strains (Shors et al. [84]; Servatius and Shors [85]; Shors and Servatius [86]; Wood and Shors [87]; Beylin and Shors [11]; Shors [88]). Interestingly, stressors of medium intensity displayed no effect on conditioning, with high-to-very-high stressful conditions, (typically a restraint-tailshock procedure, unpredictable and uncontrollable, adapted from the "learned helplessness" paradigm) being required to potentiate this learning process (Shors and Servatius [86]; Beylin and Shors [11]). The enhancement of learning by prior acute high stress was observed during classical eyeblink conditioning of both hippocampal-dependent and independent learning tasks. It could be triggered within minutes of the stressful event and lasted for days.

Acquisition of fear conditioning has also been shown to be highly susceptible to modulation by prior stress exposure. Prior shock exposure has been shown to greatly enhance subsequent contextual fear conditioning in a different context (Fanselow and Bolles [89]; Fanselow et al. [90]). Likewise, previous exposure to an acute restraint session increased contextual fear conditioning (Cordero et al. [91]; Rodriguez Manzanares et al. [92]). Moreover, using the BALBc strain of mice, Radulovic et al. [93] showed that restraint stress, in addition to its facilitating effects in contextual conditioning, it also enhances auditory-cued fear conditioning processes.

Conclusions

Therefore, high extrinsic stress facilitates Pavlovian fear conditioning. Although a systematic study should be performed, we propose that extrinsic stress shifts the dose-dependent impact of the unconditioned stimulus to the left (see Figure 4(a)).

Neurobiological mechanisms

The enhancement of both types of Pavlovian learning discussed here, eyeblink conditioning (Beylin and Shors [94]) and fear conditioning (Cordero et al. [91]), involves glucocorticoids. In the eyeblink conditioning task, endogenous

glucocorticoids were shown to be necessary and sufficient for transiently facilitating acquisition of new associative memories, and necessary but insufficient for persistently increasing their acquisition after exposure to acute stress (Beylin and Shors [94]). In the contextual fear conditioning task, animals that had been previously submitted to a single restraint session showed increased corticosterone levels following training, which suggested that increased glucocorticoid release at training might be implicated in the mechanisms mediating the memory facilitating effects induced by prior stress experiences (Cordero et al. [91]).

Anxiety mechanisms have also been related to the enhancing effects of prior stress in Pavlovian conditioning. Recent evidence provided by Bangasser et al. [95] implicated the bed nucleus of the stria terminalis (BNST) in the facilitating effects induced by stress in eyeblink conditioning. Interestingly, in humans, high degrees of trait or state anxiety have also been linked with increases in eyeblink conditioning (reviewed by Shors [9]). In the restrain stress-induced facilitation of fear conditioning, changes in GABAergic mechanisms in the amygdala have been implicated, that is, stress was shown to induce an attenuation of inhibitory GABAergic control in the BLA, leading to neuronal hyperexcitability and increased plasticity (Rodriguez Manzanares et al. [92]).

4.1.2. Spatial learning

The same acute stress procedure that was repeatedly shown by Shors et al. (see above) to facilitate eyeblink conditioning was found not to have any effect in performance during learning in the Morris water maze (Warren et al. [10]; Healy and Drugan [96]; Kim et al. [97]) (but note that in one of these studies, animals were subsequently impaired in their retention levels for the platform location (Kim et al. [97])). Similarly, exposure to cat stress before training did not affect the rate of acquisition of platform location in a radial arm water maze (Diamond et al. [98]) (but note again that this pretraining stress resulted in impaired spatial memory when tested 24 hours later). Furthermore, this lack of effect does not seem to be restrictive to stressful water maze tasks. By using a nonspatial object-recognition memory task and the same inescapable restraint and tail-shock stress procedure as mentioned above, similar results have been reported by Baker and Kim [99]. Rats stressed before being exposed to the task showed normal memory when tested 5 minutes after first exposure to objects, but were impaired when tested 3 hours afterwards. Control rats display a preference for a novel object (over a familiar one) when they are tested at different time delays (5 minutes and 3 hours). As opposed to these unstressed controls, at the 3-hour posttraining test, stressed animals spent comparable time exploring novel and familiar objects.

However, we should mention that work in mice has pointed out the importance of individual differences in the impact of acute extrinsic stress on spatial learning. Francis et al. [100] evaluated the effect of daily exposure to uncontrollable footshocks before spatial orientation. They found that such treatment did not affect the acquisition or performance of this response in three strains (DBA/2J, C57BL/6J, BALB/cByJ), but provoked a modest disruption of reversal performance in DBA/2J mice and markedly impaired reversal performance in BALB/cByJ mice. The authors emphasized the importance of individual differences in the susceptibility to stress and speculated that uncontrollable stress would not disturb response-outcome associations, but may induce a perseverative response style. Therefore, a potential effect of stress in reversal learning cannot be neglected.

Conclusion

Learning new spatial associations (i.e., when an individual is confronted for the first time to find a reward in a particular spatial setting) is a process highly resistant to the effect of prior stress (even when involving high to very high stress conditions). However, the more flexible process of reversal learning (i.e., when there is a change in the location of a reward in a particular spatial setting, from a former place to a new one, and the individual is then confronted to reverse the strategy) to find a reward seems to be more vulnerable to disruption by prior stress.

4.2. Effects of acute extrinsic stress on the consolidation of information

4.2.1. Pavlovian conditioning

There are only a few examples in the literature focusing on the impact of posttraining acute stress on consolidation of Pavlovian conditioning, and the results are less homogeneous than for acquisition.

Using the eyeblink conditioning paradigm in rats, Beylin and Shors [11] showed that the same high intensity stressor that facilitates conditioning when applied before training does not influence further retention levels when it is delivered after animals have been conditioned.

Social isolation stress given immediately after training rats in the contextual fear conditioning task impaired subsequent retention levels (if given up to 3 hours after training, but not at 24 hours) (Rudy [101]; Rudy et al. [102]), but did not have any effect if applied to the auditory fear conditioning paradigm (Rudy [101]). However, auditory fear conditioning was facilitated by the administration of mild to medium intensity stressors (handling or subcutaneous vehicle injection) after training (Hui et al. [103]).

Retention levels for a particular type of classical conditioning paradigm, the conditioned taste aversion task (Garcia et al. [104]; Bermudez-Rattoni [105]), were also shown to be inhibited if a high stressor (forced swim) is given shortly after conditioning (Bourne et al. [106]).

Conclusion

The lack of homogeneity in the very few available studies for this category does not allow formulating any conclusions for the impact of posttraining extrinsic stress in Pavlovian conditioned memories.

4.3. Effects of acute extrinsic stress on the retrieval of information

4.3.1. Spatial learning

A series of experiments has presented evidence for impairing effects of stress when it is given during a brief delay period between the acquisition of information and a subsequent retrieval challenge. Such delay normally lasts between 30 minutes and 4 hours, and therefore stress during such period can be influencing a variety of mechanisms, including consolidation, short-term memory, and retrieval. Using both conventional (Diamond et al. [107]) and water (Diamond et al. [108]; Woodson et al. [109]; Sandi et al. [110]) radial arm mazes, Diamond et al. have consistently shown that stress applied during such delay period interferes with subsequent retrieval of the previously acquired information. In most of their studies, the stressor applied was exposure of rats to a cat that, therefore, can be considered of high or very high intensity.

The same treatment was also effective to inhibit recall when it was given just immediately before the 24-hour memory test trial (Diamond et al. [98]). This finding fits with previous work in the Morris water maze, in which exposure to brief shocks 30 minutes, but not 2 minutes or 24 hours before testing (de Quervain et al. [111]). The same deleterious effect in retrieval of spatial information was observed by injecting corticosterone 30 minutes before retention testing (de Quervain et al. [111]). Further studies indicated that the impairing effects of glucocorticoids on retrieval of long-term spatial memory depend on noradrenergic mechanisms in the hippocampus, and moreover, that neuronal input from the BLA (and particularly norepinephrine-mediated BLA activity) is essential for the hippocampal glucocorticoid effects on memory retrieval to occur (Roozendaal et al. [112, 113]).

Convincing evidence indicates that the level of difficulty of the task (memory load) is a critical factor in observing the detrimental effects of stress on retrieval processes. Using the radial arm water maze, Diamond et al. [108] showed that exposure to a cat during a 30-minute delay period between training and testing for the platform location (the platform was located in the same arm on each trial within a day and was in a different arm across days) had no effect on memory recall in the easiest RAWM, but stress did impair memory in more difficult versions of the RAWM. By lesioning the hippocampus, the authors also confirmed that the radial arm water maze is a hippocampal-dependent task. In addition to the importance of memory load (difficulty or memory demand of the task), it seems that flexible forms of memory are particularly susceptible to show disrupted retrieval by stress, as opposed to more stable ones that remain largely unaffected (Célérier et al. [114]). This might reflect the differential susceptibility of different memory systems to be affected by stress.

Evidence for impairing effects of acute stress on subsequent/delayed retrieval has also been provided in humans, with emotionally arousing material being especially sensitive to this disruptive effect (Domes et al. [115]; Kuhlmann et al. [116]). As in animals, memory load is also an important factor for stress-induced retrieval impairments in humans (de Quervain et al. [117]).

Conclusion

The results reviewed here indicate that experiencing an acute, highly stressful, situation can interfere with information processing linked to retrieval of previously (recently) stored information. Although there is no information with regard to the impact of such extrinsic stress in tasks involving low intrinsic stress levels, we speculate that the inverted-U shape for the relationship between intrinsic stress and spatial information processing (Figure 3(b)) will be displaced to the left by the effect of extrinsic stress (see Figure 4(b)). Thus extrinsic stress would impair the retrieval of stressful spatial information (as described above), but would facilitate recall of spatial information linked to less arousing experiences. However, the left part of the curve remains speculative, and we cannot discard the other two possibilities of not finding an effect or even observing impaired spatial retrieval when extrinsic stress is applied before spatial tasks involving low intrinsic stress.

4.4. Neurobiological mechanisms involved in the acute effects of extrinsic stress on memory

The great sensitivity of the hippocampus to the disrupting effects of extrinsic stress in cognition is revealed by the profound suppression of hippocampal synaptic plasticity after acute exposure to stressors (Foy et al. [118]; Bennett et al. [119]; Diamond et al. [120]; Alfarez et al. [121]) or increased glucocorticoids (Alfarez et al. [121]). A crucial role for the medial temporal lobe (and the hippocampus in particular) in mediating these stress-induced retrieval impairments is also supported by human neuroimaging studies (de Quervain et al. [117]). In addition to the hippocampus, there is also evidence that acute stress-induced memory impairing effects can also be mediated by activation of dopaminergic (Murphy et al. [122]; Arnsten and Goldman-Rakic [123]) and noradrenergic (Birnbaum et al. [124]) transmissions in other structures known to be involved in high-order (including working memory and executive function) processing, such as the prefrontal cortex.

As to the potential molecular mechanisms, only a few studies have been reported. Reduced expression of NCAM in the hippocampus and prefrontal cortex after cat stress exposure was recently described to correlate with stress-induced retrieval deficits in the radial arm water maze (Sandi et al. [110]). These observations of a drastic reduction of NCAM in stressed memory-impaired rats is consistent with an increasing body of data indicating that NCAM is important for optimal circuit functioning and synaptic plasticity (Kiss et al. [125]; Welzl and Stork [126]; Washboume et al. [127]).

5. THE IMPACT OF CHRONIC EXTRINSIC STRESS ON MEMORY FUNCTION

Prolonged exposure to stress is recognized as a condition that can induce deleterious effects on brain structure and cognition (McEwen [128, 129]), as well as increasing the risk to develop neuropsychiatric disorders (Mazure [130]; de Kloet et al. [131]; Nemeroff et al. [132]).

Nowadays, the study of chronic stress is probably the most popular in the field of stress' interactions with cognitive function. In the vast majority (if not all) of studies dealing with chronic stress, it is extrinsic stress, experienced in a prolonged manner, that is studied, and therefore, most of the studies on chronic stress and memory fall into this definition. As previously, we should start by defining how the above-mentioned factors account for chronic extrinsic stress conditions.

(a) Stressor duration: in this subsection, we deal with *chronic* stress.
(b) Stressor intensity: the contribution of this factor to the impact of chronic stress has not being systematically studied. When possible, we will try to estimate the stressor intensity in the different chronic stress protocols under discussion, according to the range used above: *low*, *medium*, *high*, and *very high*.
(c) Stressor timing with regard to memory phase: although, in theory, one could imagine situations in which chronic stress is experienced at different times with regard to the different memory phases, virtually all studies in the literature applied stress procedures before exposing animals to any cognitive challenge. Therefore, we will group them in this review under the subheading of acquisition of information, even though all different memory phases could still be affected when stress is applied before learning.
(d) Learning type: we will deal with examples for both *Pavlovian conditioning* and *spatial learning*.

Summarizing, in this subsection, we will evaluate how chronic stress experienced before the learning challenge affects memory (both implicit and explicit types of memory) function.

5.1. Effects of chronic extrinsic stress on the acquisition of information

5.1.1. Pavlovian conditioning

To our knowledge, the impact of chronic stress in Pavlovian conditioning in rodents has only been tested in fear conditioning protocols. Chronic restraint stress has been repeatedly shown to potentiate both contextual (Conrad et al. [133]; Sandi et al. [134]; Cordero et al. [135]) and auditory (Conrad et al. [133]) fear conditioning in rats. In all cited cases, the chronic stress procedure applied can be considered of high stress intensity (restraint stress: 6 h/day) and was applied during 21 consecutive days. Shorter exposure to chronic restraint stress (1 week) was ineffective to affect subsequent auditory fear conditioning; however, it impaired fear extinction applied 24 hours after conditioning (Miracle et al. [136]).

Conclusion

Chronic stress (high stressor intensity, 21-day duration) seems to facilitate fear conditioning processes (Figure 5(a)).

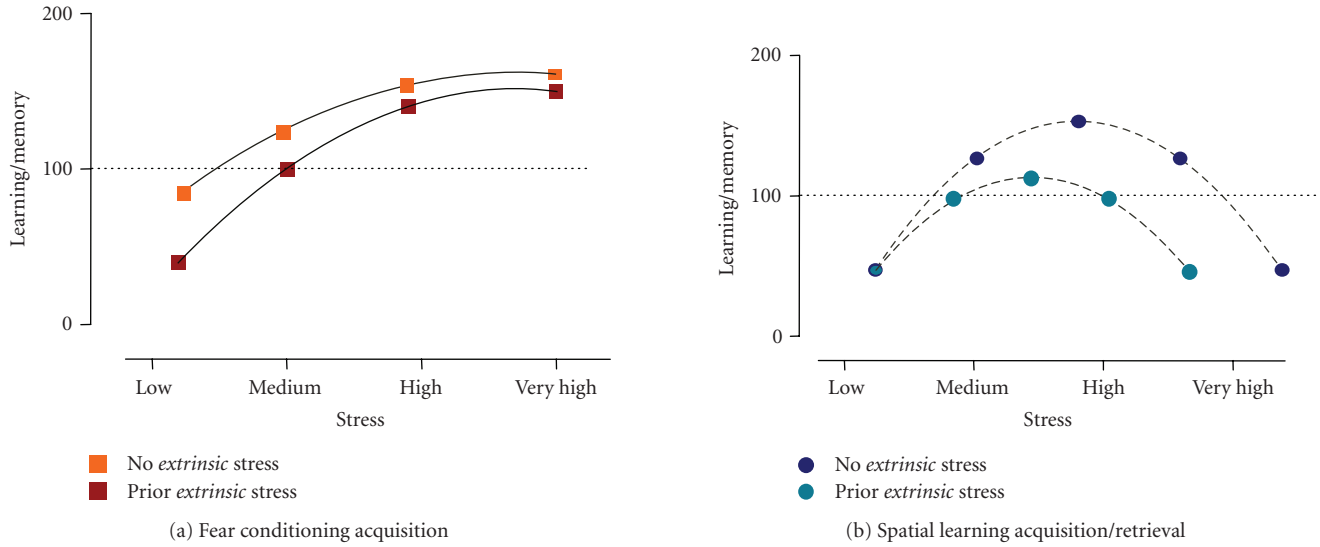
Neurobiological mechanisms

In the facilitating effect of fear conditioning induced by chronic stress, corticosterone has been proposed to play a mediating role (Conrad et al. [137]). At the neurobiological level, increasing evidence at the cellular and molecular levels suggests a connection between neuronal remodeling in the amygdala and the development of anxiety-like behavior (Vyas et al. [138, 139]; Mitra et al. [140]), which fits with the role of the amygdala in emotional behavior and fear (Phelps and LeDoux [141]). Restraint stress has been reported to enhance anxiety, and also to cause an increase in dendritic length and spine density in the BLA, but a reduction in the medial amygdala (Vyas et al. [138, 139]; Mitra et al. [140]). At the molecular level, recent evidence indicates that the serine protease tissue-plasminogen activator (tPA) (a key mediator of spine plasticity which is also required for stress-induced facilitation of anxiety-like behavior (Pawlak et al. [142])) plays a permissive role in the reported stress-induced spine loss in the medial amygdala (Bennur et al. [143]).

5.1.2. Spatial learning

Since chronic stress was originally reported to damage hippocampal structure (McEwen [128, 129]), the possibility that chronic stress affects hippocampal-dependent learning has been extensively tested over the past years. Chronically stressed male rats were shown to exhibit learning and memory deficits in a variety of spatial tasks, including the radial-arm maze (Luine et al. [144]), the Y-maze (Conrad et al. [56]), and the Morris water maze (Venero et al. [145]; Sandi et al. [146]). Similarly, psychosocial stress consisting of rats' exposure to a cat for 5 weeks and randomly housed with a different group of cohorts each day was shown to exhibit impaired learning and memory in the radial-arm water maze (Park et al. [147]). Reversal learning in spatial tasks, a cognitive operation that in addition to the efficient use of spatial information requires flexibility to relearn a new platform, seems to be compromised following treatments involving chronic (21–28 days) glucocorticoid elevations (Sandi [4, 5]; Cerqueira et al. [148]).

There is no consensus as to whether periods of stress exposure shorter than the more or less standard protocol of 21 days would result in impaired learning. Luine et al. [149] reported that when restraint stress was given for 6 h/day for 7 days and spatial learning in the eight arm radial maze was evaluated on days 10–13 post stress, no effect on performance was noted; however, daily restraint stress for 13 days



(a) Fear conditioning acquisition

(b) Spatial learning acquisition/retrieval

Figure 5: Impact of "chronic extrinsic" stress on memory formation. Chronic stress potentiates fear conditioning (a) and impairs spatial and reversal learning processes (b).

induced a medium enhancement of performance on days 10–13 post stress. More recently, Radecki et al. [150] showed that chronic immobilization stress (2 h/day × 7 days) in Long-Evans rats significantly impaired spatial performance in the Morris water maze, elevated plasma corticosterone, and attenuated hippocampal LTP.

Conclusion

Chronic stress (high stressor intensity, 3–5-week duration) seems to impair spatial and reversal learning.

Neurobiological mechanisms

Given that the hippocampus was originally found to be a main target of glucocorticoids and to be responsive to stress, much work on the neurobiological impact of stress has focused on this brain region. The idea behind is that, to certain extent, structural and molecular alterations (see below) induced by chronic stress in this brain area will account for the impairing effects of stress in hippocampus-dependent memory tasks (notably including spatial learning). Moreover, recent work is providing increasing evidence for parallel alterations induced by chronic stress in the prefrontal cortex, which could account also for some of the behavioral alterations described above and, specially, for stress-related impairments in reversal learning.

Briefly, cumulative work indicates that chronic stress markedly affects the hippocampal morphology. Stress and high glucocorticoid levels can suppress neurogenesis in the dentate gyrus (Gould and Tanapat [151]) and compromise cell survival (Sapolsky [152]). In the CA3 area, chronic stress has been shown to result in the following structural alterations: (i) dendritic atrophy of apical pyramidal neurons (Watanabe et al. [153]; Magariños and McEwen [154]); (ii) synaptic loss of excitatory glutamatergic synapses (Sousa et al. [155]; Sandi et al. [146]); (iii) a reorganization at the microstructural level within mossy fibre terminals (Magariños et al. [156]); (iv) a reduction in the surface area of postsynaptic densities (Sousa et al. [155]); and (v) a marked retraction of thorny excrescences (Stewart et al. [157]). In the CA1 area, the structural changes reported after chronic stress include (i) a general decrease of the dorsal anterior CA1 area's volume (Donohue et al. [158]); (ii) alterations in the lengths of the terminal dendritic segments of pyramidal cells in rat CA1 (Sousa et al. [155]); and (iii) an increase in the surface area of the postsynaptic density and volume in CA1 stratum lacunosum moleculare (Donohue et al. [158]).

Intriguingly, recent studies have suggested that spatial memory deficits may arise from HPA axis dysregulation following hippocampal damage, rather than being a direct effect of hippocampal injury. Thus, spatial memory deficits following CA3 hippocampal lesion could be prevented with a single injection of metyrapone, a corticosterone synthesis blocker, just before performance in the water maze (Roozendaal et al. [159]). Furthermore, the deleterious effects induced by a 21-day chronic restraint stress procedure in the Y-maze have been proposed to depend on corticosterone elevations at the time of behavioral assessment, since impaired performance was inhibited by pretesting metyrapone injections (Wright et al. [160]).

As to the prefrontal cortex, major neuronal remodeling occurs in its medial part as a consequence of chronic stress or prolonged glucocorticoid treatment, including dendritic atrophy (Wellman [161]; Cook and Wellman [162];

Radley et al. [163]; Liston et al. [164]) and spine loss (Cerqueira et al. [148]; Radley et al. [165]) in layers II/III.

Finally, given that the amygdala can exert important modulatory actions in hippocampus-dependent memory tasks (McGaugh [36]), further studies are needed to assess whether sensitization of amygdala activation induced by chronic stress (see above) might also participate in the reported spatial memory impairments.

At the molecular level, a large list of molecular mechanisms appears to contribute to the impairing actions of stress in brain structure and cognitive function. They include excitatory amino acids and a variety of signal transduction pathways, neurotrophic factors, and cell adhesion molecules (Sandi [4, 5]; McEwen [128]; Sapolsky [152]; Molteni et al. [166]).

6. DISCUSSION AND CONCLUSIONS

The results reviewed here emphasize the great importance of integrating different factors into a model of stress actions in memory formation. The five factors proposed and analyzed (see Section 2) seem to be critical to define the outcome of stress effects in memory processes.

The factor source of stress, distinguishing between *intrinsic* and *extrinsic* stress is the key to understand the complexity of effects and mechanisms involved. *Intrinsic* stress facilitates memory consolidation processes, whereas the effect of *extrinsic* stress in memory consolidation seems to be quite heterogeneous, and therefore, specifying the source of stress helps clarifying the claimed differential effects of stress/glucocorticoids in memory consolidation versus retrieval (Roozendaal [20]).

A second highly critical factor is the learning type under study, with high stress (both intrinsic and extrinsic) consistently facilitating *Pavlovian conditioning*, while high-to-very-high stress generally impairing the processing of *spatial information* (or relational and explicit types of learning). The latter proposal (i.e., that high-to-very-high stress impairs learning) is quite controversial since some researchers criticize the simplistic view that stress impairs learning by noting that the physiological stress response is a mechanism to optimize survival, and they propose that it is the behavioral strategy that changes under high stress conditions (de Kloet et al. [2]; Joëls et al. [8]). Although we basically agree with such interpretation, we should also recognize that when spatial learning/retrieval is under study, high-to-very-high stress conditions result in impaired performance in this type of tasks. It would be interesting to investigate whether such deleterious effect is in benefit of a facilitation of alternative learning (notably, emotional learning) types.

The factor "stressor intensity" is useful and allows making interexperiment comparisons. It also helps understanding how different magnitudes of challenge interact with cognition. Whereas the whole grading of stressor intensities is important to define the impact of intrinsic stress (see, e.g., Figure 3), it is high stress conditions which are particularly effective and representative of the impact of extrinsic stress in memory function.

The factor stressor timing with regard to memory phase is also critical, as we concluded that different memory phases show different vulnerabilities to stress. Although this was noted in many instances, a clear example is the susceptibility of Pavlovian conditioning to be facilitated when extrinsic stress is given before learning, but not afterwards (see Figure 4(a)), whereas it is the retrieval phase of spatial learning which seems to be particularly vulnerable to the impact of (acute) extrinsic stress.

Finally, the factor "stressor duration," distinguishing between acute and chronic stress situations, although it give a similar outcome when observing its impact in memory function (cf. Figures 4 and 5), it makes a clear contribution when we talk about performance during "acquisition" of information. Whereas chronic extrinsic stress frequently has an impact on spatial learning, acute extrinsic stress normally does not affect spatial learning, but has been revealed to be more efficient to disturb retrieval.

Given the importance of other factors already mentioned throughout the review, such as the amount of effort/load included in the information processing (Diamond et al. [108]; Célérier et al. [114]), or individual differences in personality or other stress-relevant factors (Touyarot et al. [167]; Márquez et al. [29]), future integrative attempts should be directed to analyze and integrate these or other factors with the final goal of developing an integrative and reliable model that accounts for the whole complexity of stress interactions with cognition.

Summarizing on those conditions in which we have enough information to compare the integrated impact of the different factors analyzed, we could conclude that high stress levels, whether intrinsic or extrinsic, tend to facilitate Pavlovian conditioning (in a linear-asymptotic manner), while being deleterious for spatial/explicit information processing (which with regard to intrinsic stress levels follows an inverted U-shape effect). We consider this integrative model more explanatory than classifications performed among individual factors (see Section 1).

As to the neurobiological mechanisms, a common observed feature seems to be a key role of glucocorticoids in mediating both the facilitating and impairing actions of stress in different memory processes and phases. Among the brain regions implicated, the hippocampus, amygdale, and prefrontal cortex were highlighted as critical for the mediation of stress effects. Further work is needed to develop a mechanistic explanatory model at the neurobiological level that accounts for the different interactions and factors discussed above.

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Review Article

The Role of the Medial Prefrontal Cortex-Amygdala Circuit in Stress Effects on the Extinction of Fear

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Stress exposure, depending on its intensity and duration, affects cognition and learning in an adaptive or maladaptive manner. Studies addressing the effects of stress on cognitive processes have mainly focused on conditioned fear, since it is suggested that fear-motivated learning lies at the root of affective and anxiety disorders. Inhibition of fear-motivated response can be accomplished by experimental extinction of the fearful response to the fear-inducing stimulus. Converging evidence indicates that extinction of fear memory requires plasticity in both the medial prefrontal cortex and the amygdala. These brain areas are also deeply involved in mediating the effects of exposure to stress on memory. Moreover, extensive evidence indicates that gamma-aminobutyric acid (GABA) transmission plays a primary role in the modulation of behavioral sequelae resulting from a stressful experience, and may also partially mediate inhibitory learning during extinction. In this review, we present evidence that exposure to a stressful experience may impair fear extinction and the possible involvement of the GABA system. Impairment of fear extinction learning is particularly important as it may predispose some individuals to the development of posttraumatic stress disorder. We further discuss a possible dysfunction in the medial prefrontal cortex-amygdala circuit following a stressful experience that may explain the impaired extinction caused by exposure to a stressor.

1. INTRODUCTION

Pavlovian fear conditioning is an extensively studied model for stress and anxiety-like disorders [1]. In this form of learning, an animal is exposed to pairings of a neutral conditioned stimulus (CS) such as a light or tone, with a fear-inducing unconditioned stimulus (US), such as a mild foot shock, and comes to exhibit a conditioned fear response (CR) to the CS. The CR includes freezing, increased startle reflexes, autonomic changes, analgesia, and behavioral response suppression. Experimental extinction is a behavioral technique leading to suppression of the acquired fear, that is, a decrease in the amplitude and frequency of a CR as a function of nonreinforced CS presentations. Experimental extinction is assumed to reflect an active learning process that is distinct from acquisition of fear and requires additional training to develop [2–5].

While clearly of importance to survival, the expression of emotional associations may become disadvantageous when the conditioned cue ceases to predict the appearance of danger. In that respect, the ability to extinguish emotional responses in the face of a no-longer relevant conditioned cue is an essential part of a healthy emotional memory system, particularly with respect to phobias, panic disorders, and posttraumatic stress disorder [PTSD; [4, 6–8]]. Thus, the suppression of the fear response (i.e., extinction) receives increasing attention, since it could become an effective intervention for the treatment of fear-related disorders.

Extinction suppresses, rather than erases, the original CS-US association. For example, even the completely extinguished fear can be recovered spontaneously after the passage of time [9, 10], or be "reinstated" by presentations of the US alone [11, 12], or be renewed by placing the animal in a context different from the one in which it was extinguished [13]. This is congruent with the notion that extinction is a form of relearning (of a CS-no US or "inhibitory" association) rather than unlearning (of the CS-US association) [14]. Accordingly, one suggestion put forward that extinction suppresses

the expression of an intact underlying fear response, and extinction memory is labile and weak compared with the fear conditioning itself. Hence, understanding the factors that facilitate or impair extinction may aid in accelerating behavior therapy for the treatment of anxiety disorders.

Despite the efficacy of behavior therapy for human anxiety disorders, extinction-like treatments require repeated cue exposures and are vulnerable to reversal by a number of environmental factors, particularly stress.

The effects of stressful experiences on cognition are manifested through the activation of multiple mechanisms and operating over different time courses and have been linked to the onset of a variety of affective disorders. Stress can produce deleterious effects on the brain and behavior, and it contributes towards impaired health and an increased susceptibility to disease and mental disorders [15, 16]. Investigations into the interaction between stressful experiences and memory haves focused mainly on the behavioral and neural mechanisms of memory acquisition (i.e., fear conditioning), but not on memory extinction, even though extinction is used for the treatment of psychiatric conditions based on learned fear, such as phobias, panic, generalized anxiety, as well as PTSD.

Extensive evidence indicates that the amygdala and the prefrontal cortex are key structures in the response to stress and its effects on learning and memory. Importantly, it has been shown that extinction of fear memory requires plasticity in both the medial prefrontal cortex (mPFC) and the basolateral amygdala [BLA; [17–19]]. In this review, we will discuss the relevance of the prefrontal cortex-amygdala circuit as a key mechanism for understanding stress-induced alterations occurring during the extinction of fear.

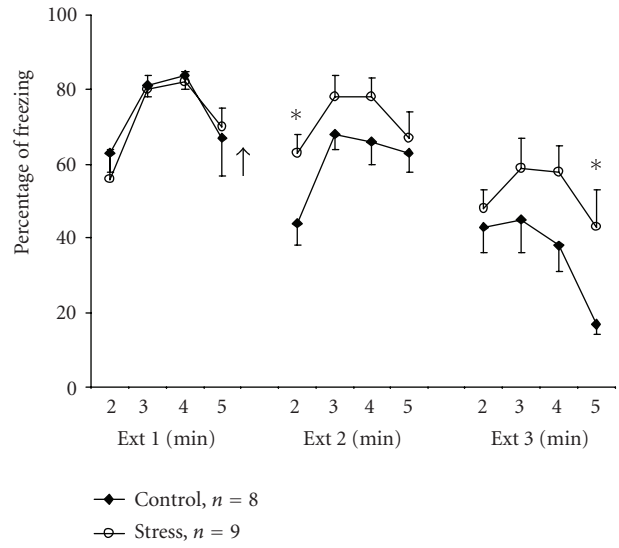

Figure 1: *Stress impairs extinction of contextual fear conditioning.* Rats were given 3 mild foot shocks in the conditioning chamber. On the next day, the rats were placed in the extinction (Ext) chamber for 5 minutes and no shock was administered (Ext 1; the last 4 minutes are presented since all animals showed high levels of freezing in the first minute). Immediately afterwards, the animals were returned to their home cage (control) or placed on an elevated platform for 30 minutes (stress). Animals were exposed to additional 5 minutes in the extinction chamber, without shocks, on days 3 (Ext 2) and 4 (Ext 3). The stressed animals showed significantly higher levels of freezing compared with the control group during the second minute of Ext 2 ($*$; $P < .05$) and the fifth minute of Ext 3 ($*$; $P < .05$). Arrow denotes time of exposure to stress.

2. STRESS AND EXTINCTION

There are intricate relationships between stress and cognitive processes [20]. On the one hand, cognitive processes are necessary to cope adequately with a stressor, both actively and passively, in that a subject has to be aware that there is a stressor and at the same time it has to learn that the stressor can be controlled by an appropriate response. Adaptation to stress occurs when the acquired response is successful in reducing the impact of the stressor. If not, maladaptation may occur. On the other hand, there is strong evidence that stress and stress hormones play an important role in the modulation of cognitive processes. It should be noted that in the fear conditioning paradigm, stress plays a role during conditioning and at least during the first stages of extinction training. Thus, we differentiate here between the aversive situation in the learning paradigm itself, for example, exposure to a foot shock, and the effects of additional exposure to an out-of-context stressor on fear extinction.

When examining the effects of exposure to an out-of-context stressor on fear extinction, we found that the stressor increased resistance to extinction (H. Reizel, I. Akirav, and M. Maroun, unpublished observation; Figure 1). Specifically, after contextual fear conditioning (using a US of 3 foot shocks of 0.5 mA each), control rats gradually extinguished their freezing (CR) when placed in the extinction box (CS) for 3 consecutive days, for 5 minutes each time. By contrast, the experimental rats were exposed to the out-of-context stressor on being placed on an elevated platform for 30 minutes immediately after the first extinction session. Animals placed on the platform exhibited behavioral "freezing," that is, immobility for up to 10 minutes, defecation, and urination [21, 22]. This stressor was found to increase plasma corticosterone levels by 38% as compared with naïve rats [23] and we have recently found that it impairs long-term potentiation in the CA1 area of the hippocampus and in the BLA-medial prefrontal pathway [24]. In the contextual fear extinction experiment, the stressed rats showed increased levels of freezing in the extinction box even 48 hours after a single exposure to the elevated platform. This suggests that exposure to the stressor had the long-term effect of impairing the extinction of fear.

We found that exposure to stress had a similar effect on consolidation of the extinction of auditory fear conditioning (see later, see below, or ahead). The impairing effects of the elevated platform on auditory fear extinction also persisted for 48 hours following exposure to the stressor. Consistent with our results, Izquierdo et al. [25] reported that exposure to three episodes of stress ending 24 hours before fear

conditioning significantly attenuated the rate of cued fear extinction relative to nonstressed controls. Shumake et al. [26] showed that rats that were selectively bred for increased susceptibility to learned helplessness show resistance to extinction of conditioned fear. Furthermore, Kellett and Kokkinidis [27] showed that amygdala kindling, which enhances emotionality, impaired the extinction of fear-potentiated startle, and rats showed increased levels of fear. They also found that electrical stimulation of the amygdala restored extinguished fear responses and that the fear reinstatement was specific to the extinction context. In a study with rainbow trout, Moreira et al. [28] compared two lines of fish that exhibit divergent endocrine responsiveness to stressors: the high-responders (HR) and low-responders (LR; the "stressed"). Postconditioning, the fish were tested by presentation of the CS at weekly intervals for 4 weeks, with no further reinforcement, and the extinction of the CR in the two lines was compared. The number of individuals within each line whose plasma cortisol levels indicated a stress response when exposed to the CS was significantly greater among the LR than HR fish at 14 and 21 days, with no HR fish falling into the stress-response category at 21 days. Thus, the stressed fish did not extinguish as well as the HR fish.

It is important to understand why exposure to stress impairs extinction learning, and here we put forward four possible explanations. One possibility is that extinction memory is labile and weak compared with fear conditioning itself, and thus exposure to a stressful experience interferes with the process of extinction learning or with the retrieval of information. Second, it has been shown that a stressful experience following or preceding a threatening or fear-related learning event enhances retention [29]. However, in extinction, the animals need to learn to suppress their fear response that is associated with the CS. Thus, the aversiveness of the stressful experience may counteract the extinguished emotional response. Further, it is possible that preexposure to the stressful experience increases resistance to extinction through sensitization, leading to the occurrence of a conditioned fear response even to a less intense "reminder" of the original US. Thus, retrieval of the CS-US association (i.e., acquisition) overcomes the CS-no US association (i.e., extinction) following the sensitization effect, making extinction more difficult to learn. However, this can hardly explain why exposure to an unrelated stressful experience, such as an elevated platform, should sensitize the animals to respond as if to the US during extinction training. A fourth possibility is that resistance to extinction is not related to sensitization or to the enhancement of an unspecific fear response. Accordingly, if the enhanced fear memory is expressed only when stressed animals are exposed to the CS, it may indicate that this response is sustained by associative learning, and thus the increased freezing behavior of stressed animals could be attributable to an attenuation of the extinction process, rather than to enhanced fear acquisition, although the latter remains a possibility [4].

It is usually assumed that stressful life events interfere with our ability to acquire new information. Yet, previous exposure to both acute and chronic stressful events can positively affect classical conditioning tasks, including fear conditioning [29–33]. Reports to date regarding the effects of stress on fear extinction show that exposure to stress increases resistance to extinction, that is, it impairs extinction acquisition and consolidation, which reduces the extent to which extinction is able to offset a fear response. In contrast, studies addressing the relationship between stress and the acquisition of new fear memories show that exposure to a stressful experience facilitates fear learning, so further enhancing the fear response. For example, previous exposure to a restraint session increased fear conditioning in a contextual fear paradigm [33]. Similarly, Rau et al. [34] have shown that preexposure to a stressor of repeated foot shocks enhanced conditional fear responses to a single context-shock pairing. Cordero et al. [29] have shown that a single exposure to an aversive stimulus is sufficient to facilitate context-dependent fear conditioning, and suggested increased glucocorticoid release at training in the mechanisms mediating the memory-facilitating effects induced by prior stressful experiences. These studies corroborate others showing that if an animal learns a stressful task, then the consolidation of this task may be enhanced by stress and that its end product, corticosterone, may be secreted during the task [35–37]. This was found to be the case in a variety of emotionally arousing tasks, such as inhibitory avoidance, spatial learning, discrimination learning, and fear conditioning [38–44].

3. THE NEURAL BASIS OF FEAR EXTINCTION

The basolateral amygdala (BLA) plays a pivotal role in the consolidation of memories related to fear and emotions, and in the initiation of responses to stressful events [37, 45–50]. Moreover, the BLA is significantly involved in both the formation and extinction of fear memory [17, 51–54]. For example, microinfusions of a protein synthesis inhibitor to the amygdala prevented recall of extinction after 30 minutes, and infusion of N-methyl-D-aspartate (NMDA) receptor antagonists or mitogen-activated protein kinase inhibitors to the BLA prevented across-day extinction of fear-potentiated startle [17, 54–56]. In another study [57], BLA lesions severely attenuated expression of previously acquired fear memory. Also, infusion of an NMDA agonist into the amygdala facilitated fear extinction [58, 59].

Another brain structure that is known to play an important role, not only in the regulation of emotion, but also in the integration of affective states with appropriate modulation of autonomic and neuroendocrine stress regulatory systems [60], is the medial prefrontal cortex (mPFC). The mPFC provides an interface between limbic and cortical structures [61] and regulates the stress-induced activity of the hypothalamus-pituitary-adrenal (HPA) axis [62, 63].

The mPFC is important in long-term fear extinction memory. Specifically, lesions or inhibition of protein synthesis in the infralimbic part of the medial PFC impair recall of extinction of conditioned fear [18, 19, 64, 65]. Furthermore, mPFC stimulation that mimics extinction-induced tone responses reduces conditioned fear [66, 67], and stimulating

the mediodorsal thalamic inputs to the mPFC is associated with extinction maintenance [68, 69]. Moreover, functional imaging studies in human subjects indicate that the mPFC is engaged during extinction [70] and that subjects with PTSD have reduced mPFC activity during trauma recall [71]. Furthermore, Miracle et al. [72] have shown that one week of restrained stress had the effect of impairing recall of extinction of conditioned fear, and suggested that this is due to deficits in the mPFC caused by exposure to stress. Recently, it has been reported that stress exposure that impairs fear extinction also caused retraction of terminal branches of apical dendrites of infralimbic neurons [25].

4. THE ROLE OF GABA IN EXTINCTION OF FEAR

In addition to evidence indicating that extinction of fear memory requires plasticity in both the mPFC and the BLA [17–19], recent studies further point to a dysfunctional interaction between the prefrontal cortex and the amygdala in the failure to extinguish conditioned fear. These studies indicate that the mPFC has a function in the inhibition of emotions through its projections to the amygdala [73] and are in line with Pavlov's [74] view that extinction learning involves inhibitory cortical circuits that reduce the CS-evoked conditioned response.

The glutamatergic efferents from the mPFC synapse on amygdala gamma-aminobutyric acid (GABA)ergic neurons [75], and through this, may provide important inhibitory input to the amygdala. Of particular interest is the projection from the infralimbic region of the PFC (which, together with the prelimbic cortex, comprises the ventromedial PFC) to the capsular division of the central nucleus of the amygdala [76]. The capsular division of the central nucleus contains GABA-ergic intercalated cells that have been shown to exert powerful inhibitory control over central nucleus neurons that project out of the amygdala [77–79]. Infralimbic input to intercalated cells could be a pathway by which infralimbic tone responses inhibit the expression of conditioned fear (e.g., reduce freezing) [80].

The anatomical data described for the interaction between these two structures pinpoint the crucial role the neurotransmission of GABA may play in the extinction of fear. Indeed, a substantial number of studies have demonstrated that the BLA contains a powerful inhibitory circuit that uses GABA as a neurotransmitter [81–83]. Moreover, the BLA has larger amounts of benzodiazepine/$GABA_A$ receptors than any other amygdala nucleus [84], explaining why the infusion of benzodiazepines or $GABA_A$ agonists into the BLA reduces fear conditioning and anxiety [85–88]. Coincidently, local blockade of these receptors attenuates the anxiolytic influence of systemic benzodiazepines [89]. Recently, Rodríguez Manzanares et al. [33] have shown that stress attenuates inhibitory GABA-ergic control in the BLA, leading to neuronal hyperexcitability and increased plasticity that facilitates fear learning. Based on these data, it can be concluded that GABA-ergic mechanisms in the amygdala play a major role in controlling the emotional consequences of stress, and may thus affect extinction of fear.

Benzodiazepines have long been used to treat anxiety and are particularly appropriate in short-term treatment situations [8]. Direct modulation of GABA-ergic neurons, through the benzodiazepine-binding site, down regulates memory storage processes and specifically affects learned fear responses. On the other hand, benzodiazepine release could be modulated by the anxiety and/or stress associated with different types of learning [90].

Much research is directed at exploring the involvement of GABA in inhibiting learned fear responses. Although several studies support the central role GABA neurotransmission plays in extinction, there are different reports regarding whether this role is to facilitate or impair extinction [26, 91–95]. Using direct modulation of GABA-ergic neurons, it has been shown that the benzodiazepine inverse agonist FG7142, which attenuates the effect of GABA at its receptor, retards extinction of conditioned fear [91, 96]. Likewise, McCabe et al. [97] have shown that benzodiazepine agonists administered to mice following training significantly facilitated extinction during a food-reinforced lever-press procedure. Potentiation of GABA by the benzodiazepine agonist chlordiazepoxide administered prior to extinction sessions facilitated extinction in a paradigm of operant responding for food reinforcement [98]. By contrast, systemic administration of the $GABA_A$ antagonist picrotoxin, after the extinction of inhibitory avoidance learning, enhanced extinction retention during testing [93], and the $GABA_A$-positive allosteric modulator diazepam impaired extinction retention when administered before extinction in a shuttle avoidance task [95].

There are also a number of ways of modulating GABA-ergic functions indirectly. For example, cannabinoid (CB1) receptors and gastrin-releasing peptide receptors are both located on GABA-containing interneurons. Endogenous cannabinoids, acting at the CB1 receptor, facilitated the extinction of aversive memories [92], and blocking the action of gastrin-releasing peptide, by genetically removing its receptor, retards extinction of learned fear responses [26]. Recently, Azad et al. [99] have shown that CB1 receptors reduce GABA-ergic synaptic transmission in the amygdale, and consequently facilitate extinction of aversive memories. Chhatwal et al. [100] showed that gephyrin mRNA and protein levels in the BLA significantly increased after fear extinction training, suggesting that the modulation of gephyrin and $GABA_A$ receptor expression in the BLA may play a role in the experience-dependent plasticity underlying extinction.

Using a low dose of the $GABA_A$ agonist muscimol, we recently found [51] that muscimol infused to the infralimbic area before extinction training (see Figure 2(a)) resulted in long-term facilitation of extinction. By contrast, where infusion of muscimol to the infralimbic area followed extinction training, no such effect was observed, regardless of the length of the extinction training period (5 or 15 trials; data not shown). However, infusion of muscimol to the BLA following a short (5-trial) extinction session facilitated extinction for at least 48 hours post-drug-infusion (see Figure 2(b)). The differences between the temporal parameters of the effects of muscimol in the infralimbic cortex compared to the BLA suggest differential involvement of these structures

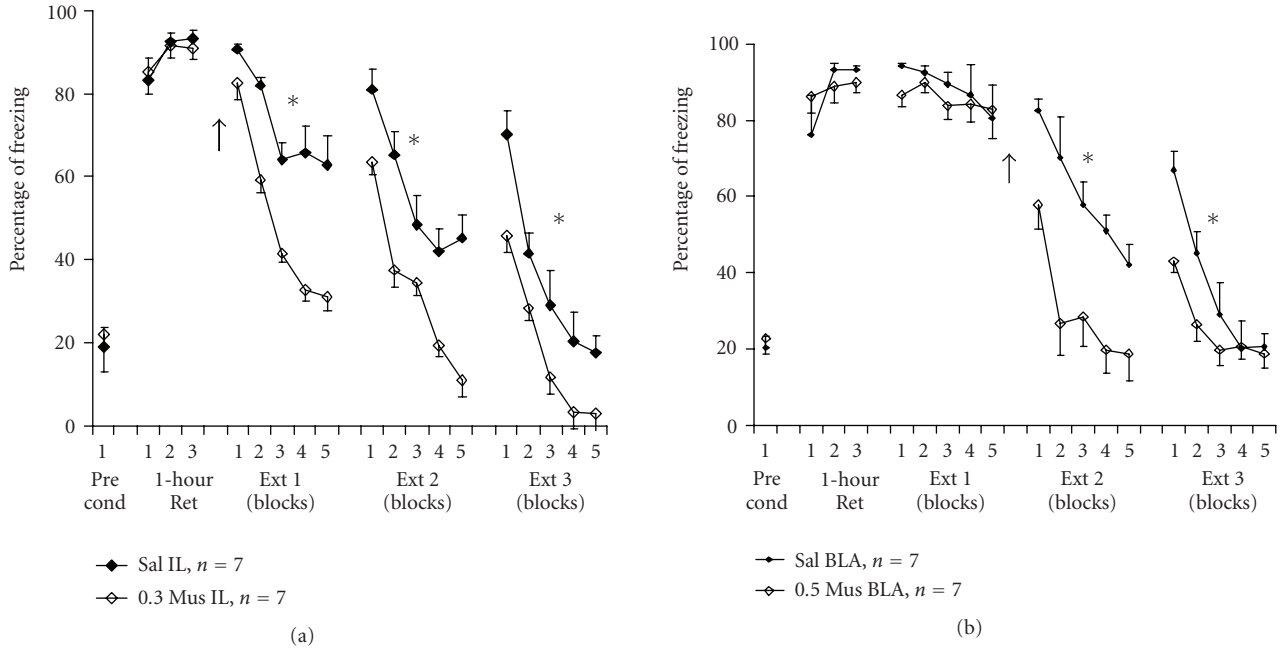

Figure 2: (a) *A low volume of muscimol microinfused into the infralimbic cortex before extinction training facilitates extinction learning.* Rats received 7 pairings of a tone with a foot shock in the conditioning chamber. After 1 hour, three tones were delivered in the absence of foot shock (1-hour Ret). On the next day, the animals were microinfused with a total of 0.3 μl saline (Sal) or muscimol (0.3 Mus) to the infralimbic cortex (IL) and were exposed to 15 tones without foot shocks (Ext 1; presented as 5 blocks of 3 trials). Animals were exposed to additional 15 tones on days 4 (Ext 2) and 5 (Ext 3), without further administration of the drug. Muscimol IL animals showed significantly lower levels of freezing compared with the saline group in Ext 1 ($*$; $P < .001$), Ext 2 ($*$; $P < .01$) and Ext 3 ($*$; $P < .05$). This supports a selective involvement of the IL in facilitating extinction of conditioned fear (see Akirav et al. [51]). Arrow denotes time of drug infusion. The Pre cond data points indicate the amount of freezing exhibited by rats prior to commencement of fear conditioning. (b) *A low volume of muscimol microinfused to the basolateral amygdala following a short extinction training session facilitates extinction consolidation.* Rats received 7 pairings of a tone with a foot shock in the conditioning chamber. After 1 hour, three tones were delivered in the absence of foot shock (1-hour Ret). On the next day, the animals underwent a short extinction training session consisting of 5 tones (Ext 1; presented as 5 trials), and were thereafter microinfused with a total volume of 0.5 μl saline (Sal) or muscimol (0.5 Mus) to the basolateral amygdala (BLA). On days 4 and 5 (Ext 2 and Ext 3, resp.), the animals were exposed to 15 tones without foot shocks (presented as 5 blocks of 3 trials). The BLA muscimol group showed significantly reduced levels of freezing compared with the other two groups during Ext 2 ($*$; $P < .001$) and Ext 3 ($*$; $P < .05$). This supports the selective involvement of the BLA in facilitating consolidation of extinction of conditioned fear (see Akirav et al. [51]). Arrow denotes time of drug infusion. The Pre cond data points indicate the amount of freezing exhibited by rats prior to commencement of fear conditioning.

in long-term extinction of fear memory. We propose that $GABA_A$ neurotransmission in the infralimbic cortex plays a facilitatory role in triggering the onset of fear extinction and its maintenance, whereas in the BLA, $GABA_A$ neurotransmission facilitates extinction consolidation.

Overall, the data suggest that manipulation of GABA transmission may have very different effects depending on whether it is administered pre- or postextinction training or before a retention test, and depending also on the behavioral paradigm used. Future studies are required to understand these discrepancies.

While examining the involvement of GABA in the effects of stress on fear extinction, we found that systemic administration of the benzodiazepine agonist diazepam reversed the resistance to extinction induced by exposure to an out-of-context stressor (see Figure 3). After classical auditory fear conditioning (3 CS-US pairings of a tone with a foot shock of 0.5 mA), control rats that were exposed to the tone without shock gradually extinguished their freezing (CR) in response to the tone during extinction training. At the end of the third extinction session, their freezing levels dropped to zero. Rats that were exposed to an out-of-context stressor (i.e., animals that were placed on an elevated platform for 30 minutes) before the first extinction training session showed increased levels of freezing in response to the tone even 48 hours after the stressor (i.e., showed resistance to extinction). A single injection of diazepam (2 mg/kg, IP) 20 minutes before exposure to the out-of-context stressor significantly facilitated extinction compared with the control and stress groups as manifested by reduced freezing levels in the first extinction session. On the second and third sessions of extinction training, the response of the diazepam-stress group was no different to that of the control group, with the former group also exhibiting significantly less freezing than the stressed rats that

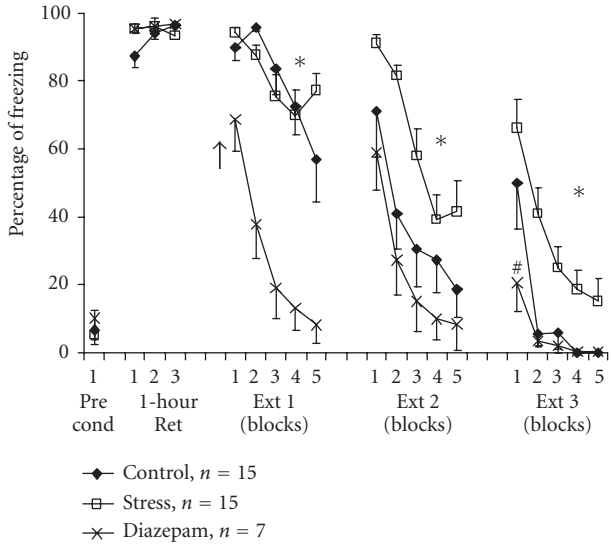

Figure 3: *Diazepam overcomes stress-induced impairment of the extinction of auditory fear.* Rats were exposed to 3 pairings of a tone with a mild foot shock in the conditioning chamber. On the next day, control animals remained in their home cages, "diazepam" group animals were injected with diazepam (2 mg/kg, IP) 20 minutes before being placed on an elevated platform for 30 minutes, while "stress" group animals were placed directly onto the elevated platform for 30 minutes, without prior administration of the drug. Immediately afterwards, animals were taken for extinction training and were exposed to15 tones (Ext 1) with no shock. Animals were exposed to an additional 15 tones on days 3 (Ext 2) and 4 (Ext 3) with no drug or shock. There were significant differences between the diazepam group and the other groups during Ext 1 ($P < .001$). On Ext 2 and Ext 3, the stress group was significantly different from the control (Ext 2: $P < .05$, Ext 3: $P < .01$) and the diazepam (Ext 2: $P < .01$, Ext 3: $P < .001$) groups. Arrow denotes time of drug infusion. The Pre cond data points indicate the amount of freezing exhibited by rats prior to commencement of fear conditioning.

had not first received diazepam. Hence, treatment with diazepam reversed the impairing effect of exposure to stress on fear extinction. Further experiments to elucidate the possible role GABA plays in the BLA and the mPFC in preventing stress-associated impairments of extinction are required.

A problem associated with the use of anxiolytic and anxiogenic compounds in studies of extinction, however, is the possibility of state dependency as opposed to a true effect on the suppression of the learning process [101]. That is, it is possible that a drug administered before or immediately following extinction produces an internal state, or drug context, that is discriminable to the animal [102]. However, in our experiment, the effect was probably not due to state dependence because the stressed animals that were treated with diazepam showed less freezing (i.e., more extinction) than the stressed animals that were treated with saline, even 24 and 48 hours after a single injection.

To conclude, the present results demonstrate that pretreatment with the benzodiazepine tranquilizer diazepam reverses the CR-enhancing effects of the elevated platform experience. These findings suggest that benzodiazepines may prevent the augmentation of the trauma-related symptoms seen in phobia and PTSD patients that are caused by exposure to a stressful experience.

5. EXTINCTION OF FEAR: INTERPLAY FOR DOMINANCE BETWEEN THE AMYGDALA AND THE PREFRONTAL CORTEX

Recent observations provide direct physiological support that the mPFC reduces fear responses by reducing amygdala output [66, 103, 104]. For example, Milad and Quirk [66] found that stimulation of the mPFC decreases the responsiveness of central amygdala neurons that regularly fire in response to the CS only when animals are recalling extinction of a fear task learned using that CS. Additionally, Morgan et al. [64] reported that rats with mPFC lesions had an increased resistance to extinction. They proposed that connections between the mPFC and amygdala normally allow the organism to adjust its emotional behavior when environmental circumstances change, and that some alteration in this circuitry, causing a loss of prefrontal control of the amygdala, might underlie the inability of persons with anxiety disorders to regulate their emotions.

If the mPFC normally inhibits the amygdala as an active component of extinction of fear conditioning, then when the mPFC is inhibited or suppressed, emotional associations mediated by the amygdala may be not inhibited during nonreinforcement. As a result, conditioned responding may be prolonged over time [64].

A combination of changes throughout this circuit is important in generating stress-induced changes in emotionality. The mPFC may have a regulatory role in stress-induced fear and anxiety-like behaviors through inhibitory effects on amygdala output and processing [105]. Indeed, extensive evidence supports the notion that the BLA is a site of plasticity for fear conditioning [104, 106], and that the BLA is extensively connected with the central nucleus of the amygdala [107, 108]. In turn, the central nucleus projects to the paraventricular nucleus of the hypothalamus [109], thereby providing the most likely route for any BLA-dependent effects on stress-induced HPA output.

We would like to take this a step further, and suggest a possible mode of action for the mPFC-amygdala circuit in fear extinction under stressful conditions. Accordingly, under normal conditions of fear suppression, the mPFC is activated and inhibits amygdala output. This dominance of the mPFC results in normal suppression of fear, and in consequence promotes extinction of fear. However, exposure to a stressful experience may reduce medial PFC inhibition of the amygdala, and as a result the amygdala takes control to assure defensive behaviors and becomes dominant. The expected consequence is interference in the suppression of the fear response, that is, impaired extinction learning. Therefore, exposure to a stressful experience would result in reduced mPFC activity leading to resistance to extinction and inappropriate and exaggerated fear responses, as seen in

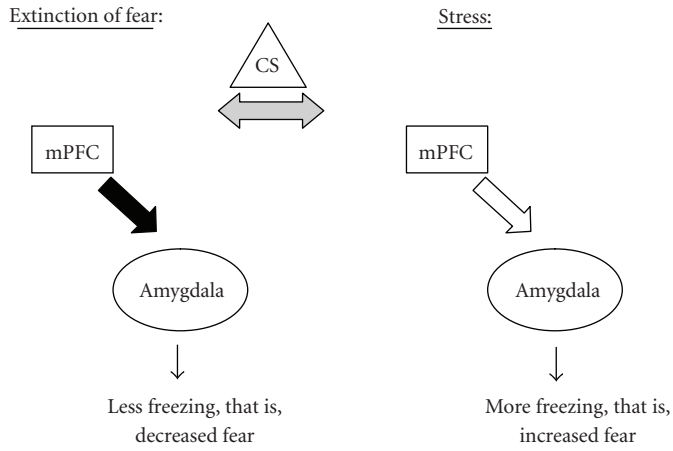

Figure 4: *A possible mode of action for the medial prefrontal cortex-amygdala circuit in fear extinction under normal and stressful conditions.* Under normal conditions of fear suppression, the medial prefrontal cortex (mPFC) is activated and inhibits amygdala output (filled arrow). This dominance of the mPFC results in less freezing in response to a conditioned stimulus (CS; i.e., extinction). However, under stressful conditions, the inhibitory action of the mPFC on the amygdala is reduced (empty arrow), the amygdala dominates (indicated by the bold circle around the amygdala) and the result is more freezing in response to a CS (i.e., impaired extinction).

PTSD patients. Indeed, abnormally low PFC activity together with abnormally high amygdala activity were found in PTSD patients, when reexposed to traumatic reminders [110]. Accordingly, deficits in extinction of conditioned fear as a result of exposure to a stressful experience are proposed to contribute to the sustained anxiety responses seen in PTSD.

Figure 4 schematically summarizes this idea and shows that during extinction of fear, the mPFC is activated and acts to inhibit the amygdala in order to reduce fear, resulting in less freezing (i.e., extinction). However, exposure to stress at a critical time with respect to extinction learning activates the amygdala to increase fear and the result is more freezing (i.e., resistance to extinction). Therefore, according to our proposed model, the stressor shifts the dominance from the mPFC to the amygdala and, as a consequence, extinction of fear is impaired.

Our model is consistent with the data shown in Figure 1, which demonstrate that exposure to a stressful experience results in resistance to extinction in the stressed group compared with the nonstressed group. Whether this effect is due to a reduction in mPFC modulation of amygdala output, and to the involvement of GABA-based mechanisms acting on the PFC-amygdala circuit, still needs to be examined. Our model is also consistent with the suggestion put forward by Quirk and Gehlert [111] that deficient inhibitory tone in the amygdala due to decreased inhibition from the prefrontal cortex could lead to overexpression of conditioned responses, producing pathological states such as anxiety disorders and drug-seeking behavior.

6. PERSPECTIVES

Pathological fear and anxiety, such as that exhibited by PTSD sufferers, may be the manifestation of abnormal modulations in the activity of the amygdala and the mPFC, and in their interaction. PTSD is defined as symptoms of reexperiencing the trauma, avoidance of associated stimuli and hyperarousal symptoms, suggesting a heightened fear response, and it has been proposed that PTSD symptoms reflect amygdala hyperresponsivity to fear-related stimuli, with a concomitant lack of "top-down" prefrontal inhibition. This proposal is supported by neuroimaging studies of PTSD patients, which observed abnormal reductions in mPFC activity [71, 112, 113], as well as enhanced and distinctive amygdala engagement [114, 115], particularly for combat PTSD veterans [113]. In line with this, fMRI and PET data have shown significant inverse correlations between the functional activity of the mPFC and the amygdala [116, 117]. Collectively, these data provide strong support for the hypothesis that PTSD is characterized by a failure of the mPFC to sufficiently inhibit the amygdala.

There is clinical interest in the effects of stress on fear extinction learning as a model for the mechanisms operating in PTSD, as well as interest in means to improve therapeutic outcomes following fear-extinction-based strategies. Future therapies aimed at increasing the inhibitory tone in the amygdala, either locally or via the prefrontal cortex, may accelerate extinction and may help in the treatment of anxiety disorders.

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Review Article

Neurobiology of Memory and Anxiety: From Genes to Behavior

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Interaction of anxiety and memory represents an essential feature of CNS functioning. This paper reviews experimental data coming from neurogenetics, neurochemistry, and behavioral pharmacology (as well as parallel clinical findings) reflecting different mechanisms of memory-anxiety interplay, including brain neurochemistry, circuitry, pharmacology, neuroplasticity, genes, and gene-environment interactions. It emphasizes the complexity and nonlinearity of such interplay, illustrated by a survey of anxiety and learning/memory phenotypes in various genetically modified mouse models that exhibit either synergistic or reciprocal effects of the mutation on anxiety levels and memory performance. The paper also assesses the putative role of different neurotransmitter systems and neuropeptides in the regulation of memory processes and anxiety, and discusses the role of neural plasticity in these mechanisms.

1. INTRODUCTION

Pathologic anxiety is a complex stress-related disorder which includes generalized anxiety, panic, social anxiety, agoraphobia, posttraumatic stress, and obsessive-compulsive disorders [1–5]. There are many animal (experimental) paradigms that model different subtypes of human anxiety [6–10]. In addition to anxiety, stress has long been known to affect animal and human cognitions [11–14], raising the possibility that memory and anxiety interact.

Numerous studies have outlined behavioral, physiological, pharmacological, and genetic aspects of memory-anxiety interaction [13, 15–20]. Since memory consolidation and anxiety both require brain arousal, it has been considered as promnestic and anxiogenic, whereas brain inhibition is amnestic and anxiolytic; review [12, 21, 22]. However, classic works of Yerkes and Dodson [14], as well as many subsequent studies [23–30], have shown that memory and stress interplay in a more complex, type-specific, and nonlinear manner. Here we will analyze the available clinical and experimental data in order to examine (with a particular focus on neurogenetics) the links between anxiety and memory functions.

Transgenic and mutant animals, including tissue-specific and inducible knockout mice, represent a valuable tool for biomedical brain research [31–34] powered by extensive on-line databases [8, 9]. Table 1 summarizes anxiety and memory/learning phenotypes in various genetically modified mouse models, including mutant mice lacking or overexpressing receptors of various neuromediators, neuropeptides, and some brain proteins mediating neuroplasticity. Several important conclusions can be made based on these findings. A common situation when the same mutation leads to altered anxiety and memory phenotypes (Table 1) confirms overlapping of the two domains at genetic (in addition to behavioral and pharmacological [12, 13]) levels. While many mutants show synergetic alterations of memory and anxiety, there are also data on reciprocal effects of some mutations (Table 1), confirming a complex nonlinear nature of memory-anxiety interplay. Moreover, as can be seen in this table, different subtypes of memory seem to be differentially influenced by altered anxiety, further contributing to the complexity of the problem discussed here. While this paper will not offer a simple solution for complex animal or human phenotypes, its aim is to discuss how different brain systems may interact in determining anxiety and memory phenotypes.

2. NEUROCHEMISTRY AND NEUROGENETICS OF MEMORY AND ANXIETY

Cholinergic synaptic transmission has long been implicated in learning, memory, and anxiety [36, 92]. Neuronal nicotinic (N) acetylcholine (ACh) receptors are hetero-oligomers

Table 1: Mouse mutagenesis data on memory and anxiety phenotypes [8]; see text for details. KO: knockout (−/−), HZ: heterozygous (+/−) mice. (↑: increased, ↓: reduced, 0: no effects, ↔: mixed or unclear results. CRF: corticotropin-releasing factor, MAO: monoamine oxidase A/B, FXR1: fragile X-related protein 1, PACAP: pituitary adenylate cyclase activating polypeptide, Rab3a: *ras*-associated binding 3a protein.)

Mouse models		Effects on		References
		Anxiety	Memory/learning	
Neurotransmitters Acetylcholine	N-receptor α4 subunit KO mice	↑	↓ within-trial habituation	[35]
	N-receptor α7 subunit KO mice	0 (↓)	0 fear conditioning, spatial learning	[36]
	N-receptor β2 subunit KO mice	—	↓ avoidance learning, 0 spatial learning	[37]
Serotonin	5HT-1B receptor KO mice	↓	↑ long-term and short-term memory, 0 habituation	[38–42]
	5HT-1A receptor KO mice	↑	↓ hippocampal-dependent learning, 0 habituation	[40, 43–45]
	5HT-5A receptor KO mice	↓	0 inter- and within-trial habituations	[46]
	Serotonin transporter KO mice	↑	↔ within-trial habituation	[47]
GABA (also see Table 2)	GABA-A α5 subunit KO mice	0	↑ hippocampal-dependent trace conditioning, 0 delayed or contextual conditioning	[48]
	GABA-A γ2 subunit HZ mice	↑	↑ cued fear conditioning, 0 spatial memory	[49]
Histamine	Histamine H3 receptor KO mice	↓	0 habituation, ↑ spatial memory and learning, higher resistance to amnestic effects of scopolamine	[50, 51]
Glycine	Glycine transporter 1 brain-selective disruption	0	↑ aversive Pavlovian conditioning	[52]
Glutamate	B subunit ionotropic receptor KO mice		↓ olfactory memory (rescued by selective expression in hippocampus)	[53]
	Metabotropic subtype 7 receptor KO mice	↓	↓ cued fear response and conditioned taste aversion	[54]
	A type receptor KO mice	↑	↓ spatial working memory (alternation)	[55]
Related models	MAO B targeted inactivation	↑	0 working memory, ↓ long-term memory	[56]
	MAO A/B KO mice	↑	0 within-trial habituation	[57]
Neuropeptides and other brain proteins	CRF receptor 1 KO mice	↓	↓ spatial recognition memory	[58]
	Thyroid hormone α1 receptor mutations	↑	↓ olfactory recognition memory, contextual fear conditioning	[59, 60]
	Neuropeptide Y KO mice	↓	↓ attention training test performance	[61]
	Brain-derived neurotrophic factor (mice)	↔	↔	Table 3
	Glial protein S100B KO mice		↑ fear conditioning, spatial memory	[62]
	Protein kinase Cγ KO mice	↓	↓ spatial and contextual learning	[63, 64]
	FXR1 KO mice	↓	↓ fear conditioning, spatial memory, 0 habituation	[65]
	Modified β-amyloid precursor KO mice	↑	↓ spatial learning, habituation	[66]
	PACAP-type 1 receptor KO mice	↓	↓ associative learning	[67, 68]
	Rab3a KO mice	0 ↓	↓ cued fear conditioning 0 acquisition, mild ↓ spatial reversal learning and spatial working memory	[69] [70]
	Rab3a loss-of-function mutant mice	↓	↓ cued fear conditioning	[69]

(formed by five of 11 known α and β subunits) mediating anxiolytic-like effect of nicotine [35]. Their loss has also been noted for Altzheimer's and Parkinson's patients with impaired cognitive functions [35], collectively implicating these receptors in both memory and anxiety. In line with this, increased anxiety and impaired memory were reported in mice lacking α4 subunit of N-type Ach receptor (Table 1). Mice lacking the receptor's β2 subunits (predominant in hippocampus) showed impaired avoidance learning, but normal spatial learning in Morris water maze [37]. Surprisingly, ablation of α7 subunits (also rich in hippocampus) leads to no or very mild alterations in anxiety (open field test) and memory (unaltered acoustic startle habituation and Pavlovian conditioning, but faster finding a platform in the Morris water maze) [36]. Taken together, this suggests that various subtypes of ACh receptors may play different roles in memory-anxiety interplay. Notably, RS-1259, a newly synthesized inhibitor of acetylcholinesterase [93], elevated ACh levels in hippocampus and improved memory in mice, suggesting that targeting brain ACh may lead to effective therapy of neurodegenerative disorders. The same drug also inhibited serotonin transport [93], implying that altered

Table 2: Clinical and preclinical data linking common GABAergic brain areas to pathogenesis of anxiety and depression.

Clinical data	Animal data
Amygdala (anxiety, memory)	
Activation in patients with posttraumatic stress disorder [71], during anticipatory anxiety [72], in adults and adolescents viewing fearful faces; also positive correlation of amygdalar activation and social anxiety scores [73–75].	Reduced anxiety and memory in rats following muscimol injection [76–78]. Reduced expression of GABA-A receptor associated protein[(a)] after fear conditioning in rats [79]. Increased c-fos expression[(b)] in rats following anxiogenic drugs [10]. Correlation between anxiety phenotype and reduced GABA-A receptor densities, benzodiazepine binding, and $\gamma 2$ subunit mRNA levels in mice and rats [80–82]. Altered amygdalar electric activity during fear conditioning in mice [83]. Reduced extracellular GABA in mice exposed to conditioned fear stimulus [84].
Hippocampus (memory, anxiety)	
Reduced blood flow in anxious volunteers during phobogenic (versus neutral) visual stimulation [85]. Decreased blood flow in right hippocampus in women with posttraumatic stress disorder [86]	Reduced expression of $\alpha 2$ GABA-A receptor subunit 6 hours after fear conditioning in rats [79]. Correlation between anxiety and altered benzodiazepine binding in rats [27, 82]. Reduced expression of $\alpha 1$ and $\alpha 2$ subunits mRNA in punished rats [87]. Altered volume in anxious HAB (versus low-anxiety LAB) rats [88]. Increased c-fos expression in rats following administration of anxiogenic drugs [10]. Reduced hippocampal allopregnanolone levels in anxious high-vocalizing rats [89]. Correlation between mouse spatial learning abilities and GABA-A receptor densities [90]. Disrupted context-specific fear memory in rats following muscimol injection [91].

[(a)] Modulates channel kinetics and neurotransmission by promoting GABA-A receptor clustering.
[(b)] Genetic marker of neuronal activation.

serotonergic system may also contribute to these effects (see further).

Gamma-amino butyric acid (GABA) is the primary mediator of inhibitory neurotransmission, acting through ionotropic A and metabotropic B type receptors. GABA-A receptors are Cl-channels composed of five subunits (from eight families: $\alpha 1$-$\alpha 6$, $\beta 1$-$\beta 3$, $\gamma 1$-$\gamma 3$, δ, ε, π, θ, and $\rho 1$-$\rho 3$) with multiple binding sites for positive (GABA agonists, barbiturates, benzodiazepines, steroids, and ethanol) and negative (GABA-A antagonists, neurosteroid antagonists, benzodiazepine inverse agonists, and chloride channel blockers) modulators [4, 12, 94–97]. GABA has long been implicated in anxiety [80, 97–101]. In both humans and animals, positive modulators of GABA receptors generally possess anxiolytic activity while negative modulators produce anxiogenic-like effects. Moreover, various GABA analogs and agents affecting transmitter metabolism to enhance GABAergic tone have been reported to exert anxiolytic effects [98, 102–107]. The role of GABA in learning and memory has also been widely recognized [28–30, 90, 100, 108–112]. Three comprehensive reviews particularly [12, 17, 113] emphasize the role of central GABA in memory-anxiety interplay, noting amnestic/anxiolytic effects of positive, and opposite profiles of negative, GABA modulators (also see [27–30, 111, 114, 115] for details).

Mounting neurogenetic data further implicates GABA in memory and anxiety. GABAergic genes are associated with anxiety ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\beta 1$, $\gamma 1$, and $\gamma 2$) [95, 96, 116, 117] and memory ($\alpha 5$) [48, 49, 118]; see Table 1. Downregulation of $\alpha 1$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\gamma 1$, δ genes was reported in anxious versus nonanxious rat strains [119]. Other studies show reduced expression of rat $\alpha 2$, $\gamma 1$, or δ subunits after fear conditioning [79] and chronic unpredictable stress [120]. In humans, treatment-resistant depression with anxiety was linked to a mutant $\beta 1$ subunit gene [121], whereas positive genetic associations were found between GABA-A subunits genes and neuroticism ($\alpha 6$ [122]), posttraumatic stress disorder with anxiety and depression ($\beta 3$ [123]), and hormonal/autonomic stress responses ($\alpha 6$ [124]).

Recent clinical and experimental data outline the role of GABA and GABA-ergic genes in amygdala and hippocampus (Table 2); the brain areas involved in the regulation of both memory and anxiety [125, 126]. In addition to receptors, these domains are also influenced by GABA metabolism. While specific amygdalar reduction in expression of GABA-synthesizing enzyme was observed in animals during learning [126], spatial learning was impaired in rats following anxiolytic GABA transporter inhibitor tiagabine [127]. Collectively, these findings confirm that central GABA is a key mediator regulating anxiety and memory, and that GABAergic genes, metabolism, and/or subunit-specific GABAergic drugs [100, 128–132] may modulate such interplay.

Glutamate receptors mediate most excitatory CNS neurotransmission. There are several known subtypes of metabotropic glutamate receptors which are coupled to G-proteins and exert their effects via second messenger signaling pathways. Genetic ablation of glutamate subtype 7 receptors in mice impairs their performance in two distinct amygdala-dependent paradigms [54] and inhibits hippocampal neurotransmission [133], suggesting that both structures are involved in glutamate-mediated mechanisms of memory and anxiety. Consistent with this, glutamate receptor densities

positively correlate with spatial learning abilities in mice [90].

Several recent clinical and experimental data also show that central dopaminergic system plays a role in the regulation of memory and anxiety, including fear conditioning [134, 135]. In line with this, a recent quantitative trait loci study showed that cognitive functions (intertrial habituation) of 25 inbred mouse strains were linked to a region on chromosome 15 mapping dopamine D1 and D2 receptors [136].

Serotonin and its receptors have long been implicated in memory and anxiety in both humans [38, 122, 134, 137, 138] and animals [1, 139–144]. In addition to receptors (Table 1), other factors include serotonin homeostasis and metabolism. Serotonin is removed from the synaptic cleft by a specific membrane transporter protein (SERT [31, 145]), representing an important target for various manipulations. For example, pharmacological inhibition of SERT leads to elevated hippocampal serotonin levels and improved memory [93]. While genetic ablation of SERT in mice is widely used as a model of anxiety [47, 145–148], these mice display increased poststress responsivity [149], indirectly implying a better memory for aversive stimuli. Clearly, further studies are needed to assess the link between SERT and cognitive abilities in animals, and its relevance to human brain dysfunctions. Overall, human anxiety-related traits seem to generally facilitate cognitive functions (e.g., acquisition of conditioned fear), and such interplay is partially serotonergically mediated [134].

Strengthening this notion, genetic variations in SERT have been linked to strain differences in emotional learning in rats [150]. In humans, SERT has also been implicated in anxiety and cognitions. For example, SERT polymorphisms have been associated with anxiety-related personality traits [122, 151], amygdalar reactivity [152–154], cognitive abilities [36, 155], and altered hippocampal neurochemistry [137]. In line with this, Caspi et al. [156] recently established that human SERT polymorphisms modulate the effect of life stress on stress-related CNS pathogenesis, while Fox et al. [157] found association of SERT polymorphisms with children behavioral inhibition—a temperamental construct predicting anxiety.

Importantly, brain catecholamines do not act individually in the brain, interact at different levels with each other, and with other brain molecules [147, 148]. Antipanic drug phenelzine (a nonselective inhibitor of monoamine oxidase MAO A/B which elevates brain norephinephrine, dopamine, and serotonin levels) also exerts mnemotropic effects [19]. MAO A/B knockout mice (demonstrating phenotype similar to the effect of phenelzine) display robust anxiety phenotype but unaltered working memory (Table 1), as assessed by their open field habituation [57]. In contrast, MAO B inactivation in mice leads to increased anxiety, unaltered spatial working memory in Y-maze, but reduced habituation to the forced swim test 4 weeks after the initial trial [56]. Collectively, these data confirm the notion that anxiety and memory phenotypes are heterogeneous and may be determined by interactions of various mediator systems. For example, Birzniece et al. [114] recently analyzed the interplay between GABA-active steroids and serotonin in modulating cognitive functions, and Sibille et al. [45] found reduced GABAergic tone in anxious serotonin 5HT-1A receptor knockout mice, also displaying memory deficits[44].

3. NEUROPEPTIDES AND NEURAL PLASTICITY ISSUES

In addition to mediators, brain neuropeptides play a key role in modulation of memory and anxiety. For example, mutants lacking receptors of "anxiogenic" cotricotropin releasing factor (CRF) display a predictable reduction of anxiety accompanied by reduced cognitive performance during the retrieval trial in the Y-maze (Table 1). Overall, these findings are in line with numerous data implicating CRF in both anxiety and memory, and suggest that novel antistress mnemotropic drugs may be created based on targeting central CRH system [58, 167]. In contrast, mutant mice with reduced sensitivity of thyroid receptors [60] display increased anxiety but reduced memory (Table 1), demonstrating that not always various manipulations exert synergetic effects on these two processes. Interestingly, while CRF has been traditionally linked to memory and anxiety, nonanxiogenic doses of CRF type 1 and 2 receptor agonist urocortin produced anxiety (accompanied by amygdalar hyperexcitability) after 5 daily intra-amygdalar infusions in rats [168]. These results indicate that CRF-induced synaptic plasticity, in addition to anxiety and memory processes, may be involved in pathogenesis of emotional disorders (also see [169] for review).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is another important regulator of synaptic plasticity, neurotrophins, neuromediators, and neuronal differentiation [67, 68]. It binds to a highly selective type 1 receptor (PAC1), widely distributed in the limbic system, including amygdala and hippocampus. Since mice lacking PAC1 demonstrate reduced anxiety and impaired memory (Table 1), PACAP/PAC1 system may be directly involved in the regulation of memory-anxiety interplay. Clearly, further studies are needed to explore this interesting aspect in detail, including its relation to PACAP/PAC1-mediated neuroimmuno-modulation and neuroprotection [170] and impairment in mossy fiber long-term potentiation [68].

Glial Ca-binding protein S100B also plays an important modulatory role in memory. S100B knockout mice display strengthened synaptic plasticity, enhanced long-term potentiation, and spatial memory in Morris water maze, while mice over-expressing this protein exhibit the opposite phenotype [62]. Importantly, these findings show that both neurons and glial cells modulate brain synaptic plasticity, and that glial-neuronal interactions must also be considered in examining memory-anxiety interplay in the CNS.

Protein kinase C (PKC) γ is an enzyme highly expressed in the limbic system—the brain structure that regulates both memory and anxiety [63, 64]. Since PKCγ plays an important role in neural plasticity, modulation of neurotransmitter release, and neuronal excitability, its genetic ablation in mice predictably affects their anxiety and learning

Table 3: Summary of data showing the role of BDNF in memory and anxiety. KO: knockout (−/−), HZ: heterozygous (+/−) mice. (?: unclear effects. ∗: although authors claimed that anxiety was unaltered in this study, it contradicts the original anxiogenic interpretation of the social defeat model (also see [158]).)

Model	Effects on		References
	Anxiety	Memory/learning	
BDNF HZ mice	0	↓ learning (but 0 spatial learning and memory, fear conditioning)	[159], but see [160, 161]
Repeated aggression accompanied by increased BDNF expression in mice	↑∗	↑ long-term social aversion	[162]
Mesolimbic-specific BDNF knockdown	↑∗	↓ long-term social aversion	[162]
BDNF intrahippocampal injection in rats	↓↑	↑ short-term spatial memory	[163]
BDNF injection to the cortex in rats		↑ long-term memory	[164]
BDNF receptor overexpression in mice	↓	↑ spatial memory and learning	[165]
Forebrain-specific BDNF KO mice	0 ↑?	↓ spatial and nonspatial discrimination learning, 0 contextual fear	[166]
Brain conditional BDNF KO mice	↑	—	[33]

(Table 1). Mechanisms underlying these effects are still unknown but most likely include postsynaptic modulation of central GABA-A and serotonergic 5HT2 receptors [64].

From various brain proteins essential for synaptic vesicle trafficking, ras-associated binding proteins, such as Rab3a [70, 171], deserve special attention in relation to memory and anxiety. Using Rab3a knockout (−/−) and Ebd (loss-of-function) Rab3a mutant mice, a recent study has shown that Rab3a −/− mice display reduced cued fear conditioning, while Ebd mutants show both reduced anxiety and cued fear conditioning (Table 1), accompanied by altered hippocampal and cortical expression of Rab3a [69]. D'Adamo et al. [70] reported that Rab3a −/− mice display deficits in short- and long-term synaptic plasticity in the mossy fiber pathway, normal acquisition but several mild impairments in other memory tasks (Table 1), accompanied by increased locomotion and reduced anxiety. Collectively, these data implicate protein modulators of synaptic transmission (such as Rab3a) in the regulation of memory and anxiety, also enabling further dissection of molecular domains involved in their regulation.

Another recent study demonstrated that Rab3a is required for brain-derived neurotrophic factor (BDNF)-induced synaptic plasticity [172], implying functional interplay between the two molecules involved in brain plasticity. Indeed, BDNF is a key neurotrophic factor, acting through trkB receptor to regulate brain growth, differentiation, and functioning [32, 160, 173]. While an early study showed no anxiety or memory effects of BDNF genetic ablation in mice, numerous other data did reveal such actions (see Table 3 for details), also implying BDNF role in aversive memories [158, 162]. Consistent with this, spatial learning induces BDNF and trkB expression in activated brain areas, while BDNF inactivation markedly impairs spatial learning [32, 165]. In addition, mutant mice with reduced BDNF levels display impaired learning and memory in some tasks [159], whereas increased mouse BDNF signaling by trkB overexpression improves memory [165].

BDNF is rich in hippocampus and amygdala, and its administration improves rat short-term spatial memory and reduces anxiety [163]. In contrast, the same study revealed increased anxiety on trial 2 in BDNF-treated rats, suggesting that different types of anxiety may differently interplay with BDNF-modulated memories. In line with this, increased BDNF signaling in mice over-expressing trkB produced anxiolysis [165], while stress and anxiety correlate with memory deficits and reduction in brain BDNF [174, 175]. Moreover, Rattiner et al. [176, 177] have recently outlined the crucial role of BDNF and its receptors in hippocampal and amygdala-dependent learning (including fear conditioning—another potential mechanism underlying BDNF modulation of memory and anxiety).

Overall, human data strikingly parallel animal data on BDNF role in memory and anxiety (Table 3). For example, functional BDNF polymorphisms have been associated with anxiety-related personality traits [178], hippocampal volume in healthy volunteers [179], and episodic memory [180]. Taken together, these data confirm the important role of BDNF in memory, anxiety, and their interplay. Given the important role of BDNF in brain plasticity [173], behavior-modulating properties of this molecule seem to be particularly intriguing.

Importantly, brain mediators seem to cooperate with BDNF in modulating brain functions. For example, BDNF interacts with cholinergic, dopaminergic, serotonergic systems, and SERT [181–184] whose involvement in memory and anxiety has already been discussed. Analyses of human quantitative trait loci associated with cognitive functions also pointed to genes encoding BDNF, ACh, and glutamate receptors [185]. From this point of view, it is interesting that heterozygous BDNF knockout mice display unaltered or little anxiety and rather mild alterations in memory (Table 3),

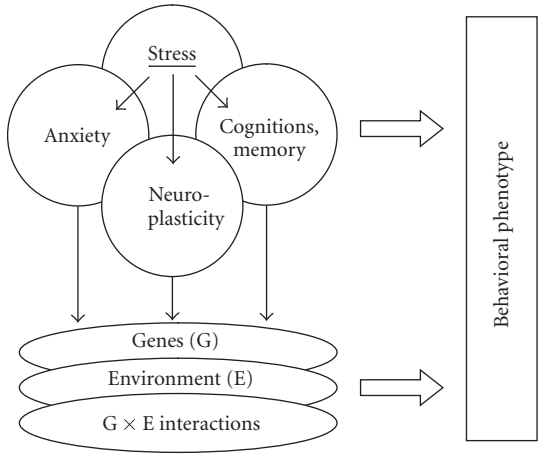

Figure 1: Stress, memory, and anxiety interplay.

accompanied by altered hippocampal ACh but unaltered catecholamine levels [160]. In contrast, simultaneous ablation of BDNF and SERT alleles exacerbates anxiety in double knockout mice and reduces hippocampal serotonin levels [147, 186], confirming an important functional interplay between BDNF and serotonin in the brain [181]. Extending original findings of Caspi et al. [156], a recent study has examined BDNF/SERT genes' interactions in depressed children, reporting that a combination of met-BDNF allele with two short SERT alleles was associated with higher depression in maltreated children [187]. Notably, this situation strikingly resembles experiments of Ren-Patterson et al. [186] in mice, indirectly supporting the notion that depression as well as specific anxiety-related traits (i.e., social anxiety or posttraumatic stress) may also be involved in BDNF-SERT interplay; also see [158, 162] for discussion.

4. CONCLUSIONS

As already mentioned, memory and anxiety do not always follow synergetic "high anxiety-better memory" rule, indicating that more complex nonlinear relations exist between these behavioral domains. Moreover, not always altered anxiety is seen together with altered memory, as vise versa (Table 1), suggesting that under certain circumstances both domains may be affected independently. Likewise, memory (as well as anxiety) must not be considered as a single entity, and clearly represents a complex multidimensional domain. However, it is important to understand that memory and anxiety represent two overlapping CNS processes that closely interact at different levels, including brain neurochemistry, circuitry, pharmacology, and various genes, as discussed here in detail. For such interactions, clinical findings strikingly parallel animal experimentation data, showing how these factors (in addition to environmental influences) may affect memory and anxiety. Both neuronal and glial cells, as well as brain mediators, neuropeptides, and other key proteins, cooperate in the regulation of memory and anxiety (Figure 1). Finally, brain plasticity factors (Figure 1) appear to play an important role in fine-tuning of memory-anxiety interplay, collectively contributing to the complexity of behavioral phenotypes.

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Review Article

The Importance of Cognitive Phenotypes in Experimental Modeling of Animal Anxiety and Depression

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Cognitive dysfunctions are commonly seen in many stress-related disorders, including anxiety and depression—the world's most common neuropsychiatric illnesses. Various genetic, pharmacological, and behavioral animal models have long been used to establish animal anxiety-like and depression-like phenotypes, as well as to assess their memory, learning, and other cognitive functions. Mounting clinical and animal evidences strongly supports the notion that disturbed cognitions represent an important pathogenetic factor in anxiety and depression, and may also play a role in *integrating* the two disorders within a common stress-precipitated developmental pathway. This paper evaluates why and how the assessment of cognitive and emotional domains may improve our understanding of animal behaviors via different high-throughput tests and enable a better translation of animal phenotypes into human brain disorders.

1. INTRODUCTION

Cognitive processes play a key role in stress-related neuropsychiatric disorders, including emotional disorders such as anxiety and depression [1–5] (Figure 1). Abundant clinical and animal evidences strongly support this notion, suggesting that disturbed cognitions per se are an important part of affective illnesses, helping integrate the two disorders within a common stress-precipitated pathogenesis [6–10]. Indeed, strong negative memories play a key role not only in different subtypes of anxiety (especially in post-traumatic stress disorder or specific phobias) [6, 11–14], but also in depression and suicidality [15–20]. These findings are further supported by recent data from psychiatric genetics [2, 21–25] and brain imaging [26–29], showing how altered cognitions, associated with genetic contributions and inherited brain anatomy and physiology traits, modify emotional regulation of stress, anxiety, and depression.

Animal experimental models of brain disorders are an indispensable tool in today's biomedical research [5, 30–32]. Animal memory-anxiety and memory-depression interplays, as well as the genetics, pharmacology, and neurophysiology of this interplay, have been comprehensively evaluated in several reviews [33–36], further strengthening the importance of memory assessment in behavioral phenotyping [37–41].

Do we routinely do this? Clearly not, as there exist several objective and subjective reasons. First, there is a traditional dichotomy between "emotional" domains (such as anxiety and depression) and "cognitive" domains (such as memory and learning) in behavioral neuroscience. Albeit relatively artificial, these boundaries somehow seem to preprogram researchers, who often enter (and remain loyal until the retirement party) the field as either "stress scientists" or "memory researchers." While some inquisitive scholars may subsequently move from one "cast" to another during their careers, in many cases it is the initial professional choice, triggered by personal preferences and reinforced by age-dependent conservatism, that dictates the whole line of subsequent behavioral research of a scientist. Sadly, such heterogeneity often further divides behavioral neuroscientists, who sometimes tend to attend only specialized meetings within their "own" domains, concepts and paradigms.

Another reality is that "anxiety" or "depression" laboratories rather rarely study memory and learning phenotypes in depth (and vice versa), and do so mostly when a gross cognitive deficit is apparent and seems to influence all outgoing

animal behaviors. In many such cases, memory testing becomes rather formal, is limited to selected "reference" memory tests, and does not focus on complex *interactions* between memory, anxiety, and depression domains (see, however, several encouraging exceptions discussed further).

Likewise, despite a growing recognition of the deleterious consequences of restricted behavioral battery usage [42, 43], current routine problems of an average behavioral laboratory include limits in testing and animal holding space, the lack of proper behavioral training, personnel, limited research budgets, or all of them together. Collectively, this leads to an extensive use and reuse of animals in high-throughput batteries [44–46]. In reality, this means that emotionality (e.g., anxiety and depression) tests are routinely run in the same cohorts of animals with relatively little attention to possible cognitive mechanisms or alterations that are triggered by such batteries, and that may, in fact, influence dramatically the subsequent behavioral scores of "anxiety" and "depression" [44]. Furthermore, learning and memory per se may also be affected by such batteries [44], further complicating behavioural phenotyping, and most likely exerting secondary effects on anxiety and depression.

Is this of concern? Can our routine laboratory practice lead to confounded findings and, even worse, potential misinterpretations of data? The aim of this paper is to analyze why and how an in-depth assessment of cognitive and emotional domains may improve our understanding of animal behaviors in different high-throughput tests, and their translation into human behavioral disorders.

2. TARGETING MEMORY-ANXIETY INTERPLAY IN ANIMAL BEHAVIORAL MODELS

Learning, memory, and anxiety have long been known as interactive dimensions in both animal and clinical studies [47, 48]. The importance of in-depth assessment of memory and anxiety together is further illustrated in Table 1. The interplay of these two domains in this table may hypothetically lead to multiple alternative states, whose misinterpretations in different behavioral tests (as well as psychopharmacological data obtained in such models) would generally be unavoidable if only single domains were assessed (also see: [31, 32] for discussion). In a similar vein, a recent review [41] has evaluated anxiety and memory/learning phenotypes in various genetically modified mouse models, including mutant mice lacking various receptors or other brain proteins. A common (but not mandatory) situation noted in this study, when the same mutation leads to simultaneously altered anxiety and memory phenotypes, illustrates the overlap between these two key domains, and demonstrates the extent to which their interplay may affect other animal outgoing behaviors.

In fact, some of phenotypes that we do observe in different models strikingly parallel hypothetical situations modeled in Table 1 (see, for example, altered anxiety and cognitions in 5-HT1a and 5-HT1b receptor knockout mice, and the ways to dissect their possible interplay, in [30–32]). Adding further complexity to the problem, it is always important to consider potential heterogeneity of memory subtypes, as the same mutation (such as 5-HT1b receptor knockout) may impair one type of memory (e.g., habituation) while improving another (e.g., spatial memory) [30].

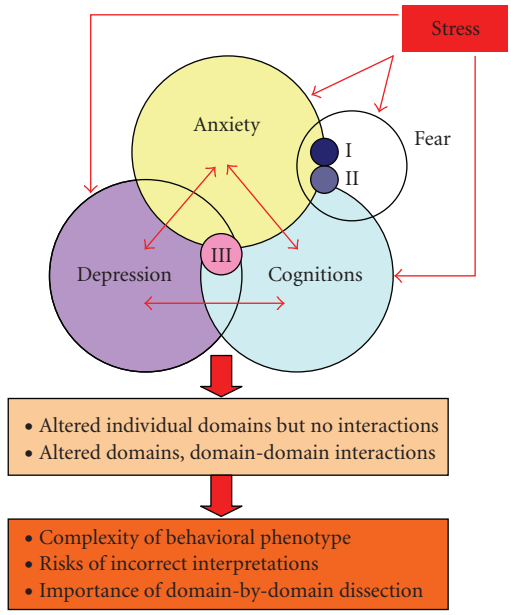

Figure 1: Interplay between fear, anxiety (including posttraumatic stress (I), and phobic disorders (II)), depression (including recurrent depression associated with negative memories (III) and cognitive domains in experimental models of neuropsychiatric disorders

Several other interesting directions of research may be considered further, based on specific targeting of memory-anxiety interplay. For example, as some subtypes of anxiety problems, such as post-traumatic stress disorder (PTSD), are based on strong aversive memories, genetic and behavioral models with both high anxiety and memory components[41, 49, 50] may lead to more valid experimental models of PTSD. However, some difficulties may also be likely with such models, as PTSD-like hyperarousal, commonly observed both clinically and in animals [49], may possibly be misinterpreted as increased locomotion (suggestive of anxiolytic-like phenotype). In any case, researchers should be aware of such interpretational difficulties, and make their conclusions with necessary caution and after testing several alternative hypotheses (see Table 1 for examples).

Finally, genetic models may target reciprocal interplay between these domains that are potentially relevant to mechanisms of stress resistance. Likewise, mice with both reduced anxiety and memory (see [41] for review) may lead to genetic models focused on mechanisms of resistance to PTSD and other types of anxiety associated with recurrent negative cognitions (see [6, 47]).

3. MODELING MEMORY-DEPRESSION INTERPLAY

The importance of cognitive mechanisms in clinical depression has long been known in the literature [51]. Indeed, we need to remember our past traumas and frustrations in order to become properly depressed. Memory and learning

Table 1: Examples of possible interplay between memory and anxiety domains, and how this may lead to misinterpreted animal behavioral and drug-induced phenotypes (effects: ↑ increased, ↓ reduced behavior). Note that real animal models have multiple other factors and domains, and the complexity (and risks of incorrect interpretation) of their phenotypes is much higher.

Domains		Anxiety	
Memory, learning	Elevated	Unaltered	Reduced
Elevated	Likely phenotype: ↑ initial anxiety (↓ activity) with ↑ habituation (anxiolytics would ↓ hypoactivity and habituation). Possible misinterpretation of baseline phenotype: hyperanxiety; ↓ sensitivity to repeated stressors (while, in fact, having ↑ vulnerability to chronic stress).	Likely phenotype: ↑ habituation [anxiolytics would ↑ activity and ↓ habituation]. Possible misinterpretation: ↓ exploration (↑ anxiety). Anxiolytics would ↓ habituation (however, this may be mistaken for ↓ anxiety)	Likely phenotype: ↓ initial anxiety with ↑ habituation (anxiolytics would ↓ habituation) Possible misinterpretation: initial hyperactivity followed by ↑ freezing ("↑ anxiety"). Anxiolytics will ↓ habituation (however, this may be mistaken for mild psychostimulant action)
Unaltered	Likely phenotype: ↑ anxiety (↓ exploration), normal memory. Anxiolytics may ↓ anxiety and memory. In some tests phenotype may be misinterpreted as baseline hypolocomotion		Likely phenotype: reduced anxiety (↑ exploration), normal memory. Anxiolytics may impair memory without affecting (already low) anxiety. In some tests baseline phenotype may be misinterpreted as hyperactivity
Reduced	Likely phenotype: ↑ initial anxiety with ↓ habituation. Anxiolytics may ↓ anxiety and further impair memory. Possible misinterpretation of baseline phenotype: hypersensitivity to repeated stressors (while, in fact, having ↓ vulnerability to chronic stress). Effects of anxiolytics may be mistaken for psychostimulant action	Likely phenotype: ↓ habituation. Anxiolytics may further impair memory. Possible misinterpretation of baseline phenotype: ↑ exploration (↓ anxiety). Effects of anxiolytics may be mistaken for psychostimulant action	Likely phenotype: ↓ initial anxiety with ↓ habituation (anxiolytics may ↓ memory). In some tests may be misinterpreted as persistent hyperlocomotion. Effects of anxiolytics may be mistaken for psychostimulant action

have also been considered in animal models of depression (e.g., see[52]). How can we apply this understanding to our experimental models and do it correctly? Table 2 summarizes a hypothetical situation where two interplaying domains (depression and memory) may lead to multiple alternative states, whose misinterpretations in different behavioral tests seem to be highly likely.

Some interesting experimental models of neuropsychiatric disorders may arise from specific targeting of memory-depression interplay. For example, since recurrent intrusive negative memories frequently accompany clinical depression [53–56], animal models based on simultaneously increased memories and depression-like phenotypes [52, 57–59] may be clinically relevant to modeling affective disorders associated with negative cognitions. In contrast, mouse models with cooccurring memory deficits and reduced depression-related behaviors (such as 5-HT1a knockout mice, see [60]) may be potentially useful to understand mechanisms of resistance to depression associated with chronic negative memories [61].

4. MODELING WITHIN AND BEYOND

With recent strategies of behavioral modeling of anxiety and depression (see [62]) supporting expansion beyond "pure" anxiety and depression domains, experimental models based on targeting these plus cognitive domains represent further important directions of research. One strategy may be to apply more extensively the models and tests that simultaneously profile anxiety (or depression) and memory functions. Conceptualized as behavioral "models-hybrids" [62, 63], this approach allows minimization of the unwanted behavioral consequences of test batteries, and provides an extensive high-throughput phenotyping of animals with a fewer number of procedures. For example, increased anxiety in the elevated plus maze and the loss of benzodiazepine anxiolytic efficacy upon repeated testing [48] may be used to indirectly assess memory functions in different mutant or drug-treated animals, as evaluated by the presence or absence of the above-mentioned "one trial tolerance" phenomenon. Likewise, the

Table 2: Examples of possible interplay between memory and depression domains, that may lead to misinterpreted animal behavioral phenotypes (effects: ↑ increased, ↓ reduced behavior; OCD-obsessive-compulsive disorder). Given high research pressure on behavioral labs, consider the likelyhood of incorrect interpretation of behavioral data.

Domains		Depression	
Memory, learning	Elevated	Unaltered	Reduced
Elevated	Likely phenotype: hypoactivity (or stereotypic hyperactivity in some tests) but ↑ sensitivity to repeated stressors. Possible misinterpretation of baseline phenotype: ↑ anxiety/freezing (or ↓ habituation, spatial memory in acute stress models)	Likely phenotype: ↑ habituation and ↑ sensitivity to repeated stressors. Possible misinterpretations: ↓ exploration (↑ anxiety) and ↑ despair depression	Likely phenotype: active locomotion with ↑ habituation and sensitivity to repeated stressors. Possible misinterpretations: initial hyperactivity followed by gradually ↑ anxiety, or ↑ "despair" depression (which, in fact, reflects ↑ learning)
Unaltered	Likely phenotype: ↓ hypoactivity (or stereotypic hyperactivity in some tests). Possible misinterpretation: ↑ anxiety/freezing (or ↓ habituation, spatial memory)		Likely phenotype: active locomotion. Possible misinterpretation of this phenotype: no or ↓ anxiety
Reduced	Likely phenotype: marked sustained hypoactivity (or stereotypic hyperactivity) with ↓ habituation and sensitivity to repeated stressors. Possible misinterpretations: ↑ anxiety (and/or OCD-like behavior) or ↓ despair depression	Likely phenotype: ↓ habituation. Possible misinterpretation: ↑ exploration (↓ anxiety)	Likely phenotype: active locomotion with ↓ habituation and sensitivity to repeated stressors. In some tests this may be misinterpreted as persistent hyperlocomotion

forced swim test (measuring "despair" depression domain) may be used to assess within- and between-trial habituation (spatial working and long-term memory) and *learned* helplessness. Fear conditioning, including active avoidance tests [64, 65]) are highly relevant to both fear (anxiety-related) and cognitive (learning) domains. Y- and T-mazes allow parallel assessment of spatial memory, exploration (anxiety), and spontaneous alternation. Morris water maze, a traditional hippocampal memory test, can also be used to study depression-like traits (e.g., immobility in [66, 67]). Finally, various elevated mazes can be used to profile cognitive domains (memory, learning) as well as animal anxiety [68, 69].

In general, there may be other combinations of anxiety, depression and memory tests, or even more sophisticated hybrid models, that could be used more extensively for high-throughput behavioral phenotyping. However, another reason to use these models more widely in behavioral research is the possibility of performing an *integrative* (versus more traditional, domain-oriented) experimental modeling of brain disorders. This approach, based on targeting commonalities (rather than differences) of disorders, will allow researchers to parallel their animal models with recent trends in clinical psychiatry, where "continuum" or "spectrum" theories are beginning to challenge the existing "heterogeneous" Kraepelinian paradigms [70–72].

An important step in this direction may be the use of rodent models that simultaneously evaluate "comorbid" anxiety and depression and also focus on cognitive (dys)functions in these models. For example, selectively bred HAB mice [52] and thyroid hormone receptor knockout mice [9] display inherited anxiety- and depression-like phenotypes, and their cognitive functions merit further studies (see, e.g., aberrant memory in the latter model). Similarly, olfactory bulbectomy, traditionally known to produce depression in rodents, has been recently reported to be relevant to comorbidity of anxiety and depression, and is accompanied by specific memory deficits in animals that resemble cognitive dysfunctions in humans with comorbid anxiety and depression [5].

Further important information can also be obtained through in-depth ethological analyses of behavioral strategies, including cross-species and cross-strain comparisons [73, 74] of animal behaviors in different tests–an approach consistent with recent endophenotyping and cross-species trait genetics concepts in animal behavioral modeling [75, 76]. Finally, expanding far beyond anxiety and depression domains may also be a rational strategy of research, as it allows modeling of complex schizo-affective and neurodevelopmental disorders based on increased anxiety, depression and altered memory, and other cognitions [77–80].

5. CONCLUDING REMARKS

To optimize behavioral phenotyping research, the neuroscientific community may need to encourage behavioral neuroscientists to produce data on memory and learning phenotypes in their papers that report anxiety- and depression-related behaviors (e.g., [30, 31, 60]). As a practical solution, "can my findings be a result of merely altered memory or learning?" should be one of the first questions asked in studies on animal emotionality and affective behaviors. In cases when both cognitive and emotionality domains seem to be affected (e.g., [81, 82]), we next need to establish the nature of their interactions, and how they might codetermine the behavioral phenotype observed. Finally, in addition to studying behavior x gene x environment interactions, we may benefit from focusing on behavior x cognitions x gene x environment interactions. "Work hard and marry a talent"–advised R. Blanchard in one of his interviews, sharing with fellow colleagues the recipe for a successful career in science. Following such wise advice, diligent behavioral neuroscientists working with anxiety and depression may benefit from joining forces with (and even perhaps marrying) their talented colleagues studying memory and learning.

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Review Article

Hippocampal Neurogenesis, Depressive Disorders, and Antidepressant Therapy

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There is a growing body of evidence that neural stem cells reside in the adult central nervous system where neurogenesis occurs throughout lifespan. Neurogenesis concerns mainly two areas in the brain: the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone, where it is controlled by several trophic factors and neuroactive molecules. Neurogenesis is involved in processes such as learning and memory and accumulating evidence implicates hippocampal neurogenesis in the physiopathology of depression. We herein review experimental and clinical data demonstrating that stress and antidepressant treatments affect neurogenesis in opposite direction in rodents. In particular, the stimulation of hippocampal neurogenesis by all types of antidepressant drugs supports the view that neuroplastic phenomena are involved in the physiopathology of depression and underlie—at least partly—antidepressant therapy.

1. NEUROGENESIS

Although some occasional reports of postnatal neurogenesis in mammals have been published during the first half of the twentieth century (see [1] for a review), it was only in the early 1960s that the first evidence of a postnatal neuronal proliferation was reported in various brain regions in adult rats, including the dentate gyrus of the hippocampus [2], the neocortex [3], and the olfactory bulb [4]. However, no consensus on this adult neurogenesis was reached at this period and these findings were somewhat forgotten for the next two decades mainly because of their apparent lack of functional relevance, and also because the definitive demonstration that the adult-generated cells were neurons rather than glia was not provided. It was only in the nineties that several technical developments allowed a clear-cut demonstration of neurogenesis in adult brain. It was then established that neural cell proliferation occurs throughout the lifespan in various species including rodents [5], monkeys [6], and humans [7], and is particularly important in two regions of the brain, the dentate gyrus of the hippocampus [5, 8] and the subventricular zone [9]. In the hippocampus, new granule cells are formed from progenitors located in the hilus of the dentate gyrus. During maturation and differentiation steps, newly generated cells enter the granule-cell layer, migrate through the layer towards the fissure, and get integrated into the basic circuitry of the hippocampus, notably through synaptic contacts with pyramidal neurons in the CA3 field [10, 11]. In the subventricular zone, neurogenesis gives rise to neurons that migrate through the rostral migratory stream and integrate the olfactory bulb as interneurons [12, 13].

To label dividing cells, the earliest studies used [^{3}H]-thymidine, which incorporates into replicating DNA during the S-phase of the cell cycle and can be detected by autoradiography [14]. An important technical improvement was the introduction of the synthetic thymidine analogue BrdU (5-bromo-3-deoxyuridine) that substitutes for thymidine in neosynthetized DNA of proliferating cells [15]. BrdU incorporated into DNA can then be easily visualized with immunocytochemical techniques using specific anti-BrdU antibodies. This technique allows quantitative analysis of proliferation, differentiation, and survival of newborn cells by varying the time interval between the pulse administration of BrdU and the sacrifice of animals [16–18]. The determination of the time and site of origin of newly generated cells in the CNS requires euthanasia shortly, generally between 1 and 3 hours, after the administration of BrdU, before newly born neurons have migrated out [19] (Figure 1).

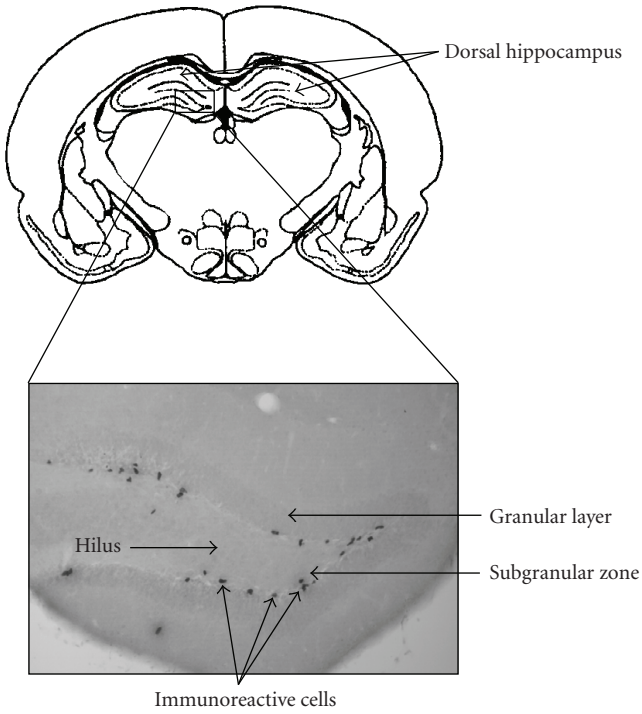

Figure 1: Photomicrograph of BrdU-positive cells in the subgranular zone of the dentate gyrus 2 hours after BrdU administration in an 8-week-old C57BL/6J mouse. Magnification: 100.

For study of cell migration, immunocytochemical labeling has to be performed at various post-injection times, between 4 and 10 days, and finally, the fate and survival of the newly generated cells can be determined 21 days after BrdU injection, once migration has been achieved [5, 10, 20, 21].

Although DNA labeling by BrdU is currently the most commonly used method for studying adult neurogenesis, the potential toxic effect of this thymidine analogue should not be ignored as it might be a confounding factor in some experiments. This led to the use of other markers of the cell cycle, such as proliferating nuclear antigen (PCNA) and Ki-67, to analyze cell proliferation in situ [22]. PCNA, a cofactor of DNA polymerase, is expressed during the S-phase of cell cycle and quantification of both PCNA- and Ki-67-immunopositive cells has been shown to reliably reflect cellular proliferation, like BrdU labeling, in the adult DG [23].

In the rodent brain, approximately 9000 new neurons per day (i.e., 270 000 per month) are generated [24], and survive with a half-life of approximately 28 days [25]. This constitutive neurogenesis declines with age, as evidenced in rodents [26] and rhesus monkeys [27]. Although earliest studies on songbirds provided data in support of a functional role of adult neurogenesis in seasonal song learning [28], the possible functional significance of this process remains to be formally determined in mammals. However, the fact that hippocampal neurogenesis can be modulated by various factors including hormones, neurotransmitters, or environment suggests its real implication in physiological mechanisms and not its occurrence as a nonfunctional residual phenomenon in mammals [29]. In particular, glucocorticoids (including cortisol) have been shown to exert a negative influence that may account for the marked reduction in granule cell proliferation caused by stress [30], whereas, in contrast, antidepressant treatments markedly stimulate hippocampal neurogenesis [31]. The relevance of these data for pathophysiological mechanisms underlying depression is critically analyzed in the following section.

2. STRESS, GLUCOCORTICOIDS, AND NEUROGENESIS

Numerous studies have emphasized that stress can be the most significant causal agent, together with genetic vulnerability, in the etiology of depression. In addition, neurons in the hippocampal formation are among the most sensitive to the deleterious effects of stress. Consequently, stress-induced decrease in hippocampal neurogenesis might be an important feature associated with depression episodes.

Stress may be caused by any environmental change, whether internal or external, that disrupts the maintenance of homeostasis, and initiates a series of neuronal responses to prepare the organism to adapt to this new environmental challenge. Under environmental or psychological stressful conditions, neurons in the paraventricular nucleus (PVN) of the hypothalamus secrete corticotropin-releasing hormone/factor (CRH/CRF) and arginine-vasopressin (AVP), which in turn, stimulate the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH promotes the synthesis and the release of glucocorticoids from the adrenal cortex which allows the appropriate adaptation of the organism to stress, mainly through their vascular and metabolic effects [32].

The principal glucocorticoids are cortisol in humans and corticosterone in rodents. They both influence metabolism, cognitive processes, and emotions, especially fear and anxiety. To prevent deleterious effects of excessive levels of circulating glucocorticoids, the hypothalamic-pituitary-adrenal (HPA) stress axis is under tight control [32] through mineralocorticoid (MR) and glucocorticoid (GR) receptor negative feedback regulation [33]. Chronic stress frequently results in glucocorticoid and CRF hypersecretion associated with decreased sensitivity to glucocorticoid-mediated feedback inhibition. In vulnerable individuals, chronic stress may lead to excessively long lasting HPA responses that may precipitate psychopathologies such as anxiety and depression [34, 35].

Both basic and clinical studies have shown that stress can be associated with morphometric brain changes, neuronal atrophy, and decrease in the proliferation of progenitor cells in the hippocampal dentate gyrus. Whether these modifications really contribute to the development of depression is still a matter of debate [31, 36–39].

During the last decade, a series of reports indicated that major depression is frequently associated with significant atrophy within the hippocampus, which can persist for several years after remission from depression episodes [40–42]. In addition, prolonged depressions appeared to be associated with more severe atrophy [43]. Since the hippocampus

plays a central role in learning and memory, these data suggested that such morphological alterations might be related to the cognitive deficits observed during depressive episodes [44, 45]. More recently, Stockmeier et al. [46] reported a reduction in both the average soma size of pyramidal neurons and neuropil, which may contribute to the volume retraction noted using fMRI in the hippocampus of patients with major depressive disorders. These morphometric alterations are most often attenuated or even reversed by antidepressants [46, 47].

Extensive preclinical investigations recently provided some keys toward understanding biological mechanisms causally related to hippocampus atrophy in severely depressed patients. In rodents, adrenal steroids were the first endogenous compounds to be identified as factors affecting hippocampal neurogenesis [48]. To date, adrenal steroids are well known to regulate both proliferation and differentiation of new neurons in the dentate gyrus [49]. In rats, a sustained increase in plasma corticosterone causes a decrease in neurogenesis while, reciprocally, adrenalectomy increases this process [50]. Indeed, removal of the adrenals accelerates neural cell proliferation and delays the death of newly formed neurons. Giving excess corticoids (e.g., corticosterone) has converse effects and consequently decreases the formation and survival of progenitor cells [51]. Treatment of adult male rats for 21 days with exogenous glucocorticoids has also a remodeling effect on dendrites in hippocampal neurons [52, 53].

In congruence with observations in depressed patients, both a reduction in hippocampal volume and a decrease in neurogenesis have been reported in subordinate tree shrews subjected to social interaction stress, which consists of a daily psychosocial conflict by introducing a naive animal into the cage of a socially experienced one [54, 55]. Changes in cell morphology, apical dendrite length, and branching of CA3 pyramidal cells were also observed in the same species under closely related experimental conditions [56]. Furthermore, chronic restraint stress for 21 days in rats led apical dendrites of CA3 pyramidal neurons to atrophy [57] and strongly reduced proliferation of dentate gyrus precursor cells [58]. Prenatal stress also decreases neurogenesis in the adult hippocampus along with increased anxiety-like behavior, hyperactivity of HPA axis, and reduced learning ability in rats [59] and exacerbated emotional behavior in rhesus monkeys [60]. On the other hand, inescapable stress leads to a reduction in neurogenesis that correlates with behavioral despair several days after exposure to stress in the learned helplessness model of depression [61]. Very recently, chronic mild stress, a validated paradigm to induce depression-like symptoms, has been shown to decrease survival (but not proliferation) of new born cells in adult rat hippocampus [62].

3. SEROTONIN, ANTIDEPRESSANTS, AND NEUROGENESIS

Serotonin, a key regulator of cell division, has been shown to modulate different processes such as neurogenesis, apoptosis, axon branching, and dendritogenesis during brain development [63]. This leads to propose for this neurotransmitter a critical role in the control of adult neural cell proliferation. In adult rats, the first study aimed at assessing the effect of 5-HT on neurogenesis was carried out using d,l-fenfluramine, which releases 5-HT throughout the central nervous system. Thus, Jacobs et al. [64] noted that d,l-fenfluramine increased cell division by two- to three-fold in the dentate gyrus. Subsequent studies confirmed the proliferating effect of 5-HT within the subgranular zone of the dentate gyrus [65], where both progenitor cells and a dense innervation by serotonergic fibers are observed [66]. Furthermore, a decrease in 5-HT content after either a lesion of serotonergic neurons by 5,7-dihydroxytryptamine (5,7-DHT) [67] or an inhibition of 5-HT synthesis by parachlorophenylalanine (PCPA) [67, 68] produced long-term deficits in the proliferation of hippocampal cells, and raphe grafts (which are enriched in 5-HT-producing neurons) reversed these deficits, very probably by replenishing endogenous 5-HT stores and restoring 5-HT functions [69].

The preferential involvement of 5-HT_{1A} receptors in the 5-HT effects on cell proliferation was first suggested by Jacobs et al. [64], who showed that d,l-fenfluramine -induced increase in neurogenesis was prevented by the selective 5-HT_{1A} receptor antagonist, WAY 100635. Later on, the promoting effect of 5-HT_{1A} receptor activation on hippocampal neurogenesis was confirmed by other groups. In particular, Santarelli et al. [70] noted that the 5-HT_{1A} receptor agonist, 8-OH-DPAT, caused an increase in cell proliferation in wild-type mice, but was ineffective in 5-HT_{1A} receptor knock-out mice, indicating that the action of 8-OH-DPAT was entirely mediated by 5-HT_{1A} receptors. However, other types of serotonergic receptors were also shown to be involved in the effects of serotonin on hippocampal cell proliferation. This is notably the case of 5-HT_{2A} and 5-HT_{2C} receptors whose activation by selective agonists enhanced neurogenesis in the rat dentate gyrus [65]. A simulatory effect was also noted with 5-HT_{1B} receptor agonists but only after 5-HT depletion [65]. Whether receptors of the 5-HT_4, 5-HT_6, and 5-HT_7 types are also implicated in the regulation of hippocampal neurogenesis is still an open question to be addressed.

The clinical benefit of antidepressants that increase serotonergic neurotransmission such as selective serotonin reuptake inhibitors (SSRIs) drove several teams to analyze the effects of these drugs on cell proliferation and neurogenesis. A three-week systemic treatment with fluoxetine was first found to increase by 70 percent cell proliferation in the dentate gyrus in rodents [30, 31]. Because this effect was not observed in 5-HT_{1A} receptor knock-out mice, it could be inferred that 5-HT_{1A} receptor activation actually mediated fluoxetine-induced neurogenesis [71]. Several groups then confirmed that chronic, but not acute, antidepressant treatments exert a stimulatory influence on hippocampal neurogenesis [38, 61, 72]. Interestingly, all classes of antidepressant drugs tested so far, including NA and 5-HT reuptake inhibitors [30], atypical antidepressants such as tianeptine [54], electroconvulsive seizures, mood stabilizers such as lithium [31, 73], were shown to increase the proliferation and survival of new neurons in the dentate gyrus.

The lack of antidepressant-like effect of fluoxetine in x-irradiated mice, in which neurogenesis was abolished, led to the claim that clinical effectiveness of antidepressants is directly related to their promoting effect on hippocampal cell proliferation [71]. Interestingly, chronic treatments with CRH-R1 and V1b receptor antagonists, which are endowed with antidepressant-like properties in validated animal models [74, 75], also exerted a positive influence on hippocampal granule cell proliferation, thereby reversing the reduction in this process which had been caused by chronic mild stress [76]. Furthermore, the new antidepressant agomelatine also exerts a stimulatory influence on cell proliferation within the hippocampus. Chronic administration of this mixed MT1/MT2 melatonin receptor agonist and 5-$HT_{2B/2C}$ receptor antagonist significantly increased the number of new born cells in the hippocampus of adult rats [77], and reversed the deficit in granule cell proliferation that had been induced at adult stage in rats born from a mother subjected to repeated stress during gestation [78]. In line with these observations, preliminary data from our laboratory showed that chronic treatment with fluoxetine or agomelatine compensated for the deficit in neurogenesis observed in transgenic GR-i mice (a murine model of depression, [79]), and raised this process up to the level observed in healthy paired wild-type mice [80]. These data are compatible with the idea that all types of antidepressant treatments apparently share the capacity to enhance cell proliferation and neurogenesis in the dentate gyrus of the hippocampus, thereby antagonizing the reduction in this process that has been regularly observed in validated animal models of depression (GR-i mice, learned heplessness, genetically helpless mice, chronic psychosocial stress, etc.) and is very likely occurring also in patients during a severe depression episode.

5-HT and corticotrope systems are closely cross-regulated under normal physiological conditions in mammals [81, 82] and their interactions are of particular relevance when considering pathological conditions such as depression, in which dysfunctioning of both systems has been consistently documented [83–86]. Although the exact mechanisms by which stress and glucocorticoids on the one hand, and antidepressants and serotonin on the other hand, affect neurogenesis have not been completely elucidated, evidence has been reported that modifications in the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus might be part of the causal event [87]. BDNF is a major neurotrophic factor in brain, which plays key roles in the survival and guidance of neurons during development, and is required for the survival and normal functioning of neurons in the adult brain [88]. Decreased levels of BDNF in response to stress could lead to a loss of normal plasticity and also to damage and death of neurons. It is conceivable that the cell loss observed in depression could result from alterations in factors that control programmed cell death including cAMP response element binding (CREB) protein. Indeed, Dowlatshahi et al. [89] reported that CREB levels are decreased in the cerebral cortex of depressed patients. Conversely, several studies demonstrated that antidepressant treatment upregulates cAMP production, and, in turn, the CREB cascade including CREB-induced expression of BDNF [90]. Interestingly, upregulation of CREB and BDNF occurs not only in response to chronic treatment with various classes of antidepressant drugs, including NA and/or 5-HT reuptake inhibitors, but also after electroconvulsive seizures mimicking electroconvulsive therapy. Accordingly, it can be inferred that the cAMP-CREB cascade and BDNF are common postreceptor targets of both glucocorticoids and antidepressant treatments [70, 91] and thus very probably participate in associated neuroplastic phenomena.

4. CONCLUSION

The data summarized in this review highlight the involvement of hippocampal plasticity in physiopathological processes linked to mood disorders. Both corticotrope and serotonin systems have largely been involved in depressive symptoms and most of the effective antidepressant therapies are known to act through them. Interestingly, these two systems induce sustained modifications in adult hippocampal neurogenesis. However, much remains to be understood about the relations between cell proliferation, the hippocampus, and depression. Although hippocampal neurogenesis appears to be necessary for antidepressant drugs to alleviate depression-related behavioral deficits, it is probably not the case for the positive behavioral effects of environmental enrichment [92] and the antidepressant therapy using transcranial magnetic stimulation [93]. Accordingly, relationships between cell proliferation and antidepressant therapy are probably much more complex than originally claimed. However, the observation that cell proliferation parallels the effects of antidepressant drugs may lead to set up new strategies to treat depressive disorders. To this aim, elucidating the cellular and molecular mechanisms of action of antidepressants on neurogenesis is the further critical steps to be achieved.

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Review Article

Endocannabinoid System and Synaptic Plasticity: Implications for Emotional Responses

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The endocannabinoid system has been involved in the regulation of anxiety, and proposed as an inhibitory modulator of neuronal, behavioral and adrenocortical responses to stressful stimuli. Brain regions such as the amygdala, hippocampus and cortex, which are directly involved in the regulation of emotional behavior, contain high densities of cannabinoid CB1 receptors. Mutant mice lacking CB1 receptors show anxiogenic and depressive-like behaviors as well as an altered hypothalamus pituitary adrenal axis activity, whereas enhancement of endocannabinoid signaling produces anxiolytic and antidepressant-like effects. Genetic and pharmacological approaches also support an involvement of endocannabinoids in extinction of aversive memories. Thus, the endocannabinoid system appears to play a pivotal role in the regulation of emotional states. Endocannabinoids have emerged as mediators of short- and long- term synaptic plasticity in diverse brain structures. Despite the fact that most of the studies on this field have been performed using in vitro models, endocannabinoid-mediated plasticity might be considered as a plausible candidate underlying some of the diverse physiological functions of the endogenous cannabinoid system, including developmental, affective and cognitive processes. In this paper, we will focus on the functional relevance of endocannabinoid-mediated plasticity within the framework of emotional responses. Alterations of the endocannabinoid system may constitute an important factor in the aetiology of certain neuropsychiatric disorders, and, in turn, enhancers of endocannabinoid signaling could represent a potential therapeutical tool in the treatment of both anxiety and depressive symptoms.

1. INTRODUCTION

Fear is an adaptive component of the acute stress response to potentially dangerous stimuli which threaten the integrity of the individual. However, when disproportional in intensity, chronic, irreversible, and/or not associated with any actual risk, it constitutes a maladaptive response and may be symptomatic of an anxiety-related neuropsychiatric disorder such as generalized anxiety, phobia, or post-traumatic stress disorder (PTSD), among others. A diversity of mechanisms, including GABAergic, serotonergic, and noradrenergic systems, appears to be involved in the regulation of anxious states which may contribute to an appropriate emotional response to aversive events [1]. In the recent years, an increasing interest in the endocannabinoid system has arisen as part of the complex circuitry that regulates anxiety and as a crucial mediator of emotional learning. Brain distribution of cannabinoid CB1 receptors is consistent with an involvement of this system in the regulation of emotional reactivity. Indeed, CB1 receptors are highly expressed in brain structures such as the amygdala, hippocampus, anterior cingulate cortex, and prefrontal cortex [2–8], key regions in the regulation of emotional responses. Moreover, the cannabinoid CB1 agonist CP 55,940 increased Fos immunoreactivity in brain structures known to be involved in anxiety and fear-related responses such as the central nucleus of the amygdala, the periaqueductal gray, and the paraventricular nucleus (PVN) of the hypothalamus [9].

Depression is a mood disorder in which the prevailing emotional mood is distorted or inappropriate to the circumstances. There are important links between chronic stress and depression. Upon exposure to acute stressful stimuli, the organism initiates a series of neuroendocrine short-term responses that are beneficial in terms of adaptation. However, exposure to chronic, unavoidable situations of stress may have deleterious consequences, including endocrine, emotional, and cognitive alterations associated with neuropsychiatric disorders such as depression. In this context,

hyperactivity of the hypothalamus-pituitary-adrenal (HPA) axis with increased glucocorticoids levels appears to be linked to major depression [10, 11]. There is evidence for an involvement of the endocannabinoid system in the regulation of neural, behavioral, and endocrine responses to aversive stimuli [12, 13] and it has been suggested that stress-induced dysregulation of specific components of the endocannabinoid system might be associated with deficits in behavioral flexibility that can be manifested in stress-related disorders such as PTSD and depression [14].

Endocannabinoids have been shown to act as retrograde transmitters at the synaptic level. Though the exact role of retrograde endocannabinoid signaling in vivo is not fully clarified yet, it is likely that by this mechanism endocannabinoids play important roles in synaptic transmission and plasticity, including modulation of emotional responses. Indeed, endocannabinoids have recently emerged as one of the most thoroughly investigated, and widely accepted, classes of retrograde messengers in the brain [15]. Cannabinoid-induced neuroplasticity may underlie diverse physiological functions modulated by the endocannabinoid system, that is, pain [16] and memory [17]. Synaptic plasticity within the amygdala appears to play a crucial role in acquisition, storage, and extinction of aversive memories, basic neural processes that serve adaptive behaviors, and the endocannabinoid system has emerged as a crucial mediator of such neuroplasticity-related phenomena. Marsicano et al. [18, 19] proposed that endocannabinoids facilitate extinction of aversive memories through their selective inhibitory effects on local inhibitory networks in the amygdala, providing evidence for a functional role of endocannabinoid release-based synaptic plasticity. Apart from the amygdala, there are some other brain areas that have been postulated as substrates for cannabinoid-induced neural plasticity such as the hippocampus and the hypothalamus where cannabinoid-dependent synaptic plasticity is involved in the regulation of the stress-response system [17, 20]. Pharmacological modulation of the endocannabinoid system has been proposed as a novel potential therapeutical strategy for the treatment of anxiety disorders and depression [21], and therapeutic interventions directed at normalization of the HPA system [11] might potentially include modulation of endocannabinoid signaling.

2. THE ENDOCANNABINOID SYSTEM AND CANNABINOID-RELATED COMPOUNDS

The endocannabinoid system includes the cannabinoid receptors, the endogenous lipid ligands (endocannabinoids), and the enzymatic machinery for their synthesis and inactivation. Endocannabinoids are important neuromodulators that appear to be involved in a plethora of physiological processes such as modulation of nociception, regulation of motor activity, cognitive processes, neuroprotection, immune function and inflammatory responses, antiproliferative actions in tumoral cells, control of cardiovascular system, and neurodevelopment, among others [22–29]. Notably, the endocannabinoid system appears to be critically involved in the maintenance of homeostasis [28, 30]. In this review, we aim to highlight its function as a stress-recovery system.

Endocannabinoids are polyunsaturated fatty acid derivatives. The ethanolamide of arachidonic acid anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most studied endocannabinoids and have been implicated in a wide range of physiological and pathological processes. Other molecules such as 2-arachidonyl-glyceryl ether (noladin, 2-AGE), O-arachidonoyl-ethanolamine (virhodamine), and N-arachidonoyl-dopamine (NADA) have been discovered more recently. The anabolic and catabolic pathways for AEA and 2-AG appear to rely on very complex enzymatic cascades and are in the progress of being elucidated. In brief, the enzime N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) synthesizes AEA from N-arachidonoylphosphatidylethanolamine (NArPE), whereas the diacylglycerol lipase (DAGL) generates 2-AG from diacylglycerol (DAG) substrates. Due to their lipophilic nature, endocannabinoids cannot be stored in vesicles. It is widely accepted that, unlike other mediators, the endocannabinoids are synthesized and released on demand, in response to diverse physiological and pathological stimuli, and appear to exert important actions as retrograde messengers. Endocannabinoid inactivating mechanisms include cellular reuptake and hydrolysis. AEA appears to be taken up by several cell types at least in part via a facilitated transport mechanism, known as the anandamide membrane transporter (AMT), which can also transport 2-AG intracellularly. Though this putative transporter has not been isolated or cloned yet, there are compounds that are considered as inhibitors of cellular uptake. A fatty acid amide hydrolase (FAAH) is the main AEA hydrolase, whereas a monoacylglycerol lipase (MAGL) is critical in degrading 2-AG. It is important to take into consideration that the actions of endocannabinoids are considered to be spatially and temporally restricted. Therefore, the effects of exogenously applied cannabinoids, which lack such selectivity, do not necessarily mimic physiological functions of the endocannabinoid system [26, 28]. Compounds that enhance endocannabinoid signaling by inhibiting endocannabinoid reuptake (e.g., VDM11, OMDM-1, OMDM-2, UCM707) or by degradation (e.g., the FAAH inhibitors URB597, AM374, or N-arachidonoyl-serotonin) are widely used in preclinical studies and appear to have a potential therapeutical interest. A profound discussion of biochemical aspects of the endocannabinoid system is beyond the scope of this paper, but the reader can find comprehensive excellent reviews (e.g., [27, 28, 31–35]) as well as recent papers on specific aspects such as alternative biosynthetic pathways for endocannabinoids [36, 37] and endocannabinoid membrane transport [38].

Cannabinoids mainly exert their pharmacological effects by the activation of specific membrane receptors. Mammalian tissues contain at least two types of cannabinoid receptors, CB1 and CB2, which are metabotropic receptors coupled to G-proteins of the Gi/o type. CB1 receptors are

localized mainly in the central nervous system, but are also present in a variety of peripheral tissues; they are among the most abundant and widely distributed G-protein coupled receptors in the brain. Transduction systems include inhibition of adenylyl cyclase and of certain voltage-sensitive calcium channels (predominately, those found presynaptically) and activation of inwardly-rectifying potassium channels and mitogen-activated protein (MAP) kinase [39]. Autoradiographic and immunohistochemical studies have shown that CB1 receptors are expressed in multiple brain areas, including the olfactory bulb, neocortex, pyriform cortex, hippocampus, amygdala, basal ganglia, thalamic and hypothalamic nuclei, cerebellar cortex and brainstem nuclei. In particular, a high density of CB1 receptors is found in cortical and limbic regions associated with emotional responses. The levels of expression vary among the various brain regions and neuronal subpopulations, and there is apparently no strict correlation between levels of expression and receptor functionality. Thus, the activity of cannabinoids at CB1 receptor depends not only on the relative receptor density but also on other factors such as receptor coupling efficiency [2, 28, 40–43]. It has been widely accepted that cannabinoids regulate GABA release by activation of CB1 receptor type, and the highest levels of CB1 cannabinoid receptors are found on the terminals of cholecystokinin-positive GABAergic interneurons [44, 45]. On the other hand, the expression of CB1 receptor in glutamatergic neurons has been vigorously debated in recent years. In fact, some authors proposed that a novel non-CB1/non-CB2 cannabinoid-sensitive receptor could be responsible for the inhibition of glutamatergic neurotransmission [46, 47]. However, it has been now well established that functional cannabinoid CB1 receptors are present on glutamatergic terminals of the hippocampal formation, colocalizing with vesicular glutamate transporter 1 [48], as well as in other cortical areas (see, e.g., [26, 49, 50]). These evidences do not exclude that a non-CB1 receptor might exist in the brain, but there is to date no molecular evidence for such novel receptor.

Cannabinoid CB2 receptors are mostly peripherally located on immunological tissues, and therefore implicated in immunological functions. However, they have also been found within the central nervous system on neurons and glial cells with their expression mainly related to conditions of inflammation [51–53]. More recent immunohistochemical analyses have revealed immunostaining for CB2 receptors in apparent neuronal and glial processes in diverse rat brain areas, including cerebellum and hippocampus [54, 55]. These results change the classical view of peripherally located CB2 receptors and suggest broader functional roles for these receptors.

It has been shown that some of the effects of anandamide are mediated by the transient receptor potential vanilloid type-1 channel (TRPV1), formerly called vanilloid receptor VR1 [39]. These receptors have been traditionally known for their function in sensory nerves where they mediate perception of inflammatory and thermal pain, but they are also expressed within the brain contributing to other important physiological functions. Co-expression of cannabinoid CB1 and TRPV1 receptors was found by using immunofluorescence techniques in diverse brain areas involved in the regulation of emotional responses. In particular, within the hippocampus, CB1/TRPV1 was detected on cell bodies of many pyramidal neurons throughout the CA1–CA3 subfields and in the molecular layer of dentate gyrus [56]. Interestingly, TRPV1 knockout mice (TRPV1-KO) showed less anxiety-related behavior in the light-dark test and in the elevated plus-maze than their wild-type littermates as well as less freezing to a tone after auditory fear conditioning and stress sensitization. TRPV1-KO also showed impaired hippocampus-dependent contextual fear together with a decrease in long-term potentiation (LTP) in the Schaffer collateral-commissural pathway to CA1 hippocampal neurons. These data provide first evidence for fear-promoting effects of TRPV1 with respect to both innate and conditioned fear and for a decisive role of this receptor in synaptic plasticity [57]. Collectively, these findings open new avenues for the study of possible functional relationships between CB1 and TRPV1 receptors, in particular regarding stress, fear, and anxiety responses.

Recently, an additional G-protein-coupled receptor (GPCR) GPR55 has been proposed as a possible new cannabinoid receptor that might play a physiological role in lipid or vascular biology [58].

3. BASIC PRINCIPLES OF ENDOCANNABINOID-MEDIATED SYNAPTIC PLASTICITY

One of the most salient features of the nervous system is its plasticity, including structural and functional changes in individual neurons and synapses. This characteristic is present both during brain development and in the adult life. Synaptic plasticity allows changes in the strength and number of synaptic connections between neurons. It is considered as one of the major mechanisms underlying learning and memory and appears to mediate several other functions in the central nervous system. The resulting changes in synaptic efficacy are thought to be crucial in experience-dependent modifications of neural function. A closely related concept is behavioral flexibility that allows an organism to adapt to variable environmental demands and produce adaptive responses.

Given the prominent presynaptic localization of cannabinoid CB1 receptors, together with its mainly inhibitory actions, cannabinoids have been proposed as local retrograde modulators, with an important role in modulating essential physiological functions and contributing in diverse synaptic plasticity phenomena [59–62]. The endocannabinoid system seems to affect neuronal excitability participating in the maintenance of homeostatic conditions in the brain [26, 63, 64]. In this respect, data obtained from conditional CB1 mutant mice suggest that the endocannabinoid system may protect neurons against excessive activity, and consequently against excitotoxicity. Marsicano et al. generated conditional mutant mice that lacked expression of the CB1 receptor in principal forebrain neurons but

not in adjacent inhibitory interneurons. In mutant mice, the excitotoxin kainic acid (KA) induced excessive seizures in vivo, and the threshold to KA-induced neuronal excitation in vitro was severely reduced in their hippocampal pyramidal neurons. Moreover, KA administration rapidly raised hippocampal levels of anandamide and induced protective mechanisms in wild-type principal hippocampal neurons, whereas these protective mechanisms could not be triggered in mutant mice. These findings indicate that neural excitability is increased in CB1-deficient mice and that the endocannabinoid system may act as a neuroprotective system against abnormally increased discharge activity [26, 65]. The CB1 receptor-mediated neuroprotective effect in the kainate model is apparently mediated by decrease of excitability of glutamatergic hippocampal neurons [48].

Activation of postsynaptic receptors, at diverse neuronal types, induces the release of endogenous cannabinoid compounds that move backwards across the synapse, until reaching the cannabinoid CB1 receptor, to which they bind, therefore inhibiting further neurotransmitter release. Endocannabinoid-mediated synaptic plasticity can be transient or long lasting and can be found at both excitatory and inhibitory synapses in diverse brain structures. Endocannabinoid-mediated short-term synaptic plasticity includes two electrophysiological phenomena, depolarization-induced suppression of inhibition (DSI), and depolarization-induced suppression of excitation (DSE). DSI is due to a presynaptic action that reduces GABA release, while DSE results from presynaptic inhibition of glutamatergic release. There is also an involvement of the endocannabinoid system in long-term forms of synaptic plasticity. Long-term potentiation (LTP) is a long-lasting increase in the strength of a synapse, while long-term depression (LTD) is a long lasting weakening of synaptic strength. Both are mechanisms of synaptic plasticity that can persist for hours to weeks and have important implications on various forms of learning and memory. Endocannabinoid-induced long-lasting inhibition of neurotransmitter release has been found in diverse brain structures and at both excitatory and inhibitory synapses (for exhaustive discussion of these phenomena, see [15, 26, 64, 66, 67]).

4. EFFECTS OF CANNABINOIDS ON ANXIETY-RELATED RESPONSES

The main feature of the recreational use of cannabis is that it produces a euphoriant effect. This "high" can be accompanied by decreased anxiety and increased sociability. However, cannabis can also produce dysphoric reactions, feelings of anxiety, panic, paranoia, and psychosis [68–72]. It is possible that the reasons for this lie on the bidirectional effects of cannabinoids on anxiety, with low doses having anxiolytic, and high doses having anxiogenic-like effects. The previous history of the individual and the environmental context may also critically influence the induced cannabinoid effects. Data from animal models provide further evidence for the complexity of the scenario. Low doses of several cannabinoid receptor agonists, nabilone [73], CP 55,940 [74, 75], and Δ^9-tetrahydrocannabinol (THC) [76] induced anxiolytic-like effects in both the elevated plus-maze and the light-dark box. In contrast, high doses of the cannabinoid agonist HU-210 produced anxiogenic-like responses in the defensive withdrawal test [77] and enhanced emotional responding to tactile stimulation [78], and mid-high doses of CP 55,940 showed anxiogenic-like effects in the plus-maze [74, 75, 79, 80] and in the social interaction test [81].

It has been shown that exposure to chronic stress enhances the anxiety-like responsiveness to cannabinoids in rats [82], a phenomenon that is also observed in humans. Accordingly, Patel et al. [83] have recently analyzed the interactions between cannabinoids and environmental stress in the regulation of amygdalar activation in mice. The combination of restraint stress and CB1 agonist administration produced robust Fos induction within the central amygdala, indicating a synergistic interaction between environmental stress and CB1 receptor activation. These data suggest that the central amygdala could be an important neural substrate relevant to the context-dependent effects of cannabinoids on emotional/affective responses.

It is worth noting that, in addition to anxiety, there are other behavioral responses, such as motor activity and exploration [75, 80, 81, 84, 85], that are affected by cannabinoid agonists in a biphasic manner. In general, low doses are stimulatory, whereas high doses are inhibitory. Bimodal effects of cannabinoids might be explained by two distinct populations of presynaptic CB1 receptors, with different sensitivities to cannabinoids, particularly WIN 55,212-2 (WIN), located possibly on glutamatergic and GABAergic neurons [26, 86]. The administration of WIN resulted in a biphasic, dose-dependent effect on hippocampal acetylcholine (ACh) release: a low dose and a high dose of the compound induced a transient stimulation and a prolonged inhibition of hippocampal ACh efflux, respectively. These amphidromic responses appeared to involve the same structural entities, Gi-coupled CB1 receptors, but different neuroanatomical sites. The low-dose excitatory effects were mediated in the septum, whereas the high-dose inhibitory effects were mediated locally in hippocampus. Moreover, the stimulatory and the inhibitory effects of the cannabinoid agonist involved activation of dopamine D_1 and D_2 receptors, respectively [7]. Local infusion of cannabinoid compounds in specific brain areas might be instrumental to identify neural pathways and neuroanatomically separated CB1 receptor subpopulations that may play distinct roles and mediate opposing actions of cannabinoids, notably, anxiolytic versus anxiogenic effects [87]. This possibility might further explain why elevation of endocannabinoids levels sometimes has effects that are different from those observed with exogenous cannabinoids [26]. An additional hypothesis which might account for the biphasic effects of cannabinoids is the possible differential implication of Gs and Gi proteins in the stimulatory and inhibitory effects, respectively [88]. It would be interesting to test this hypothesis in vivo, in relation to anxiety-related effects.

5. ROLE OF THE ENDOCANNABINOID SYSTEM IN THE REGULATION OF ANXIETY

5.1. CB1 receptor knockout mice

The development of knockout (KO) mice deficient in CB1(CB1-KO) receptors has provided an excellent tool to evaluate the physiological roles of the endocannabinoid system, and particularly its possible implication in the regulation of anxiety. The CB1-KO mice showed an increase in the aggressive response measured in the resident-intruder test and an anxiogenic-like behavior in the light-dark box, the elevated plus-maze test, and the social interaction test [89, 90]. On the other hand, Marsicano et al. [18] did not find an anxiogenic-like response in the plus-maze in their CB1-KO mice. Discrepancies might be attributed to differences in the genetic background of mutant mice, and also to differences on baseline anxiety levels and to context-dependent stress elicited. In particular, CB1-KO mice exclusively showed an anxiogenic-like behavior under high-stress conditions: light in the plus-maze and unfamiliar environment in the social interaction test [18, 89–91]. An impaired action of anxiolytic drugs, such as bromazepam and buspirone, has been also observed in mutant mice [90]. This latter result suggests that functional integrity of cannabinoid CB1 receptors is necessary to achieve a complete efficacy of anxiolytic drugs, which may have consequences in the treatment of mood-related disorders, including those derived from cannabinoid abuse.

5.2. Pharmacological blockade of CB1 receptors

Evidence for an endogenous anxiolytic cannabinoid tone also comes from certain effects of the CB1 receptor antagonist rimonabant (SR141716A). This drug has anxiogenic effects in adult rats submitted to the defensive withdrawal test and the elevated plus-maze [79, 92]. The cannabinoid receptor agonist CP 55,940 reduced ultrasonic vocalization in rat pups separated from their mother, indicating an anxiolytic effect, and rimonabant not only reversed this effect, but also enhanced pup ultrasonic vocalizations when administered alone [93]. These results further support the view that there is an endogenous regulation of emotional states mediated by the cannabinoid system that might be present since early developmental stages. As for CB1-KO animals, certain results obtained in mice following rimonabant administration showed apparently contradictory results since this compound was found to be anxiolytic in the plus-maze [89]. These data may reflect species differences, but it seems likely that environmental context and baseline anxiety levels critically account for at least some of the discrepancies observed in the literature. The context dependency is indirectly supported by the "one-trial sensitization" phenomenon described by Rodgers et al. [94] in the plus-maze. In these experiments, the CB1 receptor antagonist had no behavioral effects in maze-naïve mice, but induced an anxiolytic-like effect in the second trial of the test.

With respect to recent clinical trials, rimonabant has been tested for its possible therapeutical application in obesity and metabolic disorders, and the most frequent adverse events resulting in discontinuation of the drug included depression and anxiety [95–97].

5.3. Inhibitors of endocannabinoids inactivation

As indicated above (Section 2), the enzyme FAAH catalyzes the hydrolysis of the endogenous cannabinoid anandamide. Pharmacological blockade of this enzyme by URB597 and URB532 produced anxiolytic-like effects in the elevated zero-maze in adult rats and in the isolation-induced ultrasonic emission test in rat pups. These effects were accompanied by augmented brain levels of anandamide and were prevented by CB1 receptor blockade. Moreover, the anxiolytic actions of URB597 were not accompanied by typical cannabinoid signs of intoxication in rodents such as catalepsy or hypothermia. These results indicate that anandamide participates in the modulation of emotional states and point to FAAH inhibition as an innovative approach to antianxiety therapy [98].

A model has been proposed to explain the possible mechanism by which the AEA-CB1 receptor system may participate in the control of anxious states. Endocannabinoids might be generated in the amygdala in response to the anxiety inducing stimulus, and would, therefore, regulate emotional states by influencing amygdala outputs [99]. This view is supported also by the fact that AEA content in the mouse basolateral amygdala rises when the animal is conditioned to expect a foot shock after hearing a tone [18]. Thus, the endocannabinoid system, and AEA in particular, might be activated in response to anxiogenic situations and this activation could be part of a negative feedback system that limits anxiety [99]. In line with this hypothesis, there are data suggesting a role of endocannabinoid signaling as an inhibitory modulator of behavioral and neuronal responses to aversive stimuli [13] and in the inhibition of stress-induced activation of HPA axis [12] (see next section). A recent paper by Patel and Hillard [100] further supports a crucial role for endocannabinoids in the induction of anxiolytic-like effects. The inhibitor of endocannabinoids metabolism, URB597, produced a linear dose-dependent anxiolytic effect. In turn, AM404 that is considered as an inhibitor of endocannabinoids uptake exerted an action that was more similar to that elicited by direct agonists, with low doses producing anxiolytic effects and the highest dose having no effect [98]. The different profiles of AM404 might be due to the fact that in addition to increasing the endocannabinoid-mediated tone, this compound can also activate TRV1 receptors [101] which, as indicated by the study by Marsh et al. quoted above [57], are also involved in the regulation of anxiety.

Collectively, a majority of evidence suggests the existence of an anxiolytic endocannabinoid tone. The modulatory role of the endocannabinoid system against stress is further supported by studies from Patel et al. [12, 13] indicating that endocannabinoids act as inhibitory modulators of both neuronal and behavioral activations during an acute stress and negatively modulate HPA axis activity (see Section 7).

6. CONDITIONED FEAR RESPONSES, AVERSIVE MEMORIES, AND FEAR EXTINCTION

Neurobiological substrates of emotional-based learning have been extensively examined in animal models that allow the study of acquisition, expression, and retention of Pavlovian fear conditioning . In this paradigm, an initially innocuous/neutral stimulus (the to-be conditioned stimulus (CS); e.g., a light, tone, or odor) is paired with an innately aversive unconditioned stimulus (US; e.g., a footshock). Following several pairings, the subject comes to exhibit a conditioned fear response to the CS. Conditioned fear behavioral and physiological responses include changes in heart rate and blood pressure and freezing or cue-induced fear potentiated startle reflex. Excessive fear and anxiety are hallmarks of a variety of disabling neuropsychiatric disorders. Adaptive strategies leading to an appropriate interplay between fear expression and fear extinction are necessary for adequate coping with aversive encounters. In experimental studies like the ones mentioned above, fear inhibition is frequently studied through a procedure in which the previously fear conditioned subject is exposed to the fear-eliciting cue in the absence of any aversive event. This procedure results in a decline in conditioned fear. In other words, repeated presentation of the conditioned stimulus alone leads to extinction of the fearful response. There are clear clinical implications of research on fear extinction. Anxiety- related pathologies such as phobias and post-traumatic stress disorder (PTSD) seem to be disorders of fear dysregulation in which inhibition of fear is absent or insufficient in situations that are patently safe. In the last years, there is an increasing interest in revealing the neural mechanisms of fear inhibition, including the regions in which extinction-related plasticity occurs and the cellular and molecular processes that are implicated in this plasticity-related phenomenon (comprehensive reviews on these mechanisms can be found in [102–105]). In the present section, we will focus on the possible functional implication of the endocannabinoid system.

The use of CB1-KO mice and pharmacological blockade of CB1 receptors have yielded information regarding the involvement of the endocannabinoid system in conditioned fear responses. It has been reported that CB1-KO mice showed strongly impaired short- and long-term extinction in auditory fear-conditioning tests, with unaffected memory acquisition and consolidation. Consistent with this finding, pharmacological blockade of CB1 receptors with rimonabant led to a similar deficit in extinction in wild-type mice [18]. The authors also found that during the extinction protocol (exposure to the tone alone), the levels of endocannabinoids were raised within the basolateral amygdala, a region known to control extinction of aversive memories, both in mutant and normal mice. In subsequent studies, Azad et al. [19] showed that low-frequency stimulation of afferents in the lateral amygdala released endocannabinoids postsynaptically from neurons of the basolateral amygdala of mice, and thereby induced an LTP of inhibitory GABAergic synaptic transmission (LTDi) via a presynaptic mechanism. In turn, lowering inhibitory synaptic transmission significantly increased the amplitude of excitatory synaptic currents in principal neurons of the central nucleus, which is the main output site of the amygdala. LTDi was blocked by rimonabant, abolished in CB1-KO animals, and significantly enhanced in mice lacking FAAH, the anandamide-degrading enzyme [19]. More recently, it has been addressed whether CB1 blockade would similarly disrupt extinction in rats, using fear-potentiated startle as a measure of conditioned fear. The authors further investigated whether pharmacologic augmentation of CB1 activation would lead to enhancements in extinction. The results indicated that rimonabant dose-dependently blocked the extinction of conditioned fear in rats, as it does in mice. Moreover, administration of AM404, an inhibitor of endocannabinoid reuptake, led to a dose-dependent enhancement in extinction and this effect was blocked almost completely by rimonabant, indicating an implication of CB1 receptors. The animals treated with AM404 also showed decreased shock-induced reinstatement of fear, suggesting that this compound may reduce susceptibility to reinstatement of fear [106]. Lin et al. [107] have shown that bilateral infusion of CB1 receptor agonists into the amygdala after memory reactivation blocked reconsolidation of fear memory measured with fear-potentiated startle. These authors proposed that activation of CB1 receptors could facilitate extinction on one hand and block reconsolidation on the other.

Hölter et al. [108] have compared CB1-KO mice with their wild-type controls in an appetitively motivated operant conditioning task including food reward. During the extinction phase, when the positive reinforcement was omitted, control and CB1-KO mice showed a similar decline in accuracy of performance and total number of correct responses, accompanied by an increase in errors of omission [108]. A recent pharmacological study using rimonabant [109] further supports the notion that the cannabinoid CB1 receptor plays a pivotal role in extinction of aversive memories but is not essential for extinction of positively reinforced memories.

It has been claimed that fear conditioning in mice combines both associative and non-associative (sensitization) components and that extinction involves a significant habituation component [110]. In a more recent study, Kamprath et al. [111] have found that CB1-KO mice were severely impaired not only in extinction of the fear response to a tone after fear conditioning, but also in habituation of the fear response to a tone after sensitization with an inescapable footshock. Based on these findings, they have proposed that CB1 receptor might be critically involved in non-associative learning processes (habituation), which would contribute to the decrease in the fear response. A mouse model has been recently proposed that may allow exploring the role of the endocannabinoid system in the associative and non-associative components of fear has been recently proposed [112].

7. CANNABINOIDS AND THE HYPOTHALAMUS-PITUITARY-ADRENAL AXIS

An electrophysiological study by Di et al. [20] has revealed that glucocorticoids elicit a rapid, nongenomic suppression

of glutamate release onto parvocellular neuroendocrine cells of the hypothalamic paraventricular nucleus (PVN) by stimulating the retrograde release of endocannabinoids that would subsequently activate presynaptic cannabinoid CB1 receptors. By this mechanism, endocannabinoids may be involved in the modulation of a number of peptidergic systems, including CRH. Patel et al. [12] have addressed a role of the endocannabinoid system in the modulation of stress-induced adrenocortical activity in vivo. These authors confirmed previous studies showing that rimonabant was able to increase serum corticosterone concentrations under basal conditions. Moreover, the CB1 receptor antagonist potentiated restraint stress-induced HPA axis activation, whereas pretreatment of mice with either a low dose of the CB1 receptor agonist CP 55,940, the endocannabinoid transport inhibitor AM404, or the FAAH inhibitor URB597 significantly decreased or eliminated restraint-induced corticosterone release. Acute restraint-induced corticosterone release was associated with a decrease in hypothalamic 2-AG content, whereas the attenuation of adrenocortical response observed after prolonged stress was associated with an increase in hypothalamic 2-AG content. In view of the above data, the following speculative model can be suggested: during resting (baseline) conditions, the HPA axis would be tonically inhibited by endocannabinoids via CB1 receptors located in the PVN of the hypothalamus. In this way, the endocannabinoid system might keep under control the stress response. Upon an acute stress exposure, that is, when the stress response is needed, a reduction of endocannabinoids signaling would allow the HPA axis to be activated (disinhibition). If the stress becomes chronic, endocannabinoid levels would increase again to restore a normal homeostasis.

With respect to the effects of exogenous cannabinoid agonists, in general the literature indicates that they exert a dose-dependent effect on adrenocortical activity with high doses increasing corticosterone responses [21, 84, 113]. As previously indicated, high doses of cannabinoids are also anxiogenic. However, we have found that, at certain doses, the effects of cannabinoids on anxiety can be dissociated from their effects on adrenocortical activity. Thus a high dose of the cannabinoid agonist CP 55,940 (75 μg/kg) induced both, anxiogenic-like effects in the plus-maze and stimulation of adrenocortical activity [80]. However, a dose of 50 μg/kg induced an anxiogenic-like effect in the same test, without increasing corticosterone concentrations [113].

As in the case of anxiety, literature regarding HPA axis activity supports the general concept that the pharmacological administration of exogenous cannabinoids may lead to a completely different action when compared with the physiological functions of the endocannabinoid system [26, 28, 30].

8. ENDOCANNABINOID SYSTEM AND DEPRESSION

Several lines of evidence suggest that the endocannabinoid system may play a role in the aetiology of depression and could represent a new therapeutic target for its treatment. CB1-KO mice showed altered HPA axis function [90] and a higher sensitivity to exhibit depressive-like responses in the chronic unpredictable mild stress procedure, which suggests an increased susceptibility to develop an anhedonic state [114]. These characteristics together with their heightened anxiety [89, 90] and deficits in extinction of aversive memories [18] have been proposed to be analogous to certain symptoms of melancholic depression [115].

Several cannabinoid compounds have been evaluated in behavioral tests such as the forced swimming test (FST) and the tail-suspension test (TST) that are among the most widely used screening tests of antidepressant potential of novel compounds [116]. In the rat FST, administration of AM404 (endocannabinoid uptake inhibitor) and HU-210, a potent CB1 receptor agonist, induced decreases in immobility (indicative of antidepressant activity) that were blocked by pretreatment with the selective CB1 receptor antagonist AM251. The reduction in immobility induced by the cannabinoid compounds was comparable to that seen with the reference antidepressant desipramine [117]. In turn, the FAAH inhibitor URB597 exerted potent antidepressant-like actions in the mouse TST and the rat FST, and these effects were prevented or attenuated by rimonabant [118].

During the last years, there has been an active investigation on the implications of hippocampal neurogenesis in the pathophysiology and treatment of mood disorders. Preclinical and clinical studies indicate that stress (possibly through the action of elevated glucocorticoids) and depression lead to atrophy and loss of neurons in the adult hippocampus. On the other hand, chronic antidepressant treatment up-regulates hippocampal neurogenesis which could counteract the stress-induced damage [119, 120]. An elegant study by Jiang et al. [121] revealed an important implication of hippocampal neurogenesis in the antidepressant and anxiolytic-like effects of cannabinoid agonists. They showed that both embryonic and adult rat hippocampal neural stem/progenitor cells were immunoreactive for cannabinoid CB1 receptors, indicating that cannabinoids could act on these receptors to regulate neurogenesis. A chronic (but not acute) treatment with the potent synthetic cannabinoid HU210 promoted neurogenesis in the hippocampal dentate gyrus of adult rats and exerted anxiolytic- and antidepressant-like effects. The cannabinoid–induced newborn neurons appeared to be of functional significance, since X-irradiation of the hippocampus blocked both the neurogenic and behavioral effects of chronic HU210 treatment. These evidences strongly suggest that cannabinoid agonists might produce anxiolytic- and antidepressant-like effects by promoting hippocampal neurogenesis. In line with these findings, administration of the endocannabinoids uptake inhibitor AM404 prior to exposure to predator odor stress inhibited both the stress-induced activation of defensive burying and the suppression of cell proliferation in the hippocampus [122], indicating a role for endocannabinoids in the modulation of stress-induced changes in hippocampal cell proliferation.

The efficacy of antidepressants has been linked in part to their ability to reduce the activity of the HPA axis [123]. In view of the above data, it is tempting to speculate that the

endocannabinoid system is somehow involved in the action of currently used antidepressant drugs. In favor of this hypothesis, it has been shown that chronic administration of the tricyclic antidepressant desipramine resulted in a significant increase in the density of the cannabinoid CB1 receptor in both hippocampus and hypothalamus as well as in a reduction in swim stress-induced corticosterone secretion and immediate early *c-fos* gene in the medial dorsal parvocellular region of the PVN of the hypothalamus. Moreover, acute treatment with the CB1 receptor antagonist AM251 before exposure to stress occluded the effects of desipramine on corticosterone secretion and neuronal activation [124].

9. CONCLUDING REMARKS

During the last few years, the increasing interest in the link between the endocannabinoid system and emotional responses has led to a number of interesting data derived from animal studies. These results may contribute to understand the complex scenario of cannabinoid effects in humans, and to clarify the mechanisms underlying associations between cannabis abuse and mental disorders. Results obtained from transgenic mice lacking CB1 receptors and by using CB1 receptors selective antagonists and inhibitors of endocannabinoids inactivation suggest the existence of an intrinsic endocannabinoid tone which contributes to the regulation of stress responses and anxiety. An adequate endocannabinoid function appears to be necessary for adaptive extinction of aversive memories. The endocannabinoid system might play a pivotal role in maintaining homeostasis, notably with regard to physiological and behavioral responses to acute and prolonged stress. Certain forms of endocannabinoid-dependent synaptic plasticity have been proposed as crucial mechanisms subserving these phenomena. Throughout this review, we have focused on the endocannabinoid system as a major player in the modulation of synaptic transmission and plasticity considering solely interneural communication. However, the critical functional role of glial cells in maintaining a correct brain function and their implications in diverse neuropathological conditions are now clearly recognized. The new concept of the tripartite synapse in which the glial cell (notably astrocytes) plays an active role in the modulation of neurotransmission has recently emerged [125]. Expression of cannabinoid CB1 receptors and endocannabinoid synthesis and release have been observed in different types of glial cells [126, 127]. This "glial endocannabinoid system" may have important physiological and pathological implications [128, 129] and it would be interesting to explore a possible role in the expression of synaptic plasticity in limbic and extra-limbic regions related to stress, fear, and anxiety responses.

Disregulation or malfunctioning of the endocannabinoid system might contribute to the aetiology of anxiety-related disorders and to certain symptoms of melancholic depression. In turn, the endocannabinoid system might constitute an interesting pharmacological target for the development of anti-anxiety and antidepressant therapies.

The involvement of the endocannabinoid system in the regulation of anxiety and its participation in the modulation of behavioral and physiological responses to aversive situations have other obvious implications. Cannabis abuse may be one of the causes disrupting the necessary balance for an appropriate function of the system. There are functional interactions between the endocannabinoid system and other monoaminergic and peptidergic systems also involved in the regulation of emotional responses [113, 130]. Thus, the disruption of the endocannabinoid system as a consequence of cannabis abuse may alter these other neurochemical systems contributing to the development of emotional disorders. In addition to acute aversive emotional reactions to cannabis, the chronic use of this addictive drug may result in mental disturbances and neuropsychiatric disorders. In particular, there are data suggesting that exposure to cannabis derivatives is associated with a higher risk of schizophrenia, depression, and anxiety [68–72, 131, 132]. In this review, we have highlighted the importance of endocannabinoid-based neuroplasticity phenomena in the regulation of neuroendocrine and neurochemical systems implicated in the modulation of emotional responses and extinction of perseverative behaviors and inadaptative aversive memories. Consequently, it is likely that impairment of endocannabinoid-mediated synaptic transmission and plasticity contribute to the expression of at least some aspects of these psychiatric illnesses.

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Review Article

Role of Apolipoprotein E in Anxiety

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Anxiety is most common among Alzheimer's disease (AD) patients with an age at onset under age 65. Apolipoprotein E4 (apoE4) is a risk factor for developing AD at an earlier age and might contribute to this effect. In mice, apoE plays a role in the regulation of anxiety, which might involve histamine receptor-mediated signaling and steroidogenesis in the adrenal gland. In addition, human apoE isoforms have differential effects on anxiety in adult mice lacking apoE and probable AD patients. Compared to wild-type mice, mice lacking apoE and apoE4 mice showed pathological alterations in the central nucleus of the amygdala, which is involved in regulation of anxiety. ApoE4, but not mice lacking apoE, or apoE3 mice showed impaired dexamethasone suppression of plasma corticosterone. Understanding how apoE modulates measures of anxiety might help the developments of therapeutic targets to reduce or even prevent measures of anxiety in health and in dementing illnesses.

1. INTRODUCTION

Noncognitive behavioral changes are the major cause of institutionalization of AD patients and a major concern for their caregivers [1–3]. Such changes are also a negative predictor of survival and quality of life for AD patients and contribute to increased costs [4, 5]. However, they have received much less attention than cognitive impairments. Most pharmacological strategies for controlling behavioral changes, including treatment with benzodiazepines, cause deterioration in mental performance and motor function [6]. In AD, anxiety is inversely related to mini-mental state examination (MMSE) score (i.e., worse with more severe dementia) [7]. Anxiety symptoms occur in about 50% [8] to 75% [9] of AD patients. A study describing the relationship between anxiety and nighttime behavioral disturbance in a community dwelling sample of 153 AD patients revealed symptoms of anxiety and patient awakening associated with higher levels of patient anxiety and patient impairments in activities of daily living (ADL) in 56% of the patients [10]. Individual-patient anxiety symptoms were risk factors for patient awakenings [10]. Anxiety symptoms become more common as the disease progresses and are associated with greater disability in daily activities [7, 8]. In more than half of the cases, the caregivers demand therapeutic intervention regardless of the effects on cognitive and motor function. Therefore, there is a need to better understand the mechanisms underlying increased anxiety and to develop better treatments for these conditions.

2. APOE, BRAIN FUNCTION, AND AD

ApoE plays an important role in the metabolism and redistribution of lipoproteins and cholesterol [11]. The three major human apoE isoforms are encoded by distinct alleles ($\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$). Compared with $\varepsilon 2$ and $\varepsilon 3$, $\varepsilon 4$ increases the risk of cognitive impairments and of developing AD [12]. This increased risk might involve a loss of trophic function of apoE4 or gain of toxic function of apoE4. Anxiety is most common among AD patients with a younger age at onset (under age 65) [7]. ApoE4 is a risk factor for developing AD at an earlier age [12] and might contribute to this effect.

3. ROLE OF APOE IN REGULATING MEASURES OF ANXIETY REVEALED IN *APOE*$^{-/-}$ MICE

The elevated plus maze can be used to assess measures of anxiety in mice (Figure 1). The plus maze consists of a perpendicular cross elevated above the floor. The sides of one axis are walled off. There are infrared photobeams to record movements. Mice will prefer the safety of the enclosed,

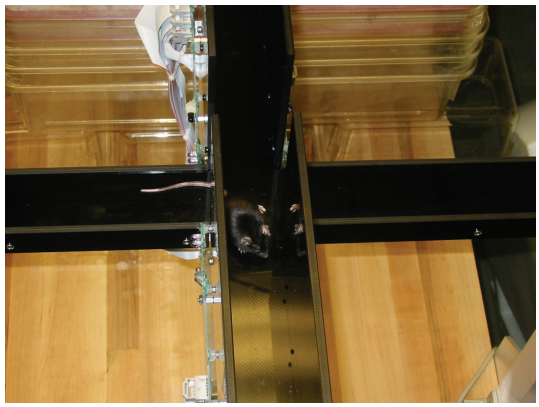

Figure 1: Elevated plus maze: the mice are tested for 10 minutes; while they are curious to explore the open areas, they are anxious to do so.

darker arms, but they like to explore the open arms and poke over the edge. Less anxious mice will venture more onto the open arms, and poke their heads more over the edges of the open arms. Male *Apoe*$^{-/-}$ (C57BL/6J-Apoe^{tm1Unc}) and wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, Me). When measures of anxiety in the elevated plus maze were assessed in 6-month-old *Apoe*$^{-/-}$ male and wild-type control mice, *Apoe*$^{-/-}$ mice showed increased measures of anxiety [13]. These changes are age-dependent and not seen in 3-month-old mice.

4. APOE AND ADRENAL STEROIDOGENESIS

Liver and brain are the major sites of apoE synthesis. However, many other tissues, particularly steroidogenic tissues such as the adrenal gland, also express apoE [14, 15]. Adrenal apoE expression is the highest in cortical cells that synthesize glucocorticoids (GCs), declines when steroidogenesis is stimulated, and increases when it is blocked [14, 15]. The function of apoE synthesized by the adrenal gland is unclear. *Apoe*$^{-/-}$ mice show an age-dependent dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis regulates the secretion of GCs through a mechanism that primarily affects the adrenal gland. In *Apoe*$^{-/-}$ mice, activation of the HPA axis is seen at the same time as increased measures of anxiety are observed in the elevated plus maze. *Apoe*$^{-/-}$ mice have an age-dependent increase in basal adrenal corticosterone content and abnormally increased plasma corticosterone after restraint stress and anxiety testing in the elevated plus maze [13]. *Apoe*$^{-/-}$ mice also show increases in lipid droplets in adrenal cortex and medulla [13]. These data are consistent with hypersecretion of adrenal corticosterone and increased adrenal corticosterone content and with the reported inverse relationship between the levels of apoE mRNA and adrenal steroidogenesis, and they suggest a key role for apoE in the tonic inhibition of steroidogenesis and adrenal cortical activity.

5. HISTAMINE AND ANXIETY OF *APOE*$^{-/-}$ MICE

Histamine increases measures of anxiety [16], and altered histamine signaling could contribute to increased measures of anxiety in adult *Apoe*$^{-/-}$ male mice. We began to assess the possible role of histamine receptor-mediated signaling in regulating measures of anxiety in *Apoe*$^{-/-}$ and wild-type mice. Drugs were dissolved in saline and administered by intraperitoneal injection 1 hour before behavioral testing at the indicated doses, selected based on preliminary studies. The person testing the mice was blinded to genotype and treatment. The histamine H_3 receptors modulate histamine release and synthesis via negative feedback. We assessed whether young (3–5-month old) *Apoe*$^{-/-mice}$, which show similar measures of anxiety in the anxiety-provoking open arms of the plus maze, respond differentially to histamine H_3 receptor ligands. Anxiety levels were assessed 1 hour after intraperitoneal administration of thioperamide (5 mg/kg) or saline. Wild-type mice treated with H_3 receptor antagonist thioperamide showed increased measures of anxiety as compared to wild-type mice treated with saline [17]. The total activity in the closed arms was comparable in the saline- and thioperamide-treated wild-type mice. These data indicate that the differences in measures in the open arms were not caused by differences in activity levels. In contrast, thioperamide had no effect on measures of anxiety in *Apoe*$^{-/-}$ mice.

Next we determined whether in wild-type and *Apoe*$^{-/-}$ mice, H_3 antagonists also have differential effects on novel object recognition [18, 19]. During the training session, mice were allowed to explore for 15 minutes an open field containing two objects. For the retention session (24 hours later), they were placed back into the same open field for 15 minutes, after one of the familiar objects had been replaced with a novel object and the other familiar object with an exact replica. The percentage of time the mice spent exploring the novel versus the familiar object relative to the total amount of time they explored either object in the retention session was used as a measure of object recognition memory. Wild-type and *Apoe*$^{-/-}$ mice received saline, thioperamide (5 mg/kg), or clobenpropit (10 mg/kg) during the training and retention sessions [17]. The recently cloned H_4 receptor [20] was found to have an affinity for H_3-specific ligands. To rule out the possible contribution of the H_4 receptor to the effects of thioperamide, we also treated wild-type and *Apoe*$^{-/-}$ mice with clobenpropit, a H_3-specific antagonist that was reported to be an H_4 receptor agonist as well [20]. In the training session, all groups of mice spent a comparable amount of time exploring each object. In the retention session, the saline-treated wild-type and *Apoe*$^{-/-}$ mice spent significantly more time exploring the novel object (wild-type: 8.37 ± 0.93 seconds; *Apoe*$^{-/-}$: 9.34 ± 2.76 seconds) than the familiar object (wild-type: 5.43±0.69 seconds; *Apoe*$^{-/-}$: 5.15±1.25 seconds), whereas the thioperamide- and clobenpropit-treated wild-type and *Apoe*$^{-/-}$ mice spent equal amounts of time exploring both objects. The similar effects of thioperamide and clobenpropit on novel object recognition indicate that they are mediated by the H_3 receptor and not the H_4 receptor.

To determine whether H_3 ligands have differential effects on emotional learning and memory in wild-type and *Apoe*$^{-/-}$ mice, passive avoidance learning was used. Mice were placed in a lighted compartment of a chamber also containing a dark compartment. They entered the dark compartment by preference where they received a brief and slight foot shock (0.3 mA for 1 second). After 24 hours, the mice were again placed in the light compartment, and the time to reenter the dark compartment was measured. Drugs were administered 1 hour before behavioral testing on both days. Both saline- and thioperamide- (5 mg/kg) treated wild-type and *Apoe*$^{-/-}$ mice showed emotional learning and memory as the time to reenter the dark chamber was significantly higher on day 2 than day 1. There was no effect of thioperamide but consistent with increased measures of anxiety of *Apoe*$^{-/-}$ mice in the elevated plus maze, the latency to enter the dark compartment on day 1 was lower in *Apoe*$^{-/-}$ than wild-type mice ($P < .05$, Tukey-Kramer).

To determine whether there are differences in H_3 receptor expression in young *Apoe*$^{-/-}$ and wild-type mice (3–5-month old) which could have contributed to their differential response to H_3 antagonists on measures of anxiety, we performed saturation analysis with [^{3}H]-Nα-methylhistamine (NAMH) in brain regions that have been implicated in cognition or emotion [17]. The total number of receptors (B_{max} in nM) in the amygdala (wild-type: 87.3±2.5; *Apoe*$^{-/-}$: 81.8± 2.3), cortex (wild-type: 119.9 ± 3.0; *Apoe*$^{-/-}$: 56.8 ± 5.8), and hippocampus (wild-type: 108.4 ± 10.5; *Apoe*$^{-/-}$: 29.1 ± 1.7) was lower in *Apoe*$^{-/-}$ than in wild-type mice. In the hypothalamus, B_{max} was not different between the groups. There was no difference in the binding affinities of [^{3}H]-NAMH in any brain region. Thus, there is no simple association between levels of H_3 receptor expression in structures associated with anxiety versus cognition, which could explain why H_3 antagonists impaired hippocampus- and cortex-dependent novel object recognition [18] but did not increase more amygdala-dependent measures of anxiety in the plus maze.

In experimental models of anxiety, stimulation of H_{1-}, but not of H_{2-}, receptors increases measures of anxiety [16, 21, 22]. In *Apoe*$^{-/-}$ mice, reduced negative feedback via H_3 receptors could increase histamine release and signaling of H_1 and H_2 receptors. To determine whether in *Apoe*$^{-/-}$ mice increased signaling of these receptors contributed to the increased measures of anxiety, 3–5-month-old wild-type and *Apoe*$^{-/-}$ mice were assessed in the elevated plus maze 1 hour after intraperitoneal administration of the H_1 antagonist mepyramine (5.6 mg/kg), the H_2 antagonist zolantidine (10 mg/kg), or saline. *Apoe*$^{-/-}$ mice treated with mepyramine, but not with zolantidine, showed reduced measures of anxiety as compared to *Apoe*$^{-/-}$ mice treated with saline [17]. The total activity in the closed arms was comparable and not significantly different between the saline-, mepyramine-, and zolantidine-treated *Apoe*$^{-/-}$ mice, indicating that the differences in measures in the open arms were not caused by differences in activity levels. In contrast, mepyramine had no effect on measures of anxiety in wild-type mice. The lack of an effect of H_1 receptor blockade on measures of anxiety in wild-type C57Bl/6J mice is consistent with the lack of effect

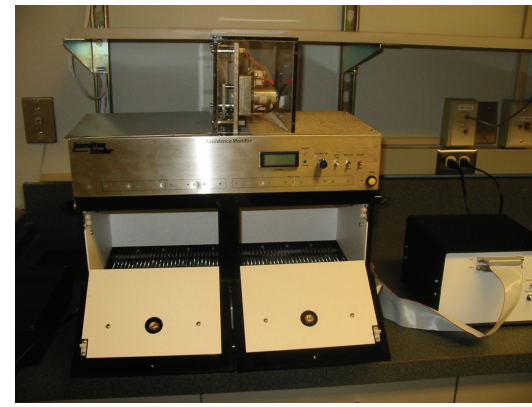

Figure 2: Passive avoidance: the mice are trained to avoid the preferred dark compartment by paring it with an aversive stimulus.

of H_1 receptor blockade on measures of anxiety in wild-type ddY mice and it supports that the H_1 receptor becomes activated at higher levels of histamine release [21]. The reduced measures of anxiety in *Apoe*$^{-/-}$ mice after H_1 receptor blockade are consistent with the reported antagonizing effects of mepyramine on experimental anxiety induced by histamine releasers [16, 21] and the anxiogenic effects of the H_1 receptor agonist and H_3 receptor antagonist betahistine [22].

In *Apoe*$^{-/-}$ mice, the effects of mepyramine on measures of anxiety in the plus maze were not associated with a reduced HPA axis response. Plasma ACTH and corticosterone levels were assessed directly after plus-maze testing [23]. Compared to saline controls, mepyramine reduced the plasma corticosterone levels in wild-type (saline: 179 ± 38 ng/mL, $n = 6$; mepyramine: 89 ± 26 ng/mL, $n = 6$; $P < .05$ Tukey-Kramer), but not in *Apoe*$^{-/-}$ (saline: 206 ± 30 ng/mL, $n = 8$; mepyramine: 224±10 ng/mL, $n = 9$) mice. There were an effect of genotype ($P < .01$) and a genotype x treatment interaction ($P = .0474$). Mepyramine also reduced plasma ACTH levels in wild-type mice (saline: 121 ± 20 pg/mL, $n = 6$; mepyramine: 77±9 pg/mL, $n = 6$; $P < .05$ Tukey-Kramer), but not in *Apoe*$^{-/-}$ mice (saline: 57±5 pg/mL, $n = 8$; mepyramine: 62±11 pg/mL, $n = 9$). These data show that in *Apoe*$^{-/-}$ mice, mepyramine does not reduce measures of anxiety by inhibiting the HPA axis response. The dissociation of the effects of H_1 receptor blockade on anxiety from those on the HPA axis in *Apoe*$^{-/-}$ and wild-type mice and the differential effects of H_3 receptor blockade on novel object recognition and anxiety in *Apoe*$^{-/-}$, but not wild-type, mice suggest that differential pharmacokinetic profiles of histaminergic drugs in the two genotypes do not underlie the behavioral results.

There are no differences in H_1 receptor expression in young (3–5-month old) *Apoe*$^{-/-}$ and wild-type mice. Saturation analysis with [^{3}H] mepyramine in brain regions implicated in cognition or emotion [17] showed similar total number of receptors (B_{max} in nM) in the amygdala (wild-type: 103.4 ± 13.16; *Apoe*$^{-/-}$: 120.9 ± 20.92), cortex (wild-type: 126.5 ± 8.472; *Apoe*$^{-/-}$: 170.0 ± 13.02), hippocampus (wild-type: 104.0 ± 10.13; *Apoe*$^{-/-}$: 98.66 ± 11.03), and

Table 1: Elevated plus-maze performance of 6–8-month-old NSE-apoE mice.

Genotype	Distance moved in closed arms (inches)	Time in closed arms (s)	Ratio time in open arms/time in open + time in closed arms[1]
Wild type ($n = 8$)	1003 ± 76	4962 ± 85	0.078 ± 0.015*
Apoe$^{-/-}$ ($n = 37$)	824 ± 30	5069 ± 113	0.035 ± 0.010
apoE3 ($n = 27$)	917 ± 40	4697 ± 100	0.087 ± 0.013**
apoE4 ($n = 17$)	886 ± 68	5204 ± 190	0.022 ± 0.007
apoE3/E4 ($n = 9$)	825 ± 66	4807 ± 77	0.053 ± 0.008

[1]There was a significance of genotype on ratio time in open arms/time in open + time in closed arms ($P = .0094$).
*$P < .05$, wild-type versus *Apoe*$^{-/-}$, apoE4, or apoE3/E4.
**$P < .05$ versus *Apoe*$^{-/-}$ and apoE3/E4, and $P < .01$ versus apoE4.

Table 2: Elevated plus-maze performance in 6-month-old GFAP-apoE male mice.

Genotype (line)	Distance moved in closed arms (inches)	Time in closed arms (s)	Ratio time in open arms/time in open + time in closed arms
Apoe$^{-/-}$ ($n = 34$)	1050 ± 43	475 ± 12	0.058 ± 0.012
apoE3 (127) ($n = 11$)	1137 ± 59	417 ± 14	0.174 ± 0.022*
apoE4 (129) ($n = 4$)	1194 ± 69	479 ± 34	0.044 ± 0.024
apoE4 (130) ($n = 9$)	1039 ± 36	475 ± 24	0.042 ± 0.016

*$P < .05$ versus *Apoe*$^{-/-}$ mice and $P < .01$ versus apoE4 (129) and apoE4 (130).

hypothalamus (wild-type: 218.7 ± 22.33; *Apoe*$^{-/-}$: 159.4 ± 29.00) of *Apoe*$^{-/-}$ and wild-type mice and similar binding affinities of [^{3}H]-mepyramine in each brain region.

6. HUMAN APOE ISOFORMS AND MEASURES OF ANXIETY

We hypothesized that human apoE isoforms have differential effects on measures of anxiety in adult (6–8 months of age) *Apoe*$^{-/-}$ mice expressing human apoE3 or apoE4 at similar levels. *Apoe*$^{-/-}$ male mice without human apoE expression and apoE4 mice showed increased measures of anxiety in the elevated plus maze, whereas apoE3 male mice behaved like wild-type controls (Table 1). These differential effects of apoE isoforms on anxiety were age-dependent and not seen in young (2–4-month-old) male mice.

The isoform-specific effects of apoE are independent of the cellular source of apoE. When anxiety levels in the elevated plus maze were assessed in a cohort of 6-month-old GFAP-apoE male mice, in which the expression of apoE3 or apoE4 is targeted to astrocytes, GFAP-apoE3, but not GFAP-apoE4, male mice showed less measures of anxiety in the elevated plus maze than *Apoe*$^{-/-}$ mice (Table 2). Similar results were seen in the elevated zero maze, in which the mouse does not need to turn around in the open areas in order to return to the closed areas [24].

The elevated plus maze is different from tests that involve unavoidable anxiety-provoking stimuli, such as acoustic stimuli. By assessing the acoustic startle response, we determined that apoE also has age- and isoform-specific effects on anxiety elicited by unavoidable acoustic stimuli. To measure startle reflex, we used the SM100 startle monitor system (Hamilton-Kinder) (Figure 3). At 3 months of age, there were no effects of apoE isoforms on the acoustic startle response. However, there were differential effects of apoE isoforms on the acoustic startle response at 6 months of age. *Apoe*$^{-/-}$ mice showed a higher acoustic startle response than age-matched wild-type mice. This was not present in apoE3 mice and was present to a lesser extent in apoE4 mice. There was a difference in acoustic startle response between apoE3 and apoE4 mice and between *Apoe*$^{-/-}$ and apoE3 or apoE4 mice [25]. Thus, the differential effects of apoE isoforms on measures of anxiety are not limited to avoidable anxiety-provoking stimuli. There were no differences in hearing threshold or fear-potentiated startle in the 6-month-old male groups.

7. DEXAMETHASONE SUPPRESSION AND APOE4

Impaired suppression of cortisol levels after administration of dexamethasone is reported in AD [26]. Therefore, we examined whether dexamethasone could suppress plasma corticosterone in adult apoE transgenic mice [25]. Mice were injected with dexamethasone (0.1 mg/kg) or saline between 9:00 a.m. and 10:00 a.m., and trunk blood was collected 6 hours later [27]. Compared to saline, dexamethasone suppressed plasma corticosterone levels in wild-type, *Apoe*$^{-/-}$, and apoE3 mice but dexamethasone suppression was impaired in apoE4 mice. The impaired dexamethasone suppression in the apoE4 mice might relate to other perturbations of cortisol responsivity observed in AD, including reduced cortisol-mediated regulation of hippocampal glucose metabolism [28] and dexamethasone sensitivity of peripheral blood nuclear cells [29].

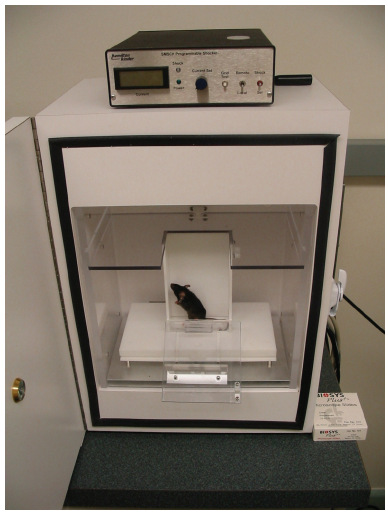

Figure 3: Acoustic startle: the mice are placed on a sensing platform and their response to acoustic stimuli is recorded.

8. THE AMYGDALA AND APOE4

The differential effects of apoE on measures of anxiety were associated with neuropathological alterations in the central nucleus of the amygdala, which plays an important role in the regulation of anxiety. Compared to wild-type mice, $Apoe^{-/-}$ and apoE4, but not apoE3, mice had lower levels of microtubule-associated protein (MAP) 2-positive neuronal dendrites ($P < .05$). These changes were age-dependent. Three-month-old wild-type and $Apoe^{-/-}$ mice had similar levels of MAP 2-positive neuronal dendrites. Interestingly, in nondemented human subjects [30] and in AD subjects [31], amygdala atrophy increased with increasing $\varepsilon 4$ allele dose. However, other studies did not find effects of the $\varepsilon 4$ allele on amygdala atrophy. This might relate to differences in the mean age of the subjects in the different studies and a decrease in the effect of $\varepsilon 4$ with advanced age.

9. DIFFERENTIAL EFFECTS OF APOE ISOFORMS ON MEASURES OF ANXIETY IN PRAD PATIENTS

Consistent with the mouse studies, apoE also has isoform-dependent effects on measures of anxiety in probable AD (PRAD) patients [25]. Diagnosis of probable AD was made in each case according to NINDS-ADRDA criteria [32]. Cornell depression scale and neuropsychiatric inventory (NPI) were recorded for all subjects (mean age ± SEM; all subjects: 73 ± 1 years; $\varepsilon 3/\varepsilon 3$: 75 ± 3 years; $\varepsilon 3/\varepsilon 4$: 73 ± 2 years; $\varepsilon 4/\varepsilon 4$: 71 ± 2 years). Subjects were nonsmokers in good general health and free of past or present major psychiatric or neurological disorders (other than AD). Male $\varepsilon 4/\varepsilon 4$ subjects had higher anxiety scores than gender-matched $\varepsilon 3/\varepsilon 3$ subjects ($P < .05$). In males, but not in females, subjects with $\varepsilon 4/\varepsilon 4$ had also higher anxiety scores than those with $\varepsilon 3/\varepsilon 4$, suggesting that apoE3 can antagonize the effects of apoE4 on measures of anxiety in males but not in females. The anxiety scores did not correlate with the mini-mental state exam (MMSE) scores. Compared to $\varepsilon 3/\varepsilon 3$ male subjects, sleep disturbances were lower in $\varepsilon 4/\varepsilon 4$ ($P < .01$) and $\varepsilon 3/\varepsilon 4$ ($P < .05$) male subjects. Thus, sleep disturbances did not correlate or contribute to anxiety scores. ApoE did not have isoform-dependent effects on apathy or depression scores.

10. CONCLUSIONS

ApoE isoforms have differential effects on measures of anxiety in $Apoe^{-/-}$ mice expressing human apoE3 or apoE4 at similar levels and in PRAD subjects. The $\varepsilon 4$ allele is also associated with depression in some [33–35] but not other [36–40] studies. As noncognitive behavioral changes are the major cause of institutionalization of AD patients and a major concern for their caregivers, more research aiming at increasing our understanding of mechanisms underlying these behavioral changes is needed to advance treatment strategies to reduce these changes.

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Review Article

Anxiety from a Phylogenetic Perspective: Is there a Qualitative Difference between Human and Animal Anxiety?

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A phylogenetic approach to anxiety is proposed. The different facets of human anxiety and their presence at different levels of the phylum are examined. All organisms, including unicellular such as protozoan, can display a specific reaction to danger. The mechanisms enabling the appraisal of harmful stimuli are fully present in insects. In higher invertebrates, fear is associated with a specific physiological response. In mammals, anxiety is accompanied by specific cognitive responses. The expression of emotions diversifies in higher vertebrates, only primates displaying facial expressions. Finally, autonoetic consciousness, a feature essential for human anxiety, appears only in great apes. This evolutive feature parallels the progress in the complexity of the logistic systems supporting it (e.g., the vegetative and central nervous systems). The ability to assess one's coping potential, the diversification of the anxiety responses, and autonoetic consciousness seem relevant markers in a phylogenetic perspective.

1. INTRODUCTION

In human, anxiety is present in most psychopathological conditions [1]. The regulation and alleviation of anxiety is a key factor in the promotion of human well-being. However, anxiety is often experienced as an automatic and uncontrollable response with deep roots in our phylogenetic past. On the other hand, psychological processes like rumination that are central to human anxiety, imply high-order cognitive capacities, such as self-consciousness. It thus appears that anxiety comprises many facets, some of which having deep roots in our evolutionary history and others being properly human. From this perspective, a phylogenetic approach to anxiety might deepen our understanding of this phenomenon in human, and help to distinguish similarities and differences with alike states in animals. Further, as progress in the understanding of the neurobiological substrates of anxiety and in the discovery of new pharmacological treatments of anxiety often involves rodent models [2, 3], it is essential to be aware of the processes that are absent in the animal species used [4], in order to be aware of the limits of such models.

To achieve this goal, we used a comparative approach, which consisted in assessing in animal species the presence of the process described in psychology and thus designed for humans. Such rationale provides operational criteria for the study of emotions in animals and may be a heuristic framework for interspecies comparison, which may be used also for emotions other than fear and anxiety. This approach is necessarily theoretical and requires to review many findings obtained in animals research, trying to analyze data obtained within other frames. To this aim, the present paper first describes the different elements constituting human anxiety and examines their presence along the phylum. Then, it reviews the different neurological and physiological systems of the organism supporting the anxiety responses along the phylum. Finally, the different conjunctions in a given species of the elements constituting anxiety will be examined.

2. THE DEFINITION OF ANXIETY AND RELATED CONCEPTS

Fear, anxiety, and panic are three related concepts that need to be differentiated. Fear is considered by most emotion theorists as a basic emotion in humans (e.g., [5, 6]). As such, fear would develop on the basis of an innate emotional program that coordinates the different facets of the organism response (e.g., expressive, physiological, or behavioural

responses) when confronted with an identified threat. Some theorists have proposed that basic emotions are rather short-lived, which distinguishes them from mood [5]. In this perspective, panic has been conceived as a paroxystic fear, that is, a full-blown fear expressed and experienced at the maximum of its possible intensity [1].

In human psychology, anxiety is often thought of as a secondary emotion, this is, as an emotion in response to a primary emotional reaction [1, 7]. Anxiety would be the fearful reaction to another emotion, be it, for instance, fear or anger. For example, in panic disorder, anxiety is conceived as the fear of the panic (fear) response. In anxiety, the stressor is not always clearly identified, in contrast to what happens in fear. Such definition implies that anxiety requires more cognitive capacities than fear. Anxiety necessitates the capability to hold a representation of an emotional state and to react to it. This representation might be rudimentary, for instance, the reactivation of the emotional somatic state (e.g. the concept of somatic marker [8]), but it constitutes a necessary condition to anxiety. This implies that anxiety should appear in higher species when compared to fear. This definition parallels the conceptual construct that has been proposed by Robert and Caroline Blanchard in animal research. Indeed, these authors hold that the key factor distinguishing fear from anxiety is the immediacy (or certainty) versus the potentiality (uncertainty) of the threat and they define anxiety as an anticipatory fear [9].

Fear and anxiety are complex phenomena that articulate different components. For example, when confronted with a danger, a subject may display a specific response that includes a behavioural component (e.g., flight), a physiological one (e.g., increase in heart rate), and an expressive one (specific vocalization or facial expression).

As an emotion, anxiety supposedly orients the organism toward a specific type of interaction with its environment [10] and thus mobilizes the entirety of the organism resources. In this perspective, anxiety comprises several elements that constitute an emotion. These elements can be categorized as, on the one hand, the different facets of the emotional response, and on the other hand, the different logistic systems of the organism that provide the biological and neuronal supports to allow for these responses. In the next sections, we will present these different elements for the human species, and assess their presence across the phylum. By taking this perspective, we, by no means, imply that humans should be considered as the most accomplished species that would subsume all the evolutionary gain of other species that would be located lower in the phylum. Rather, our perspective is a pragmatic one, taking as standard the species that we know best; both from direct experience, and from accumulated scientific work on emotion. We however hypothesize that, as suggested by several emotion theorists, there might be a trend to a complexity gain when going from species situated at a low level in the phylum (protozoan or some invertebrates) to species situated at a higher phylogenetic level. This paper may thus provide a heuristic approach, indicating which aspects of the emotion phenomenon are the most relevant in a phylogenetic perspective.

3. THE FACETS OF ANXIETY AS AN EMOTIONAL RESPONSE

3.1. Action tendencies

In this section, we will present the different facets that constitute an emotion, focussing on fear, in the perspective that anxiety is the fear of an emotional state.

Emotions have been conceived as action tendencies [10] resulting from a specific appraisal of the situation. Appraisal is the process by which an emotional meaning is attributed to a situation. Appraisal does not necessarily imply complex cognitive processes; it may consist in a very rudimentary innate detection of an unconditioned stimulus. In this perspective, individuals would constantly appraise external and internal stimuli in terms of their relevance for the organism and in terms of the behavioral reactions that may be required as a response to those stimuli [11].

When a relevant stimulus is identified, physiological, motor, and expressive response systems are activated, which constitutes the action tendency. This concept refers to the inner dispositions (or their absence) of performing certain actions or achieving certain relational changes with the environment. In other words, an action tendency is the activation of a behavioural plan aiming at changing the individual-environment relation. Impulses of "moving towards," "moving away," and "moving against" are examples of action tendencies [12]. The various types of action tendencies depend upon the biological constitution of the organism. Hence the phylogeny would bring along a number of such action tendencies, organizing, for instance, defence and attack, protection, attention orientation, or inhibition. According to Frijda [10, page 409], the basic emotions in human, such as those proposed by Darwin [13], Tomkins [14], or Izard [6], are the reflection of these action tendencies inherited from the phylogeny. Of course, as it is the case for facial expression, these innate programs could be modulated and accommodated through learning.

Such actions tendencies can be found in a ubiquitous manner across the phylum. For example, avoidance of danger and flight has been observed in protozoan such as paramecia [15, 16], which suggests that a central nervous system is not necessary as to the expression of such behaviours. In almost all invertebrates such as molluscs or arthropods (insects or crustaceans), specific behavioural responses can be observed when a subject is faced by threat, including withdrawal from the danger, absence of movement, and reduction of nondefensive behaviours. For example, *Aplysia californica*, a gastropod mollusc, is able to react to a threatening stimulus by escaping locomotion [17]. Further, lack of movement can be observed in several insect species when faced by danger [18]. Finally, when confronted with threat, *Aplysia* displays a reduction of nondefensive behaviour such as feeding [17]. All these behaviours are remarkably conserved through the phylum and they are also observed in vertebrates including reptiles, fish, birds, and mammals.

It is to be noted that, in humans, action tendencies are not necessarily immediately enacted [10]. They would constitute a *preparation* of the organism to react in a certain way,

but the actual reaction would depend upon a sufficient activation of the action tendency. Thus, some species would benefit from a buffer between the activation of a response mode and its actual enactment. This is found in many species, including invertebrates. For example, it has been shown that environmental disturbances such as light, a drop of water, or a pebble dropped in the aquarium induce a modification of the ventilatory rate and the heart rate in crustaceans such as crayfish. These modifications occur before the animal would undergo behavioural activity. Further, in case the intensity of the fearful stimulus is low, the animal will not display any behavioural modification. These physiological modifications have been interpreted as indicative of an animal's intention for body movement before physical movement occurs [19].

3.2. The appraisal component

Regarding the appraisal or evaluation component, Scherer [20] proposes a specific hierarchy of mechanisms for the ongoing appraisal of the environment and he presents specific hypotheses regarding the pattern of evaluative meaning that should precede particular emotional states. His theory is particularly interesting in the present context as specific predictions are made regarding the phylogenetic trend.

In human, specific emotions would be brought into play by the operations of a series of five stimulus evaluation checks (SECs). These checks are performed rapidly by mechanisms that continually scan the objects in the perceptual field, with different patterns or outcomes of the check process seen as giving rise to different emotions. Based on logical, phylogenic, and ontological arguments, Scherer [11] postulates that the SEC sequence order is fixed, with the more fundamental SECs in terms of adaptation coming first. The first SECs could be found in very simple organisms without neocortical processing capacities [11]. Thus, Scherer [11, page 41] postulates that "rudimentary forms of the novelty, intrinsic pleasantness, and even the need/goal significance checks are 'hard-wired'," suggesting that they can be genetically transmitted, and thus conserved by evolution.

The first SEC, "novelty check," looks for potential changes in the pattern of the situation. The orientation reflex is one of its consequences. Scherer [11, page 306] states that, in human, the novelty SEC is at least partly independent of higher cortical functions and may result from preprocessing in the brain stem or limbic structures. In other species, the novelty check might be totally genetically determined and independent of any neural system. This ability exists in an ubiquitous way across the phylum, including in protozoan and invertebrates. It can for example be detected using habituation: when an animal has been exposed repeatedly to a new stimulation and has established that it is inconsequential, it is able to ignore it, a phenomenon termed as habituation. Habituation has been demonstrated in all organisms across phylogeny including single-celled protozoa [10], invertebrates such as nematode ancestral worm *Caenorhabditis elegans* (which is much studied by neurobiologists because it has a fully mapped nervous system comprising exactly 302 neurons) [22], insects such as fruit flies [23], or mollusc such as *Aplysia* [24], and vertebrates such as fish [25], rats[26], or humans [27].

The second SEC is the "intrinsic pleasantness check." On the basis of innate feature detectors or of learned associations, this second SEC evaluates the pleasantness of the stimulus or situation, hence determining approach or avoidance [11]. Scherer [11] stresses that this check has to do with the inherent pleasantness of a stimulus, and that it is not dependent on stimulus relevance to the goals of the organism. Again, in human, this SEC would be partly independent of cortical structures and some of its processes might take place in the amygdala. In other species, this check might be totally determined by automatic processes. If an animal is able to display either approach or avoidance of a stimulus present in its surrounding, or to undergo appetitive or aversive learning, one may conclude that it possesses the ability to do this check. According to some authors, the approach-avoidance distinction is also applicable to organisms as simple as the protozoa amoeba. In this case, approach and avoidance behaviours are extremely basic [28, page 2]. For example, in amoeba, a weak light will stimulate a movement in that direction, whereas an intense light will elicit a withdrawal from the light source. Approach and avoidance can also be observed in more sophisticated invertebrates including ancestral worms and insects. For example, the nematode *Caenorhabditis elegans* is able to display preferences for some stimuli over others [29], to avoid noxious chemicals, high osmolarities, acidic pH, and noxious mechanical stimuli [30], and to display aversive learning [31]. Insects such as drosophila display appetitive as well as aversive conditioning [32]. In fact, Schneirla [28] argued that organisms at all levels of complexity, ranging from protozoan to higher vertebrates, possess what he termed A-type (approach-type) mechanisms, facilitating food-getting, shelter-getting, and mating, and W-type (withdrawal-type) mechanisms, enabling defence, huddling, flight, and protection in general. He proposed that the sophistication of these mechanisms varies considerably across the phylum, those of protozoa and invertebrates being rudimentary and rigid, and those of higher organisms being more complex and flexible (see also [33, 34]). These two reactions have survival value, as they move the organism toward beneficial stimuli and away from harmful stimuli [35, page 7] and are therefore conserved from protozoan to higher vertebrates.

Goals and needs of the organism come into play in the third SEC, the "goal/need conductiveness check." It examines the extent to which the introduction of the detected stimulus or event will advance or hinder the attainment of a specific goal or the satisfaction of a need. The goal/need conductiveness check is divided into three subchecks: the relevance subcheck that examines the relevance of the stimulus or event for important goals/needs of the organism, the expectation subcheck that determines the stimulus consistency with the state expected at this point in the goals/needs sequence, and the conductiveness subcheck that determines if the stimulus is conducive or obstructive to the respective goals or needs. This check can also be entirely genetically determined.

If a given animal is able to display specific behaviour to escape stimuli that are incompatible with its survival such

as predators or high temperatures, one can consider that it has this capacity. This can be seen in almost all invertebrate species. For example, nonsegmented worms such as nematodes escape when exposed to temperature above 33°C (for a review on nematodes see [36]). Other invertebrate have specific behaviours to escape predators: cuttlefish can bury into the sand to hide themselves from predators [37], grasshopper may display immobility when confronted with a frog [38] as well as beetles when attacked by spiders [39]. This kind of behaviour is also observed in protozoan. For example, ciliated protozoans such as *Euplotes* are able to change their morphology [40] and behaviour [41] in response to predators [42]. Of course, these data do not enable to distinguish the capabilities of these species regarding the different subchecks of this appraisal component; such a detailed analysis being beyond the scope of this review.

So, this third SEC has not been altered significantly through evolution, as it is described in invertebrates, and even protozoan such as ciliates as well as higher vertebrates. This is probably related to the fact that it is essential to the survival of the different species. One should note that, at the methodological level, the distinction between the second (valence) and third (goal conductiveness) SEC might not be possible to operate in lower-order species. Beyond this methodological limitation, an alternative hypothesis should be considered: this distinction might not be relevant. In species low on the phylogenetic scale, these two SECs might not be differentiated. Their distinction would only appear in higher-order species.

These three first checks have also been studied in an extensive and systematic way in some mammals, such as for example lambs [43, 44]. These species display specific behavioural and physiological pattern of response when subjected to environmental challenges characterized either by novelty, by intrinsic pleasantness, or by having need/goal significance.

The fourth SEC, the "coping potential check," determines the cause of the event, and the capacity of the organism to control it or to confront it, or to adjust to the final outcome. If a species is able to react in a different way in function of the predictability/controllability of a signal, one may claim that it has this ability. To our knowledge, no study has been published addressing the presence of such processes in ancestral worms or protozoan. Ancestral worms such as nematodes possess the ability to assess the rhythmicity of some events; this is necessary but probably not sufficient to possess the ability to react in function of the uncontrollability of an event. Such changes of behaviour in function of the controllability of a stimulus have been described in mammals such as dogs by Overmier and Seligman [45]. Indeed, in dogs, prior inescapable electric foot shock interferes with later escape/avoidance learning in which shock is the negative reinforcer, a process termed as learned helplessness. One may claim that if a species displays learned helplessness, it might react in a different way depending upon the predictability/controllability of the situation. Learned helplessness has been described in various mammals including dogs, rats, mice, cats, and sheep [45–53] but also in lower vertebrates such as fish [54–56]. Further, insects such as cockroach also exhibit a failure to escape shock when possible to do so following nonescapable/uncontrollable shocks [57–59] in a similar way as vertebrates displaying learned helplessness. Therefore, one may claim that the "coping potential check" may be present in several species across the animal kingdom, including all vertebrates and some invertebrates such as insects. However, no evidence exists in more rudimentary invertebrates such as worms.

Finally, the last SEC, the "norm/self compatibility check," evaluates the congruence of the event with the social and individual norms and standards such as mental prescriptions, self-concept, and self-ideal. This check needs the presence of cultural transmission. The presence of culture in animals such as nonhuman primates is still debated. Some authors claim that "proto-cultures" or "traditions" (defined as "long-lasting behavioral practices shared among members of a group partly via social learning," see [60]) can be observed in animals. This for example has been first described in the early fifties [61] in a group of Japanese macaques (*Macaca fuscata),* a species displaying acquisition of innovative behaviours, such as potato and wheat-washing, first displayed by a young female and then transmitted to social partners as well as to successive generations [62]. In chimpanzees (*Pan troglodytes),* behavioural variants (traditions) have been described in different communities, such as differences in tool usage, grooming and courtship behaviours [63]. However, all authors would not agree that these traditions can correspond to the cultural transmission seen in humans. According to Donald [64], humans have three cognitive processes (mimetic skill, language, and external symbols) not available to other primates and enabling such a transmission. Others propose that sophisticated forms of imitation that are only described in humans are necessary for cultural transmission [65]. Similarly, some argue that culture is a uniquely human form of social learning, requiring imitative learning, instructed learning (teaching), and collaborative learning, three social-cognitive processes emerging in human ontogeny [66].

The pattern of the outcome of the different SECs determines a particular emotional meaning and directly activates the corresponding action tendency. In human anxiety, the central features are that aspects of the situation are evaluated as intrinsically negative (intrinsic pleasantness check), as threatening important goals of the organism (e.g., survival, or social acceptation in a gregarious species) (goal/need conductiveness check), and as unpredictable or uncontrollable (coping potential check). Thus, to experience full-blown anxiety, a species would need to have the capacity for the first four SECs defined by Scherer's theory. As previously shown, all these four checks seem to be present in an ubiquitous manner in the different phyla, from invertebrates such as insects to lower vertebrates (fish) and mammals and even, for some of them, in unicellular organisms such as protozoan. Therefore, according to this theoretical frame, some rudimentary form of anxiety may be present from invertebrates to humans. However, as we will see, the level of sophistication, as well as of awareness of these evaluations and

of the resulting experience vary tremendously from species to species, according to their cognitive capabilities.

3.3. The physiological component

As action tendencies, emotion and anxiety recruit all the logistic capacities of the organism. The physiological systems are activated in order to support the actions and transactions with the environment called for by the emotional situation. In humans, many physiological and endocrine responses have been observed in emotion and in anxiety in particular. There is still a debate regarding whether specific emotions (and anxiety can be considered as such) have unique physiological characteristics. Despite a century long tradition of physiological research in human emotion, no definite conclusion has been reached yet [67]. Physiological responses in human emotions seem to result from a complex interaction between the demand of the situation, personality characteristics, and the type of regulation strategies used in that situation [68].

Regarding fear and anxiety, meta-analyses of the literature have documented marked changes in most peripheral responses: cardiovascular changes, respiratory changes, muscles tonicity changes, or skin temperature changes when compared to neutral states [67]. These changes are driven by the autonomic nervous system. These changes, however, are not that different from other intense emotions such as anger, with the exception that anger produces more elevated diastolic blood pressure.

Most of these reactions are present in rodents such as rats, and they can vary as a function of the behavioural response that the subject may display. For example, a flight response can occur in response to threat that is associated with increased blood pressure and tachycardia, enhanced cardiac output and respiration, increased cerebral perfusion and redistribution of blood flow to increase limb circulation [69–72]. Some aspects of these responses are also observed in lower vertebrates such as fishes. Indeed, salmons show flight associated with increased heart rate when confronted with a simulated predator attack [73]. Other components, such as variations in skin temperature or skin conductance are difficult to measure without stressing the animals, so that the few empirical studies that assessed these modifications were only done in mammals using radiotelemetry. For example, a decrease in skin temperature following alerting stimuli has been shown in monkeys in different parts of the body including the nose, nasal mucosa, ears, hands, feet, and tail [74]. Such temperature variations according to fear or anxiety are logically absent in lower vertebrates, which are poikilothermic. Other aspects of the human physiological response to threat are not present in lower vertebrates. For example, fishes, amphibians, and reptiles do not have dilatator musculature innervating the iris so that they may not exhibit mydriasis.

Even if not possessing an autonomic nervous system similar to the one enabling the physiological response to danger seen in vertebrates, invertebrates need the same rapid cardiovascular and respiratory regulation to be primed for the defensive behaviours they exhibit toward threatening stimuli. Indeed, such modifications provide the organism with the metabolic/energetic resources that will be necessary to deal with environmental challenges. Are such physiological responses observed in invertebrates when confronted with danger? Are they associated with the behavioural response? In crustaceans, perception of changes in the surroundings of the animal can induce modifications of some physiological variables such as heart rate and ventilatory rate [19]. This is also seen in molluscs such as cephalopods. For example, octopus displays cardiac arrests when exposed to a stressful situation [75]. Thus, the physiological responses observed in some invertebrates such as crustaceans or molluscs faced by threatening stimuli are very close to the responses of vertebrates mediated by the autonomic nervous system [76]. In other invertebrates such as insects, the energy necessary to cope with threat is provided to the organism by other means. For example, in insects, the blood flow to the different tissues is not regulated by an increase of the heart rate. Indeed, insects have an open circulatory system that differs from the closed circulatory system (in which blood is always contained within vessels) found in vertebrates. In an open system, blood (termed as hemolymph) flows freely within the body and establishes direct contact with all internal tissues. In case of danger, hemolymph delivery to the tissue is directly increased, without a modification of heart rate. However, even if modifications in heart rate have not been documented in fear-challenging situations, behavioural activity induces modification in heart rate (C. Lazzari, personal communication). As fear is associated with modification of activity, it can thus be that it is related to heart rate modifications.

Thus, it is possible that the representation of the body changes occurring during danger may be very different depending on the species: mammals may perceive environmental-induced changes driven by the autonomic nervous system in their body and including modifications in heart and ventilatory rate, in skin temperature, and mydriasis, lower vertebrates (amphibians, reptiles, fish) and some invertebrates (crustaceans, molluscs) may exhibit modified heart and ventilatory rate without changes in temperature or mydriasis.

3.4. The expressive component

Emotions are not only inner states. They are also communicated to the environment, as they convey the behavioural intend of the individual. In human, the expressive component has certainly been the most studied, at least for facial expression. A series of studies has demonstrated innate and cross-cultural aspects of emotional facial expressions in humans. However, these innate facial displays are modulated by a set of cultural and display rules [77, 78]. The gist of this literature is that the nonverbal communication of emotion serves very important functions of regulation, both within the species and cross-species. It is conceived of, primarily, as a social process.

While much work has been devoted to the facial display of fear, the literature in human is almost silent regarding

a facial expression that would be specific to anxiety. Most scholars do not distinguish facial expression between these two states [6, 79]. Similarly, the studies that have investigated modulations of prosody during emotional states did not distinguish fear from anxiety [80, 81]. Yet, emotional prosody in humans has clear phylogenetic roots that have been traced back to primates [82]. This point will be developed in the following paragraphs.

An interesting phenomenon for emotion regulation, known as facial feedback, has been documented in humans [78, 79]. A wealth of research has established that holding a certain nonverbal expression was generating or reinforcing the corresponding affect. Thus holding a nonverbal expression of anxiety generates and intensifies this emotion. Phenomenon of contagion via mimicry has also been documented [83–85].

In humans, some studies have documented that different emotions were expressed by different postures (e.g., [86]). Further, Stepper and Strack [87] have documented that manipulating posture has an impact on the emotional subjective feeling state and affects later judgment of valenced material. Further, there is some evidence that body odours are modulated by emotion, including fear and anxiety. For instance, Chen and Haviland-Jones [88] have collected underarm odours on gaze pads in human subjects exposed to a joyful or a frightening movie. The authors have observed that, on the only basis of the collected odours, human participants could detect above chance level the emotion induced.

In animals too, emotional state can be communicated to the environment by specific signals, including facial, postural, vocal, or chemical ones. Further, other kind of expressive components are also documented, including more specific ones such as camouflaging.

Modification of facial expression in relationship to emotions can be seen only in species having a well-developed facial musculature. Facial musculature is highly conserved across primates [89], the one of chimpanzee being almost identical to that of humans [90]. Indeed, in this species, specific facial expressions have been described in response to danger such as fear grin. However, even if some spare evidence indicates that some mammals such as rats are able to display some specific facial expression to the affective aspects of taste [91], the facial musculature of nonprimate mammals is undeveloped or nonexistent [89, 92, 93] and may not allow more specific facial expressions.

Postural changes have been extensively described in higher vertebrates confronted with danger. For example, rodent may display a posture characterized by immobility, flattening of the ears, piloerection, and marked mydriasis. Indeed, specific postures have been repeatedly seen in vertebrates in emotional situations: they have been nicely illustrated by Darwin [13].

Specific vocalizations to threat have also been documented across the phylum. For example, vervet monkeys emit specific alarm calls to different predators such as leopards, eagles, or pythons [94]. Variation in alarm calls with the type of predator has also been described in rodents such as gerbils [95]. In other species, these calls are less sophisticated as they may indicate the presence of a danger to congeners, without giving more information on the precise nature of the threat. Specific vocalizations to danger have been described in birds [96], but also in amphibians (e.g., crocodiles [97]) and fish [98]; they are thus present across the vertebrate phylum. Further, such calls have also been described in invertebrates such as insects. For example, Wyttenbach et al. [99] showed that field crickets emit ultrasonic signals in the 25–80 kHz range when confronted with predators, inducing escape behaviour in other crickets. However, all signals emitted by these crickets do not elicit the same response: when they produce signals in the 4–5 kHz, conspecifics approach, indicating the specificity of these alarm calls. So, vocal expressions related to danger can be seen in vertebrates as well as in invertebrates.

The use of pheromones to alert conspecifics of the presence of a danger is common in many animal species. For example, in the presence of an intruder, several species of social hymenoptera secrete pheromones that cause defensive behaviour among conspecifics [100]. Such reactions can be found in vertebrates as well. For example, carnivorous mammals of the Mustelidae family use anal scent glands to produce olfactory warning, often repellents signals [101]. Fear may be communicated by odours in mice and rats as well [102]. Such reaction can also be documented in nonhuman primates. Indeed, it has been shown that the genital scent glands of two prosimian primates are involved in producing a fear scent [103].

Camouflaging can be considered as a form of behaviour intermediate between emotional expression and coping with the situation. Indeed, it often appears when a species is confronted with a danger such as a predator. The most common form of it involves the modification of the visual appearance, but calls, songs, and scents can also be changed. Different strategies of camouflaging have been described, such as crypsis, aposematism, Müllerian mimicry, and Batesian mimicry. Crypsis enables to minimize the signal to noise ratio, thus rendering the detection of the subject very difficult for a predator. It generally consists in matching colours and patterns between an animal and its background [104–106]. It is very common in invertebrates such as arthropods (e.g., in insects) or molluscs (e.g., in cephalopods) as well as in some vertebrates such as fishes, amphibians, reptiles, and birds. For example, the day octopus (*Octopus cyanea*), which forage on coral reefs, produce colour patterns capable of instantaneous matching to backgrounds from sand and reef rubble, through to spiked corals and seaweeds. More rarely, this kind of defence strategy can also be seen in mammals. For example, in the rock pocket mice *Chaetodipus intermedius* and in the deer mouse *Peromyscus maniculatus,* variation in coat colour, as a function of the colour of rock substrate, has been documented. This strategy is adaptive, providing the mice cryptic protection against predators [107]. The other camouflaging strategies (aposematism, Müllerian mimicry, and Batesian mimicry) are based on a maximization of the signal to noise ratio. Aposematism consists in displaying warning signals (e.g., conspicuous coloration) informing a potential predator that the prey is toxic or unpalatable. It exists in

many invertebrates, but also in fishes, amphibians, snakes, and birds [108]. Batesian mimicry is a form of mimicry in which an innocuous unprotected species closely resembles a noxious model species. Hoverflies that resemble bees or wasps are an example. This can involve the coloration pattern as well as some aspects of the animal's posture. For example, the Indo-Malaysian octopus can adopt a colour and a posture mimicking a poisonous sea snake. In Mullerian mimicry, two or more equally poisonous species share an identical colour pattern, thereby reinforcing the warning each gives to predators. In some cases, dynamic camouflage can be observed: some insects imitate the movements of branches or leaves in their surrounding.

3.5. Cognitive mode

In human psychology, extensive research has documented that emotion in general, and anxiety in particular, are accompanied by specific cognitive response. Threat and anxiety have been shown to powerfully affect attention allocation. Laboratory studies have documented that threatening stimuli automatically attract attention, even during subliminal exposure (very rapid presentation that cannot be consciously perceived) (for a review, see [109]). In people suffering from chronic anxiety, this pattern would be even more pronounced and aggravated by a poor capacity to disengage attention from threat. In fact, most models of human anxiety (e.g., [110]) consider that an attentional bias toward threat is an essential component of anxiety, especially of dysfunctional anxiety.

Attention bias toward anxiogenic stimuli has rarely been examined as such in nonhuman animal species. However, different phenomena have been described in animals that can be interpreted within this frame, including fear-potentiated startle, increased cognitive performance in stressful situations, anxiety-induced increased attention toward negative stimuli and a bias for threat cues in anxious mice.

Fear-potentiated startle corresponds to an increase of the amplitude of the acoustic startle response in the presence of a cue previously paired with a shock. It has been described in rhesus monkeys [111] but also in rodents such as rats [112] or mice [113]. To our knowledge, fear-potentiated startle has not been examined in nonmammalian vertebrates such as birds or fishes.

Another phenomenon that has been widely documented is the increased mnesic performance observed in anxiogenic situations: this is generally attributed to the fact that anxiogenic situations increase attention, thus increasing mnesic encoding. This facilitation has been repeatedly observed in rodents such as mice but also in birds. The processes used to increase anxiety include pharmacological manipulations, lesions studies, maternal separation in pups, genetic invalidation, and strain variations. For example, a principal component analysis showed that, in mice, higher emotional memory performance is related to heightened state anxiety [114]. Further, Venault et al. [115] showed that, in rodents but also in chickens, anxiogenic compounds increased memory in three different tasks, while anxiolytic drugs elicited opposite effects. However, this association is probably not causal, as β-CCT, a selective benzodiazepine receptor antagonist, blocks the antianxiety but not the amnesic action of benzodiazepines in mice [116], suggesting that the anxiolytic and the amnesic effects of these compounds are independent. In mice, a multiple regression analyses also revealed a relationship between attention toward salient stressful stimuli in a conditioned task and sensitivity to stress [117], suggesting that attention toward negative events may contribute to the response in stressful situations. Finally, when mice characterized by heightened anxiety-like behaviour are subjected to a fear conditioning protocol including a fully conditioned stimulus (a tone always followed by a shock) and a partial conditioned stimulus (a light, only partially related to the shock), normal mice discriminate between the partial and the full conditioned stimulus, while the anxious mice show the same response to the two stimuli [118]. This phenomenon has been interpreted as a bias for threat cues.

Most of these studies suggesting an attentional bias toward threat in anxious animals have been conducted in mammals, specially rodents, the sole exception being the pharmacological studies that were also conducted in birds. Even if the absence of such studies does by no ways mean that such processes do not exist in lower vertebrates, it suggests that it is at least difficult to assess in fish, amphibians, or reptiles. A reason for that could be that this facilitation does not occur in that species, but this remains to be confirmed by experimental studies.

3.6. The subjective feeling component

In the human literature, an important component of emotion is of phenomenological nature: the subjective feeling state. It reflects the notion that, when emotional, the individuals feel in a different state that colours their perception of the world and of themselves. Most authors agree that the subjective feeling component results from the global perception by the individual of the changes operating in the different emotion facets [119]. There is also a consensus on the fact that the subjective feeling state can vary in terms of awareness. For instance, Lane [120] has identified several levels of awareness of emotion, from a diffuse sense of bodily changes, to the reflexive awareness of observing oneself in an emotional state. These different levels of awareness are supported by different brain structures. They supposedly progressively appear during the ontogenesis, with the highest level of awareness fully mastered only at adolescence.

Reflexive emotional awareness is particularly relevant for emotion regulation in general and anxiety in particular. This capacity enables humans, not only to be reflexively aware of their on-going experiences, but also to reactivate past experiences, or to imagine future ones [121]. The capacity for self-consciousness, labelled autonoetic consciousness by Tulving [122], is the central element that allows remembering specific past experiences (i.e., episodic memory) as well as for imagining what future experience would feel like. As a form of anxiety consists in an apprehension for a future emotion (e.g., fear or anger), it implies the capacity to envision what

a future experience would feel like. Hence, possessing autonoetic awareness capacities opens many avenues for anxiety to develop. For instance, for a student, the capacity to imagine a future examination creates a source of anxiety. On the contrary, it has been observed that people who, because of cerebral damage in the frontal and prefrontal regions, lack any autonoetic capacities (for a review, see [121]) are unable to experience any anxiety.

The capacity for autonoetic consciousness is one of the last cognitive features to develop in the human ontogeny. Its first manifestation in terms of reflexive capacities to one's own experience appears around 4 years of age and it is believed to be only fully developed around 14 years of age [121]. To date, the evidence for autonoetic consciousness in nonhuman primates is still the object of a debate [123]. This debate is further fuelled by the fact that the exact cognitive processes leading to autonoetic awareness are still to be identified. However, the brain regions involved, as well as the important cognitive resources required, strongly suggest an important involvement of executive processes.

As autonoetic consciousness is a key feature of episodic memory [122], the development of episodic memory across species might shed some light on the birth of autonoetic consciousness along the phylum. Several reviews of this question have been proposed (e.g., [123, 124]). However, it should be stressed that autonoetic consciousness does not only imply the capacities to remember "what, when, and where" a specific event occurred. This latter capacity seems to be acquired early in the phylum, as it is already mastered by birds [123]. Rather, autonoetic consciousness also implies the capacity of representing oneself as the subject of the experience remembered. This latter facet implies self-awareness. This capacity seems to appear very late in the phylum. According to Gallup et al. [125], self-awareness can be reflected by self-recognition and by the ability to infer mental states in others. Indeed, according to these authors, if a subject is able to have a representation of itself, it may possess the ability to identify itself (self-recognition) and to use its own experience to infer comparable experience in others (a process termed as mental state attribution or theory of mind). Therefore, self-recognition and mental state attribution could be heuristic indicators of self-awareness. Gallup [126] developed a paradigm enabling to test self-recognition in great apes: the capacity to interpret one's own reflection in a mirror. It has been shown that mirror self-recognition exists in chimpanzees [126, 127], but also in other great apes including orangutans and bonobos [128, 129]. Interestingly, this capacity has not been seen in some great apes such as gorillas [128, 130] or in monkeys such as macaques [126]. Further, self-recognition has also been shown in great apes using other paradigms [131]; however, it was never observed in other nonhuman primates, suggesting a phylogenetic gap for this process between great apes and other nonhuman primates such as macaques.

It should however be noticed here that the assumption that great apes are able of self-recognition of their image in a mirror has been questioned by some authors, and is still matter of controversy. Indeed, according to some authors (see, e.g., [132]), the behaviour of these primates when faced with a mirror could instead have occurred by chance or result from experimental artefacts. On the other hand, evidence of mental state attribution in animals is still matter of controversy. It seems that this process appears very late in the phylum. Scarce evidence indicates that chimpanzee may be able to take into account what other chimpanzee can or cannot see [133]; however, this question remains a contentious issue [132]. So, some controversial evidence indicates that great apes such as chimpanzees, bonobos, and orangutans may possess some abilities such as self-recognition, that reflect self-awareness, a process necessary for autonoetic consciousness. However, at this point, prudence is necessary because this by no means indicates that they possess autonoetic consciousness. This just means that they have some abilities enabling this kind of consciousness.

4. THE LOGISTIC SYSTEMS OF THE ORGANISM SUPPORTING THE ANXIETY RESPONSE

In humans, the anxiety response is supported by several biological systems, including neurotransmitters such as biogenic amines, stress hormones, activity driven by the autonomic nervous system, and changes within specific brain areas. Are these different features present at all levels of the phylum?

Fear triggers the release of various biogenic amines, including the catecholamines adrenaline, noradrenalin, octopamine, and dopamine and the indolamine serotonin. Adrenaline, noradrenalin, and dopamine have been described in all vertebrates, with some variations that have been suggested to be related to an evolutive trend [134]. Indeed, high noradrenalin/adrenaline ratio appears to be characteristic of more primitive vertebrates while a lower ratio occurs in tetrapods and mammalian adults. In invertebrates, all catecholamines have been detected in several insects, but also in scorpions as well as in gastropods and cephalopods [135]. Serotonin has also been detected in several invertebrates including arthropods such as scorpions, insects, or crustaceans, or molluscs such as cephalopods [136–140]. Are these biogenic amines released under stressful situation similar to the ones triggering fear and/or anxiety? This seems to be the case. For example, stress elicits an increase in noradrenalin and dopamine in oysters: this response occurs rapidly and its intensity is correlated with the intensity of the stress [141]. Consequently, one may claim that there are only small variations across the phylum as to the biogenic amines.

Fear and anxiety also produce some specific hormonal release, related to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, including a release of several stress hormones such as corticotropic-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and glucocorticoids. Stress hormones seem also highly conserved across the animal kingdom. Indeed, CRH has been described in various mammals but also in birds such as pigeons and quails, frogs, and several fish species (elasmobranch fish, teleosts, goldfish, salmons, eel). Such molecules are not only found in vertebrates. Indeed, CRH-like molecules have been reported in some invertebrates including in the nervous system

of the annelid *Dendrobaena subrubicunda,* the insect *Periplaneta americana*, and the mollusc *Planorbarius corneus* (for a review, see [142]). ACTH release from hypothalamic centres has been observed in birds, amphibians, and teleost fish. With regard to invertebrates, ACTH-like compounds are found in the nervous system of various molluscs and insects, but also in the protozoan *Tetrahymena pyriformis* (for a review, see [142]). Therefore, this compound or its functional equivalent is present at quasi all levels of the phyla. In mammals, glucocorticoids such as corticosterone or cortisol are released by the adrenals, a gland consisting of an outer part (the adrenal cortex) and an inner part (the adrenal medulla). Nonmammalian vertebrates lack the typical anatomical adrenal gland of mammals, but they are equipped with cells resembling mammalian cells of the adrenal cortex. Corticosterone has been detected in some birds such as chickens or ducks, reptilians, amphibians, and fish but also in some invertebrates, particularly insects (for a review, see [142]). So, again, there are very few variations in stress hormones across the phylum.

The phylogeny of the autonomic nervous system has been extensively studied by Nilsson [143, 144]. It appears that this system is more or less the same in all vertebrate species, with the exception of the lower fishes (cyclostomes) that do not have the double cardiac innervation (noradrenergic and cholinergic) that all the other vertebrate species have (from higher fishes to mammals). Invertebrates do not have autonomic nervous system as vertebrates; however, past work undertaken by comparative neuroanatomists such as Zavarzin [145] drew similarities between the sympathetic nervous system of vertebrates and the unpaired nerves of insects.

Another important system supporting the human anxiety response is the facial musculature, enabling the facial expression of emotions. Such musculature is not present in invertebrates having an external skeleton, such as insects or bivalves. In nonmammalian vertebrates, this musculature is very rudimentary, enabling only opening and closing of the apertures such as mouth, eyes, and nostrils [146, 147]. Greater mobility of the lips can be seen in mammals, probably because this may facilitate suckling [148]. In primates, facial musculature gains in complexity as specific muscles appear that enable emotional facial expression (e.g., zygomaticus major, zygomaticus minor, levator labii superioris, depressor angulioris, depressor labii inferioris, and risorius) [148]. The facial musculature is innervated by neurons originating from the craniofacial motor nuclei (VII) of the brain stem. According to Sherwood et al. [146], a basic pattern of muscle representation in the craniofacial motor nuclei is strongly conserved across mammals. However, counting of the number of neurons in these areas shows that hominids (great apes and humans) have 24% more facial neurons than predicted from their medulla size, indicating a larger development of this structure in great apes and humans. Further, in old world anthropoid primates, cortical neurons originating in the motor cortex and projecting directly to cranial nerve motoneurons have been described: there is no evidence of such direct projections in other mammals [146, 147]. These projections may enhance volitional control over facial expression. So, facial musculature and the structure that control it are mostly described in higher primates such as great apes and humans.

Several functional neuroimaging studies have investigated the brain structure whose activity is modified during fearful experience. For example, activation of the amygdala has been observed during acquisition of conditioned fear [149]. This involvement of the amygdala has then been largely confirmed [150]. Further, during fear conditioning, an activation of the anterior cingulate cortex is also observed and, in case of trace fear conditioning, an additional activation of the hippocampus has been documented [151]. These authors suggest that the hippocampus may enable the storage of the spatiotemporal aspects of the fear experience, while the anterior cingulate cortex may permit to drive attentional resources toward the stimulus and to anticipate the occurrence of the fearful stimulus. Other studies focused on brain activation during anticipation of fear. They showed that during anticipation, subjects report fear experience associated with activation of the physiological variables related to fear. Further, these studies revealed that during anticipation, there was an activation of the prefrontal cortex [152] (particularly of the orbitofrontal cortex [153]), of the temporal area [153, 154], and of the insulae [153]. Finally, when subjects are requested to try to self-generate emotions by reexperiencing past events, they show a decreased activation of the hypothalamus, of the posterior cingulate cortex, and of the orbitofrontal cortex and an increased activity in secondary somatosensorial cortices, in the insulae, and in the hippocampus [155]. Interestingly, some of these modifications are observed in areas enabling the perception and the regulation of body internal states (somatosensorial areas and insulae). So, these studies show that several brain areas are engaged in humans during fear or anxiety, including subcortical ones (hypothalamus, amygdale, hippocampus) and cortical ones (prefrontal cortex, somatosensorial areas, insulae, cingulate cortex).

Is such a pattern of activation also observed in other species? How does the anatomy of these brain areas evolve across the phylum? We will answer these questions mainly focusing on vertebrates, as the nervous system is organized in a different manner in invertebrates making a comparative approach difficult.

We will first consider the phylogeny of the hypothalamus, the amygdale, and the hippocampus. The hypothalamus is a very old area and unlike most other brain structures, it has been conserved throughout phylogeny and exists in all vertebrates, including fishes. Amygdala and hippocampus have not been described as such in fishes; however, on the basis of anatomical and developmental data, it has been suggested that the fish medial and lateral regions of the telencephalic pallia might be the homologous neural structure to the mammalian amygdala and hippocampus, respectively [156–159]. Further, these areas seem to be associated with functions that are also homologous to the ones of limbic structures in higher vertebrates. Indeed, several recent studies showed that medial and lateral pallium ablation in fishes induces a deficit in fear and spatial learning, respectively [160–162].

In amphibians, similar results are obtained as the medial pallium appears to be homologous with the hippocampus of mammals [163]. Further, in these species, the basic subdivisions and connections of the amygdalar nuclei found in mammals and described [164] as structures homologous to the lateral, medial [165], and central [164] amygdala have been recently identified within the ventral part of the lateral pallium. Finally, the posterior dorsal ventricular ridge of amphibians has afferents and efferents similar to the ones of the basolateral amygdala of mammals [166]. This can also be seen in reptiles [167, 168]. In birds, the hippocampal formation is considered to be homologous to the mammalian hippocampus [169] and the posterior and medial archistriatum is considered as a homolog of the amygdala in mammals [170]. In mammals such as rodents, the amygdala as well as the hippocampus are largely equivalent to the ones of primates in their connectivity, neuroanatomy, and function. The role of hippocampus in trace and contextual fear conditioning is well established [171–174]. Further, the function of the different subdivisions of the amygdala in fear and anxiety is largely described, the lateral and central parts being involved in classical fear conditioning [175–178] and the medial nucleus being mostly related to unconditioned fear [179, 180]. So, in vertebrates, the subcortical structures implicated in fear and/or anxiety have been well conserved, the hypothalamus being present in all species, and regions homologous to the hippocampus and amygdala being present, and functionally activated during fear, in fishes. In higher vertebrates, a suborganization of these areas appears, subserving specific functions.

We now consider the phylogeny of the neocortical areas (prefrontal cortex, secondary somatosensorial areas, insulae, cingulate cortex) involved in the human anxiety. The classical view concerning the origins of the mammalian neocortex considers that it may be inexistent in nonmammalian vertebrates such as birds or reptiles. In fact, a three-layered cortex has been described in reptiles [181, 182] and some authors claim that neuronal populations homologous to the ones found in the mammalian neocortex are seen in the avian/reptilian dorsal ventricular ridge [183]. However, this view is contested. The following paragraphs discuss the presence of these areas in mammals, and mention some debates regarding their functional equivalents in birds.

In rats, the frontal cortex is subdivided into three topologically different regions: the medial prefrontal cortex (that includes the anterior cingulate), the orbital prefrontal cortex, and the agranular insular cortex [184]. Rats have also a distinct secondary somatosensory cortex. All these areas are activated by anxiogenic stimulus (see, e.g., [185]), suggesting that they are involved in fear and anxiety. However, rats may not have exactly the same neural representation of fear as primates. Indeed, recently, some features that seem to be unique in primates have also been described. For example, it has been shown that activity within the right anterior insula correlates with conscious awareness of the bodily responses occurring during emotional states (e.g., heartbeat detection) suggesting that this area may provide a substrate for subjective feeling states [186, 187]. Interestingly, this region has a specific pattern of afferents enabling this function (e.g., the thalamocortical lamina 1 pathway) that is only developed in primates [188], suggesting that awareness of visceral changes related to emotions may only exist in primates. Further, these projections are small in macaques, and their size develops mainly in great apes. In the anterior cingulate cortex, some specific neurons termed as spindle cells have been described that are present only in humans and great apes [189]; they have been suggested to be involved in emotional self-control and problem-solving capacity [190]. Further, some specific afferents of these areas such as the ancillary thalamocortical lamina 1 pathway are also specific to primates. Within the prefrontal cortex, there is also another area that is unique in great apes and humans: Brodmann's area 10. This area may be involved "in the retrieval of memories from the individual's past experience and the capacity to plan adaptive responses" [191] which may be essential to autonoetic consciousness.

5. CONCLUSIONS

Table 1 presents in a simplified way a summary of the data presented in the previous sections. A clear evolutive trend appears, as the components of the emotional processes as well as the logistical systems related to their realization gain in complexity from lower to higher levels of the phylum. Further, it can be noticed that the species located higher in the phylogenetic tree, while gaining some additive abilities (cognitive bias, autonoetic consciousness), never loose the more primitive capabilities they share with the lower invertebrates. Therefore, the human anxiety may indeed be based on aspects inherited from the evolutionary history as well as on high-order cognitive processes. Table 1 clearly shows that some very rudimentary aspects of the behavioural responses are present in unicellular organisms such as protozoan and ancestral nonsegmented worms such as nematodes (novelty, pleasantness, and goal conductiveness checks, associated with a behavioural response and with the presence of stress hormones), probably indicating the high survival potential of these aspects of emotional responses in general and of anxiety in particular. In insects, the response is enriched by an additive appraisal check (coping potential), the presence of a specific emotional expression characterized by postures, vocalisations, and pheromones, and by the release of specific monoamines in response to environmental challenges. The physiological response to danger is documented in crustaceans as well as molluscs; this enables us to distinguish the pure behavioural response from action tendencies in which a modification in the physiological indicators may appear before the behavioural response occurs. The logistic systems supporting the main facets of human anxiety appear in vertebrates (the vegetative and central nervous systems). Low-order vertebrates (fish, amphibians, and even reptiles) possess an autonomic nervous system coordinating the physiological response to stressful situations. This system is associated with the hypothalamus and brain areas that are functionally homologous to subcortical areas involved in fear in higher-order species (e.g. the amygdala and the hippocampus). In birds, specific responses related to their ability to regulate body temperature appear. In mammals, a functional

Table 1: Summary of the findings about the presence of the different emotional responses and of the different logistic systems necessary for emotions across the phylum. Grey cells indicate presence of the process or system in a given phylum. SEC is stimulus evaluation check. FE is functional equivalent.

Anxiety from a phylogenetic perspective

	Protozoan	Ancestral worms	Insects	Crustaceans	Molluscs	Fishes	Amphibians	Reptiles	Birds	Nonprimate mammals	Monkeys	Great apes[b]	Humans
Emotional process													
Appraisal													
Novelty SEC													
Pleasantness SEC													
Goal conduciveness SEC													
Coping SEC													
Cognitive bias													
Action tendencies													
Action preparedness													
Emotional expression													
Pheromones and odours													
Postures													
Facial expressions													
Vocal expressions													
Physiological responses													
Cardiovascular and respiratory responses													
Temperature changes													
Autonoetic consciousness													
Logistic systems													
Monoamines													
Stress hormones													
Facial musculature													
Vegetative nervous system													
Central nervous system													
Hypothalamus													
Hippocampus FE													
Amygdala FE													
Hippocampus													
Amygdala													
Anterior insula[a]													
Anterior cingulate cortex[a]													
Broadman 10 area													

[a] Concerns not the structure per se, but the thalamocortical lamina 1 pathway afferent of this structure.

[b] Concerns orangutans, chimpanzees, and bonobos.

SEC is stimulus evaluation check.

FE is functional equivalent

amygdala is present, with many subdivisions. Primates are characterized by their ability to display specific facial expressions in reaction to danger; they are associated with an important facial musculature. Finally, some very sophisticated facets of emotional processes such as autonoetic consciousness appear in conjunction with some specific connections of parts of the prefrontal areas necessary for the conscious perception of the visceral changes related to emotions, of emotional control, or of retrieval of memories from past experience.

At first sight, Table 1 reveals a striking phenomenon: many emotional processes related to anxiety can be executed even in the absence of the logistical structures that support them in humans. For instance, while insects already display a large range of emotional processes such as appraisal, action tendencies, and emotional expression, they are lacking many of the structures, especially in the vegetative and central nervous systems, that are governing these facets of anxiety in humans. This observation is even more pronounced in crustaceans and molluscs. This suggests that the processes and functions active in anxiety appear in lower-order species that have not developed the neural, chemical, or anatomical structures that support them in humans. In these lower species, functionally equivalent structures might organize these processes. Further, on the phylogenetic scale, the evolution would have developed *ad hoc* structures for more functional diversity and efficiency. This view is in line with a Lamarckian perspective on the phylogeny of anxiety.

Another remarkable point that can be seen in Table 1 is that insects possess the four SECs necessary to fear. Indeed, they have the ability to appraise the novelty, the pleasantness, the goal conductiveness, and the coping potential of a given

situation. Interestingly, these abilities exist independently of other features of the anxiety response, such as the physiological response to fearful situations. These processes seem independent of the presence of specific brain areas such as limbic structures that do not exist in the insect nervous system, which suggests that they may be realized via other logistical systems in these species.

Further, Table 1 also allows assessing the relationship between a given process and a given logistical structure. For example, cognitive biases are central to human models of pathological anxiety (e.g., [191]). Recent research has shown that the amygdala plays a central role in attentional biases towards threat in pathological anxiety [176]. As displayed in Table 1, it is interesting to note that empirical evidence has documented such cognitive biases only in species that have an amygdala. Hence, the present phylogenetic approach confirms that the amygdala plays a central role in cognitive biases observed in anxiety.

Different aspects of the literature reviewed above clearly suggest that anxiety as a conscious anticipation of danger only appears in great apes. This capacity, that implies autonoetic awareness, is directly related to the development of the neocortex and its connections with the limbic system and with the thalamus. This suggests that the capacity to represent oneself and one's reactions to hypothetical situations depends upon the capacity to strategically activate emotion networks or representations of emotional states. This reflexive capacity would be shared only by great apes and humans. Thus it might be that only great apes experience anxiety as humans, with its apprehension component. This does not mean that other species (e.g., other mammals such as rodents) may not have the aptitude to experiment anxiety with its anticipation dimension. However, in the case of lower mammals, this anticipation may not be conscious and may not be related to the ability to activate a representation of the situation with its possible consequences.

Finally, Table 1 also allows finding out the most relevant aspects of the anxiety response in a phylogenetic perspective. It thus seems that the coping appraisal check, the diversification of the emotional response, including the emotional expression and the physiological response, and the capacity for autonoetic awareness are the most relevant of these dimensions. Indeed, the coping potential ability enables us to separate insects from lower invertebrates, the diversification of the emotional response occurs at higher levels of the phylum (facial expressions appear in monkeys) and, finally, autonoetic consciousness appears in great apes.

To come back to our initial question, whether there is a qualitative difference between human and animal anxiety, Table 1 and our discussion of it suggest that it might not be the case. Rather, a clear phylogenetic trend appears, punctuated, thought, by important steps, as the three dimensions identified in the preceding paragraph. What is proper to human anxiety seems to be due to the well developed self-awareness capacity in that species. This feature, however, seems to be already shared, to a lesser extend, with great apes.

In conclusion, the present review proposes a general frame for discussing anxiety in the context of phylogeny. In many cases, the data necessary to assess the presence of a given process are not available and additional empirical work may be necessary to clarify this question. Still, as testified by the points highlighted in the general discussion, this approach proves to be heuristic, both for our understanding on how a phenomenon such as anxiety varies across the phylogeny, and for our understanding of the processes and logistic systems underlying anxiety.

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Research Article

Microinfusion of Pituitary Adenylate Cyclase-Activating Polypeptide into the Central Nucleus of Amygdala of the Rat Produces a Shift from an Active to Passive Mode of Coping in the Shock-Probe Fear/Defensive Burying Test

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High concentrations of pituitary adenylate cyclase-activating polypeptide (PACAP) nerve fibers are present in the central nucleus of amygdala (CeA), a brain region implicated in the control of fear-related behavior. This study evaluated PACAPergic modulation of fear responses at the CeA in male Sprague-Dawley rats. PACAP (50–100 pmol) microinfusion via intra-CeA cannulae produced increases in immobility and time the rats spent withdrawn into a corner opposite to the electrified probe compared to controls in the shock-probe fear/defensive burying test. Shock-probe burying and exploration, numbers of shocks received, locomotion distance, and velocity were all reduced by intra-CeA PACAP injection. Further, intra-CeA PACAP effects were manifested only when the animals were challenged by shock, as intra-CeA PACAP injections did not cause significant changes in the behaviors of unshocked rats. Thus, intra-CeA administration of PACAP produces a distinct reorganization of stress-coping behaviors from active (burying) to passive modes, such as withdrawal and immobility. These findings are potentially significant toward enhancing our understanding of the involvement of PACAP and the CeA in the neural basis of fear and anxiety.

1. INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP), a member of the secretin/glucagon/vasoactive intestinal peptide superfamily (Arimura and Shioda [1]), is a pleiotropic molecule with remarkable central actions on neuroendocrine and behavioral systems. Intracerebroventricular (icv) or intrahypothalamic PACAP injection results in a significant and long-lasting reduction of food intake (Morley et al. [2]; Chance et al. [3]), elevated plasma vasopressin, mean arterial blood pressure levels, and induces c-fos and vasopressin gene expression in the hypothalamus (Murase et al. [4]; Nomura et al. [5]). A marked increase in steady-state levels of CRH gene expression in the hypothalamic paraventricular nucleus (PVN) was detected after icv PACAP injection, which was blocked by coadministration of a selective PACAP receptor antagonist (Grinevich et al. [6]). PACAP nerve fibers heavily innervate the majority of corticotropin-releasing hormone (CRH) neurons in the PVN (Legradi et al. [7]) and icv PACAP administration under stress-free conditions in freely moving rats increased corticosterone levels and acutely activated PVN CRH neurons (Agarwal et al. [8]), mimicking important aspects of stress activation. Our group reported that PACAP infused into the PVN increased self-grooming behavior and suppressed ongoing exploratory activity (Norrholm et al. [9]). These data support the view that PACAP acts as an excitatory neuropeptide, recapitulating previously demonstrated behavioral effects of electrical and neurochemical PVN activation (Van Erp et al. [10]; Monnikes et al. [11]). Evaluation of time course of PACAP-induced behaviors indicated a cumulative effect of intra-PVN PACAP administration and restraint stress, thereby supporting our hypothesis

that PACAP amplifies the effects of stress on behavior (Norrholm et al. [9]).

The influence of PACAP on brain function has also been investigated in learning and memory studies. For example, icv injection of PACAP facilitated the learning, as well as retrieval, of the passive avoidance response (Telegdy and Kokavszky [12]). This finding further highlighted the potential contribution of PACAP to neurobehavioral responses to aversive or threatening stimuli, but its action site could not be determined from their study, further necessitating specific anatomical pharmacologic identification of PACAP target regions. In addition to the neuroendocrine and grooming effects mediated by the hypothalamus, PACAPergic mechanisms in stress responsivity may be processed through the amygdala. The amygdala is viewed as an interface between sensory information and defensive behavioral output, such as manifestations of fear or anxiety (Maren [13]; Davis [14]; LeDoux [15]). Whereas the lateral and basolateral nuclei are responsible for forming the association between fearful and neutral stimuli, perhaps through potentiation of synaptic transmission, the central nucleus (CeA) is implicated in the behavioral and autonomic expressions of fear (LeDoux [16]; Davis [14]). Strikingly high densities of nerve fibers immunoreactive for PACAP have been identified in the central-extended amygdala that includes the central nucleus of the amygdala (CeA) and the lateral part of the bed nucleus of the stria terminalis(Koves et al. [17]; Kivipelto et al. [18]; Piggins et al. [19]; Kozicz et al. [20]; Hannibal [21]). Likewise, medium to high densities of specific PACAP receptor (PAC1-R) expression were detected in CeA (Hashimoto et al. [22]) suggesting local physiologic role for the peptide.

The dense innervation of the amygdala by PACAP nerve fibers clearly indicates that this peptide can exert a strong, but largely unknown, influence on amygdaloid function. The present study, therefore, was designed to explore PACAP's contribution to the regulation of fear behavior, specifically at the level of the central nucleus of amygdala, using the shock-probe fear test. This method was originally developed as the defensive burying paradigm by Treit and coworkers (Treit and Pinel [23]). Findings from Treit's laboratory and others have suggested that either an increased burying response or increased withdrawal from the probe and immobility would be interpreted as qualitatively different expressions of fear behaviors evoked in response to the electrified shock probe. These two basic modes of coping have been viewed as active or passive, according to several investigators (Roozendaal et al. [24]; Treit et al. [25]; Degroot et al. [26]; De Boer and Koolhaas [27]) enabling the evaluation of aversive behaviors by quantitative, as well as qualitative, criteria. Therefore, in the current study we hypothesized that local administration of PACAP into the central amygdala would exert a strong influence on the expression of coping behaviors in rats exposed to the electrified probe.

2. MATERIALS AND METHODS

2.1. Animals and surgery

Male Sprague-Dawley rats (Taconic Farms, NY, USA), weighing 210–240 upon arrival, were used for the study (total $n = 58$). Animals were pair-housed until cannula implantation surgery and then single-housed in polycarbonate cages and maintained on a 12:12-hour light/dark cycle (lights on at 0700 hours), with food and water available *ad libitum*. Rats were handled daily for a week and habituated to the environmental conditions in the testing room. All procedures were carried out in accordance with the University of South Florida Institutional Animal Care and Use Committee guidelines regarding the care and use of experimental animals. After an initial 1-week acclimation and handling period, rats were anesthetized with Ketamine (90 mg/kg) and Xylazine (10 mg/kg) and a 24-gauge stainless steel guide cannula (Plastics One, Roanoke, Va, USA) was unilaterally implanted into the right central nucleus of amygdala under stereotaxic control (coordinates: 2.4 to 2.6 mm caudal to bregma, 4.4 to 4.5 mm lateral to the midline, and 5.5 to 5.6 mm below the skull surface) through a burr hole in the skull. Cannulae were secured to the skull with three stainless steel anchor screws and cranioplastic cement and temporarily occluded with a dummy cannula. Following surgery, ketoprofen (5 mg/kg) was injected subcutaneously to minimize post-surgical pain and inflammation. Unilateral cannulation of the right amygdala (Huston et al. [28]) was chosen since this side, compared to the left amygdala, has greater involvement in fear conditioning and anxiety responses (Baker and Kim [29]) and unilateral manipulations are surgically less invasive. Experiments were conducted 7–8 days post-surgery and during the light period of the cycle (1000 and 1400 hours).

2.2. Shock-probe fear test

For four consecutive days before behavioral experimentation, rats were given mock injections by attaching the guide cannula to an empty injection connector tubing for 2 minutes in their home cage and then the tubing was disconnected and the rats were exposed to the test chamber without the shock probe for 20 minutes. During the pretest session (the day before the experiment) individual rats were given a mock injection, and exposed to the test chamber in presence of an unelectrified shock probe for 20 minutes. Animal behavior was recorded onto digital video files at these pretest sessions.

Rats were randomly assigned into either the control or experimental groups prior to behavioral testing and infused with artificial cerebrospinal fluid (aCSF control) or PACAP (PACAP38; American peptide company, Sunnyvale, Calif, USA) using a BAS bee syringe pump system (West Lafayette, Ind, USA) connected to a 31-gauge internal cannula (Plastics one, outer diameter 0.25 mm, inner diameter 0.125 mm) with 2.5 mm protrusion below the end of the guide cannula to reach the target region. PACAP was diluted in sterile aCSF containing 0.05% bovine serum albumin (Sigma Chemicals, St. Louis, Mo, USA) and administered at a dose of 50 or 100 pmol into CeA in a volume of 0.2 μL over a 30-second period. The internal cannula remained inserted for 1 minute post injection to prevent backflow and to allow for diffusion of the peptide. The internal cannula was then withdrawn and

the animal was placed immediately into the shock-probe fear test chamber.

The shock-probe fear test apparatus consisted of a 46.6 × 28 × 26 cm Plexiglas chamber, evenly covered with 5 cm of Tek-fresh odor-absorbent bedding material (Harlan Teklad, Madison, Wis, USA). The shock-probe (8 cm long and 0.8 cm in diameter) was inserted through a hole on one wall of the chamber, 2 cm above the bedding material and helically wrapped with two copper wires through which electric current could be administered. The probe was not electrified until the spontaneously moving rat touched it with its forepaws, at which point the animal received a brief, 2 mA shock from the shock source (precision animal shocker, model H13-15, Coulbourn Instruments, Allentown, Pa, USA), remotely activated by an investigator using a footswitch. The 20-minute test began once the rat received its first shock and the probe remained electrified for the remainder of this period. To determine whether intra-CeA infusion of PACAP, without shocks, would produce alterations in behaviors, a group of rats was subjected to intra-CeA aCSF or PACAP injections and 20-minute exposure to the test chamber in the presence of an unelectrified probe. Animal behavior in the test chamber was recorded onto digital video tape and then saved as MPEG2 digital video files for subsequent observation, scoring, and automated analysis.

2.3. Verification of injection sites

Immediately after behavioral testing, animals were deeply anesthetized with Nembutal (90 mg/kg, ip) and perfused transcardially with heparinized saline followed by a solution containing 2% paraformaldehyde and 2.5% acrolein in .1 M phosphate buffer. Standard Nissl staining by cresyl violet and immunolabeling for PACAP were used to evaluate the injection sites. Tissue preparation for immunohistochemistry was performed according to a previously described method (Norrholm et al. [9]). Free floating coronal sections of the forebrain, taken at 30 μm thickness were pretreated with 1% sodium borohydride in distilled water followed by .5% hydrogen peroxide in phosphate-buffered saline and then preincubated in 10% normal horse serum. Sections were incubated for 3 days at 4°C in rabbit anti-PACAP serum (Peninsula Laboratories Inc., San Carlos, Calif, USA) diluted at 1:10,000 followed by sequential incubations in biotinylated donkey anti-rabbit IgG (1:200, Vector, Burlingame, Calif, USA) and the ABC elite kit (1:100, Vector, Burlingame, Calif, USA). Immunoreactivity was visualized with diaminobenzidine (DAB) as chromogen. A total of fifteen animals with missed cannula placements were excluded from statistical analysis. Ten additional animals were excluded for other problems such as bleeding, necrosis, or inadequate spread of synthetic PACAP immunoreactivity.

2.4. Analysis of behaviors

The following behaviors were analyzed from digital video files either by the automated tracking capabilities of Ethovision or counted using the behavior tracker (version 1.5, www.behaviortracker.com), an event-recorder software: (a) locomotion parameters: locomotion distance, defined as the total distance moved in the arena during the test period and mean velocity of locomotion, (b) probe exploration, including a stretched/attend-like posture oriented toward the probe or directly touching or sniffing the probe, (c) immobility, defined as crouching, sitting, or standing still on at least three feet, with the body motionless except for small and slow, lateral scanning movements of the head, (d) zonal preference, defined as time spent in the zone either away from the probe or near the probe, generated by dividing the length of the test chamber into two equal halves, (e) burying parameters: latency to bury, defined as the time between the first shock and the first burying event, duration of time spent on burying the probe such as spraying bedding materials toward or over the probe, the frequency of burying events and the height of bedding material over the probe at the end of session, (f) numbers of contact-induced shocks, (g) rearing time and numbers of rearing events, (h) grooming time and numbers of grooming events. The rats' reactivity to shock was scored according to a four-point scale (Pesold and Treit [30]) where "1" is head or forepaw flinch only, "2" is whole body flinch and/or walking away from the probe, "3" is whole body flinch and running from the probe, and "4" is whole body flinch and jumping (all four paws in the air), followed by running to the opposite end of the chamber (Pesold and Treit [30]; Treit and Pinel [23]). Mean shock reactivity scores were calculated for each rat by summing the shock reactivity scores and dividing them by the total number of shocks received.

All data were expressed as means ± SEM and analyzed by ANOVA, followed by post hoc analysis using the student-newman-keuls multiple comparisons test (SigmaStat 3.0, SPSS Inc., Chicago, Ill, USA). A probability level of $P < .05$ was considered to be statistically significant.

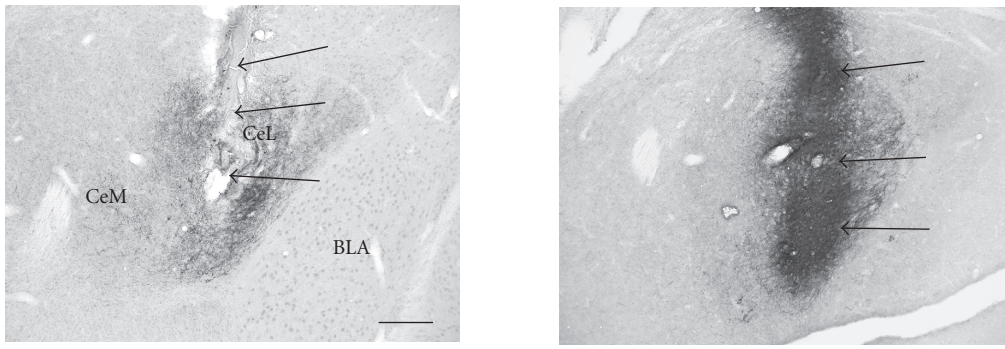
3. RESULTS

As indicators of baseline behavior, measures of exploration of the unelectrified probe were evaluated from recordings made during pretest sessions (last habituation session 24 hours before test day, as described in Section 2). No statistically significant differences were found in numbers of probe exploration events and total time spent on probe exploration among sets of rats prior to their placement into the various treatment groups ($P = .911$ and $P = .854$, resp.).

Figure 1 demonstrates typical injection sites at the level of the CeA using PACAP immunolabeling. The spread of the injected synthetic peptide was verified by the presence of a dense immunoreaction product in addition to the normal appearance of endogenous PACAP nerve fibers (Figure 1(b)).

3.1. Effects of intra-CeA PACAP microinjection on probe exploration and zonal preference and locomotion parameters in the rat shock-probe fear test

One-way ANOVA indicated that PACAP infusion into the CeA significantly decreased the frequency [$F(1, 9) = 11.05$;



(a) (b)

Figure 1: Histological verification of an injection site produced by microinjected synthetic PACAP. (a) Section from control brain, injected with aCSF vehicle. (b) Injected synthetic PACAP (50 pmol) immunoreactivity in the CeA. BLA = basolateral nucleus of the amygdala, CeM = central nucleus of the amygdala medial part, CeL = central nucleus of the amygdala, lateral part. Note the presence of high density of endogenous PACAP fibers in both (a) and (b). Arrows indicate the location of cannula track. In (b), synthetic PACAP injection is visible as an intense dark reaction product. Scale bar = 200 μm.

$P = .001$] and the duration of probe exploration [$F(1,4) = 8.15$, $P < .05$)] in shocked animals (Figures 2(a), 2(b)). A significant main effect was also found on zonal preference by intra-CeA PACAP microinjection [near zone time; $F(4,4) = 6.49$, $P < .05$), away zone time; ($F(4,4) = 6.52$, $P < .05$)] in rats tested with the electrified shock probe (Figures 2(c), 2(d)).

In addition, both total distance moved [$F(4,5) = 11.46$, $P < .001$] and movement velocity [$F(3,5) = 13.11$, $P < .001$] were significantly reduced by intra-CeA PACAP injection in shocked groups during the 20-minute test session (Figures 3(a), 3(b)). Immobility behavior was found only in shocked groups, following probe contact-induced shocks. Both the number of immobility events [$F(7,5) = 10.49$, $P = .001$] and total time spent on immobility behavior [$F(99,2) = 226.29$, $P < .001$] were significantly increased by intra-CeA PACAP-injection relative to aCSF-injected controls (Figures 3(c), 3(d)).

3.2. Burying-related behaviors

One-way ANOVA indicated a significant main effect of intra-CeA PACAP infusion on bury latency [$F(4,4) = 6.55$, $P < .05$], total duration of burying [$F(1,7) = 13.17$; $P < .001$], bury events [$F(1,5) = 16.56$, $P < .001$], and the height of bedding over the probe [$F(2,3) = 31.52$, $P < .001$] as compared to aCSF controls. Probe burying was significantly delayed in PACAP-injected rats compared to aCSF controls (Figure 4(a)). Intra-CeA PACAP-injected rats displayed significantly reduced number of burying events (Figure 4(b)). The total amount of time spent on burying the electrified shock probe was also significantly decreased by PACAP injection as compared to aCSF controls (Figure 4(c)). As a result, the height of the bedding material over the probe at the end of the test session was significantly reduced in both the 50 and 100 pmol PACAP-injected groups (Figure 4(d)).

3.3. Intra-CeA PACAP infusion reduces number of shocks without altering individual shock reactivity

Intra-CeA PACAP infusion resulted in a significant reduction in the number of shocks received, relative to intra-CeA aCSF-injected rats [$F(5,5) = 5.12$, $P < .05$] (Figure 4(e)). However, no significant differences were found in the shock reactivity index between aCSF and PACAP-injected groups (Figure 4(f)).

3.4. Intra-CeA PACAP injection does not alter exploration of the unelectrified probe or locomotion parameters in unshocked rats

No statistically significant effects were found in probe exploration in PACAP-injected unshocked groups compared to their respective aCSF-injected controls (Figures 5(a), 5(b)). No intra-CeA PACAP injection effects were found in animals tested with the unelectrified shock probe as in unshocked groups, near and away zone times were roughly equal, and unaltered by intra-CeA PACAP injection (Figures 5(c), 5(d)). In unshocked groups, intra-CeA PACAP injection did not produce statistically significant differences in total distance moved movement or movement velocity compared to their respective aCSF-injected control (Figures 5(e), 5(f)). No burying behavior directed specifically toward the probe was found in unshocked groups, regardless of treatment (data not shown).

3.5. Intra-CeA infusion of PACAP does not alter grooming and rearing behaviors in either shocked or unshocked conditions

PACAP microinjection into the CeA at either dose did not significantly alter the frequency or duration of rearing and

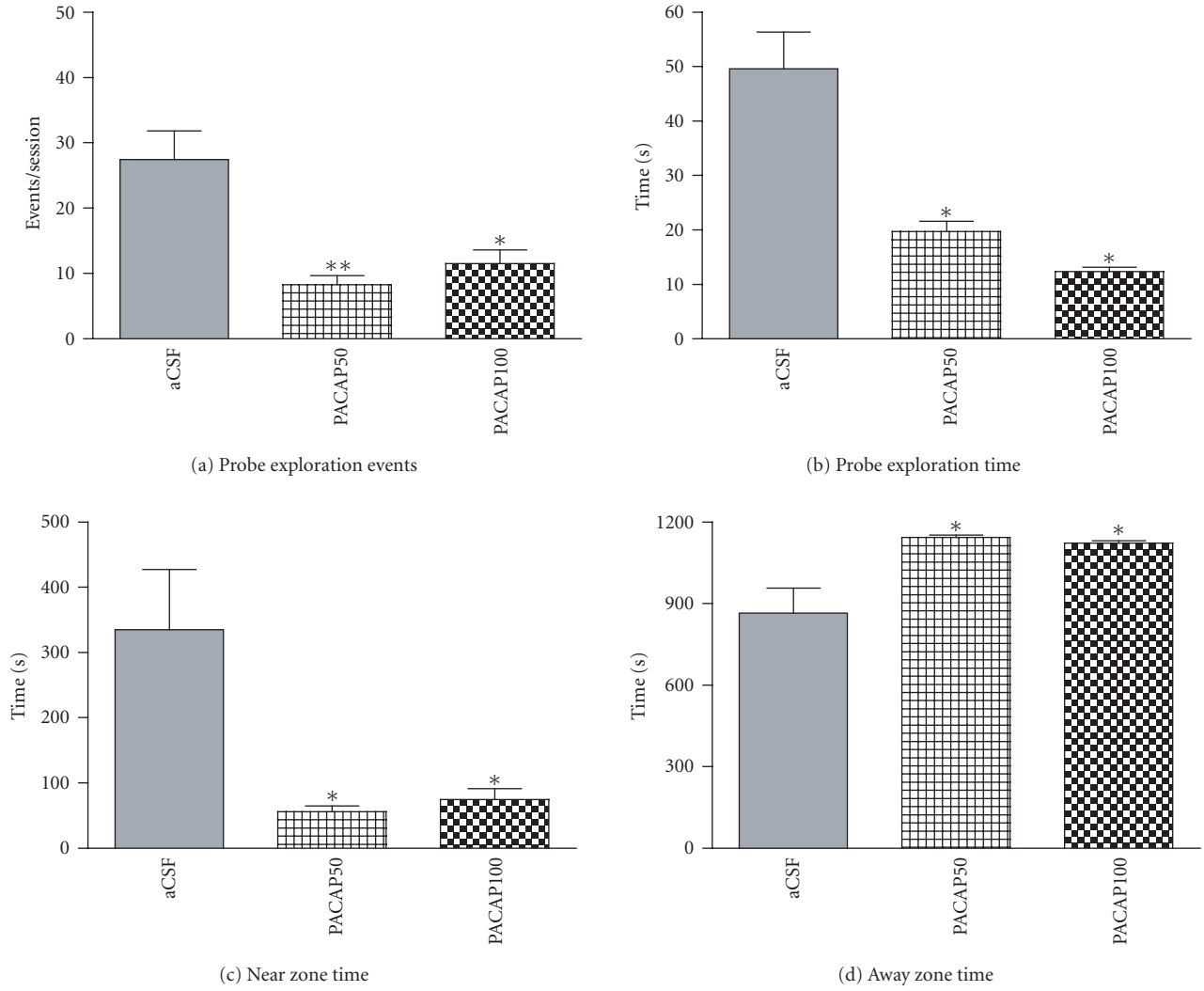
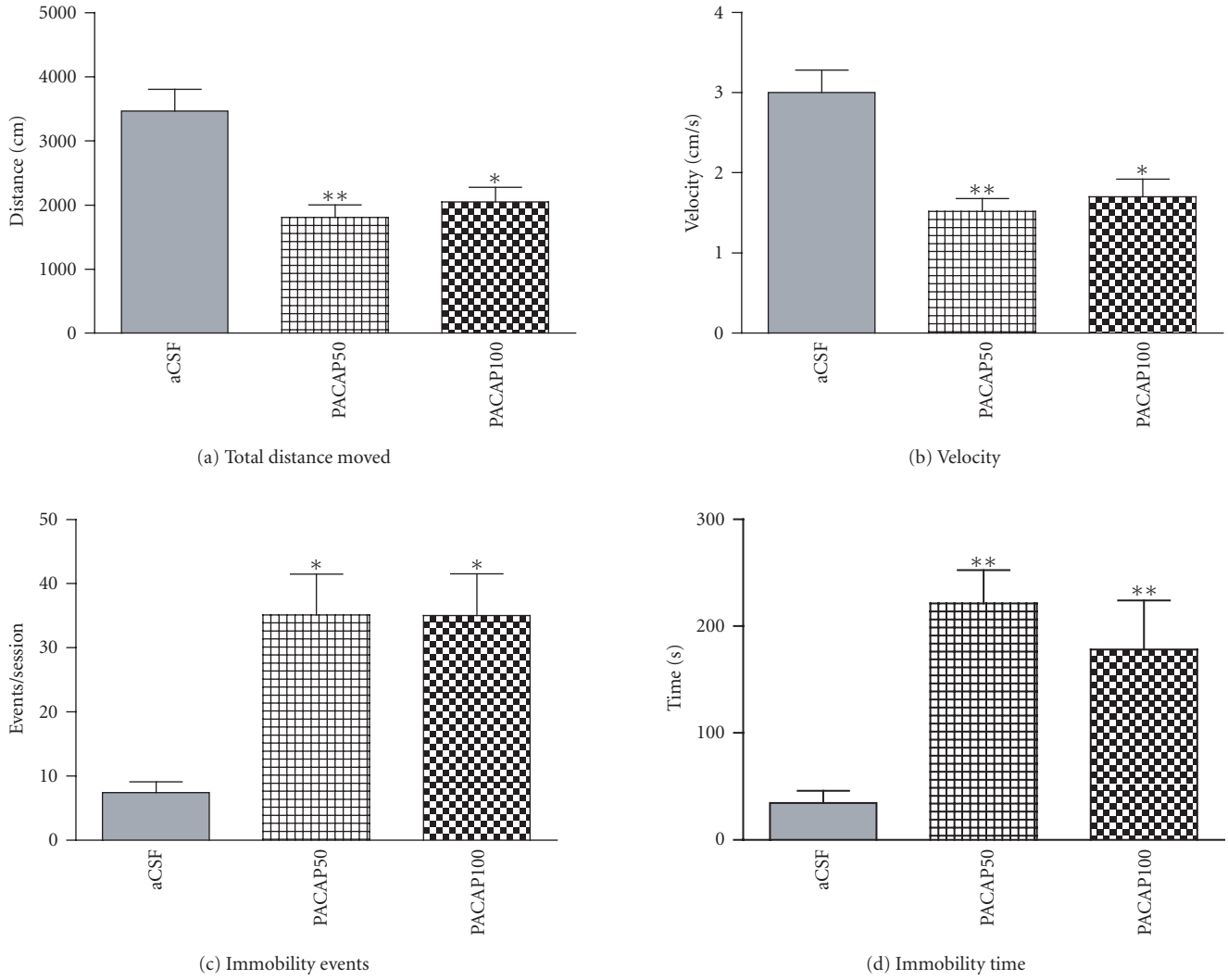


FIGURE 2: Effects of intra-CeA microinfusion of PACAP on shock-probe exploration and zonal preference in shocked rats. The numbers of probe exploration events (a) and time spent on probe exploration (b) are significantly reduced by intra-CeA PACAP. Zonal preference is altered by intra-CeA PACAP microinjection as rats spent significantly less time in the near zone (c) but more time in the zone away from the electrified shock probe (d). $^{*}P < .05$ and $^{**}P \leq .001$ compared to aCSF controls. (aCSF $n = 7$/group, PACAP50 $n = 7$/group, PACAP100 $n = 4$/group).

grooming behaviors as compared to their respective controls (Figure 6).

4. DISCUSSION

Since PACAP's discovery, experimental studies have identified roles for PACAP as a multifunctional molecule acting as a neurotransmitter/modulator, neurotrophic factor, supplementary hypophysiotropic hormone, and peripheral vasodilator (Arimura [31]; Vaudry et al. [32]) but the participation of PACAP in neural systems and behavioral functions is inadequately understood. Since strikingly high local concentrations of PACAP immunopositive nerve fibers are found in the central nucleus of the amygdala (CeA) (Koves et al. [17]; Kivipelto et al. [18]; Piggins et al. [19]; Kozicz et al. [20]; Hannibal [21]), a structure associated with the expression of aversion and fear, we hypothesized that PACAP at the level of the CeA could modulate fear-related behaviors. The present study investigated the effects of intra-CeA PACAP microinjection on behavioral responses using the shock-probe fear (defensive burying) test. In this paradigm, the animal is confronted with an electrified shock probe wrapped with uninsulated wires from which shocks are administered. When the spontaneously moving rat touches the probe by exploration, the resultant behavioral response whether active burying or passive (e.g. withdrawal and immobility) can be evaluated using automated and semiautomated observation. In the traditional interpretation of the test, increased probe burying while locomotion is unaltered indicates an anxiogenic response, and reduced burying with increased contact induced shock may indicate anxiolysis. On the other hand, increased withdrawal from the probe and reduction in contact-induced



(a) Total distance moved

(b) Velocity

(c) Immobility events

(d) Immobility time

Figure 3: Effects of intra-CeA PACAP microinjection on locomotion parameters in shocked rats. Total distance moved (a) and mean movement velocity (b) were significantly reduced by intra-CeA PACAP microinjection. Immobility events (c) and total time spent on immobility (d) following probe-contact-induced shocks were increased by intra-CeA PACAP. $^{*}P < .05$ and $^{**}P \leq .001$ compared to aCSF controls.

shocks, particularly in the version of the test used by our study where the shock source remains continuously electrified (Treit and Fundytus [33]) can also be interpreted as measures of heightened innate fear. Indeed, our results indicated that intra-CeA microinfusion of PACAP (50 or 100 pmol) enhanced certain types of aversive behaviors in the shock-probe fear test, consistent with our notion that PACAPergic neurotransmission may be linked to manifestations of stress and fear (Agarwal et al. [8]; Norrholm et al. [9]).

In the current study, intra-CeA PACAP injection produced a significant increase in the withdrawal of the shocked rats away from the electrified probe, resulting in dramatically reduced numbers of contact induced shocks. Duration of immobility and time spent in the away zone were markedly elevated in CeA-PACAP-injected animals. Time spent in the near zone, latency of the last shock, duration of burying, and the height of bedding over the probe were also greatly reduced relative to aCSF-injected animals. Measures of locomotion (total distance and time) and velocity of movement were reduced in intra-CeA PACAP-injected animals tested with the electrified shock probe. In the 4-point shock-reactivity scale, (Pesold and Treit [30]; Treit and Pinel [23]), no statistically significant differences were found between intra-CeA vehicle-injected and intra-CeA PACAP-injected rats, indicating that the observed behavioral manifestations were not overtly influenced by organismic variables such as possible changes in shock sensation. Collectively, these data highlight the importance of the CeA in the reorganization of coping strategy in CeA-PACAP-injected animals using the shock-probe fear test to elicit fear and anxiety related responses.

Thus, intra-CeA PACAP-injected animals react with a passive behavioral coping response, which reduces the numbers of shocks received. The mechanisms leading to the

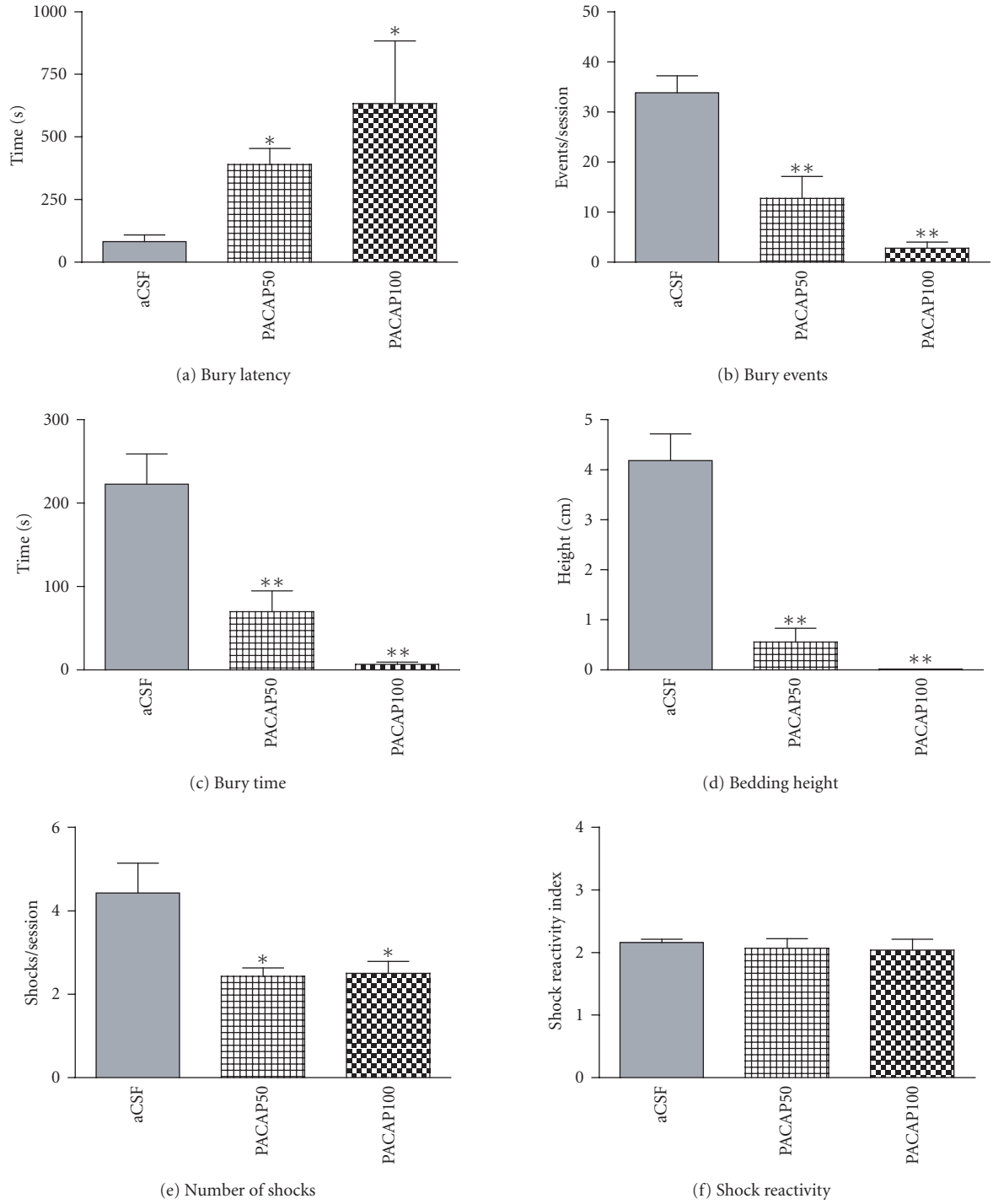
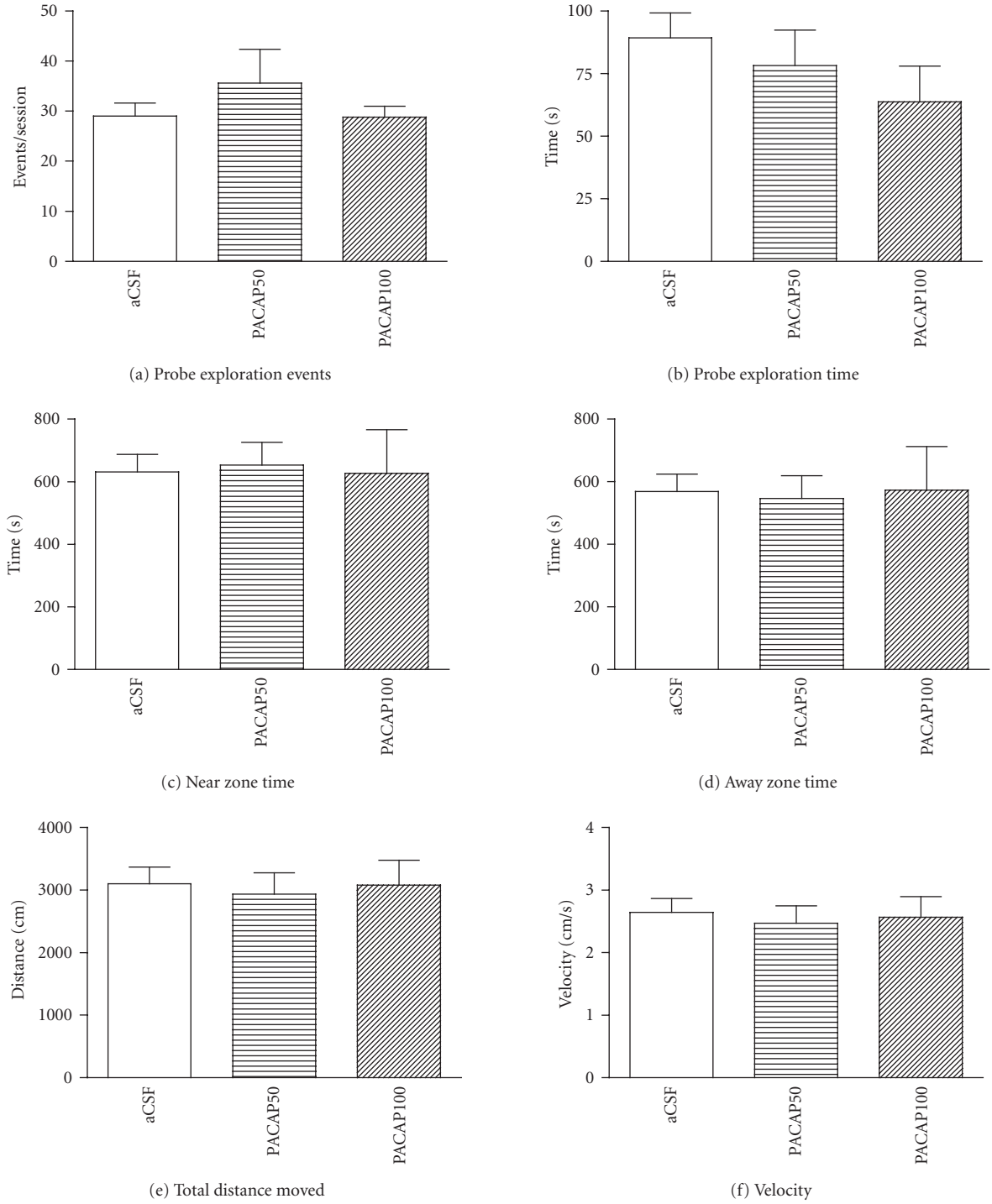


Figure 4: Effect of intra-CeA PACAP administration on shock-probe burying and shock-related behaviors. Latency to bury the electrified shock probe (a) was significantly increased in PACAP-injected animals whereas burying events (b) and time (c) and the height of bedding over the probe (d) were reduced. The number of probe-contact-induced shocks (e) was significantly reduced in PACAP-injected animals but shock reactivity (e) was unaltered. $^{*}P < .05$ and $^{**}P \leq .001$ compared to aCSF controls.

behavioral manifestations of PACAP-shock interactions are not known, but we suggest that administration of PACAP in the CeA, likely acting upon its cognate receptor which is widely expressed in the amygdala (Hashimoto et al. [22]), produces its pharmacologic effects locally, on neurons of the CeA. It is therefore possible that the observed pharmacologic effect of PACAP on the behaviors we have described here reflect a role for the endogenous PACAP nerve fibers in the



(a) Probe exploration events

(b) Probe exploration time

(c) Near zone time

(d) Away zone time

(e) Total distance moved

(f) Velocity

Figure 5: Summary of relevant behaviors of rats tested with the unelectrified shock probe (unshocked groups). Intra-CeA PACAP microinjection, in the absence of shocks, had no significant main effect on rat behaviors in the test chamber. (a) probe exploration events, (b) probe exploration time, (c) near zone time, (d) away zone time, (e) total distance moved, and (f) movement velocity. $^{*}P < .05$ and $^{**}P \leq .001$ compared to aCSF controls. (aCSF $n = 6$/group, PACAP50 $n = 4$/group, PACAP100 $n = 5$/group.)

CeA (Koves et al. [17]; Piggins et al. [19]; Hannibal [21]) in the formation of coping behaviors in response to strong aversive stimulation. Determination of the exact contribution of PACAP to responses evoked from the CeA is ultimately dependent on the nature of the target neurons influenced by this neuropeptide. Based on the high concentration of PACAP nerve fibers in the lateral, capsular subnuclei and medium density PACAP innervation in the medial

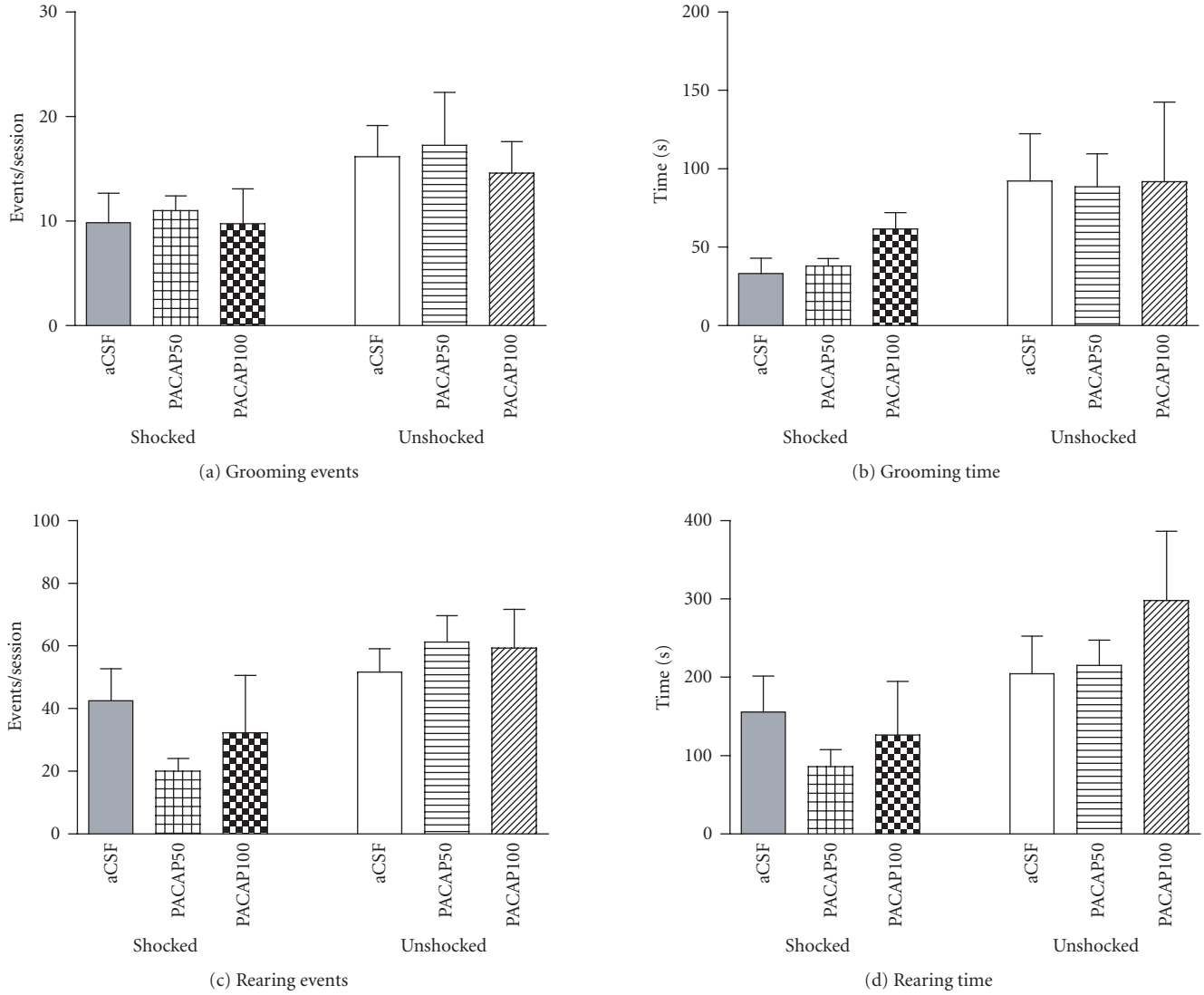


Figure 6: Grooming and rearing behaviors of shocked and unshocked rats in the shock-probe fear chamber. Intra-CeA PACAP microinjection had no statistically significant effects on grooming events (a) and time (b) or rearing events (c) and time (d) in shocked or unshocked groups of rats, relative to their respective aCSF-injected controls.

subnucleus of CeA, enkephalin, neurotensin, GABA, and CRH-containing neurons (Cassell et al. [34]) may represent natural targets of PACAP's physiologic effects. Likewise, the behavioral pharmacologic effects observed in the current study most likely reflect PACAP's actions on several classes of CeA neurons that may be interneurons and/or output projection neurons.

It has been recognized that the CeA serves as an output nucleus of the amygdala. Its efferent fibers project to the hypothalamus and brainstem areas such as the periaqueductal gray, parabrachial and caudal pontine reticular nuclei and the nucleus of the solitary tract, which are poised to mediate fear-related behaviors, including immobility and autonomic responses (Hopkins and Holstege [35]; LeDoux et al. [36]; Hitchcock et al. [37]; Saha et al. [38]). Immobility is considered as a first stage of defense when an animal is confronted with a threat, triggering increased vigilance and immobility. In this fear state, the organism has been primed to respond, but is not yet active; an exaggerated startle response is typically found (Lang et al. [39]). CeA lesions block the expression of immobility to fearful stimuli (LeDoux et al. [36]), and attenuate the development of the passive emotional and autonomic components of the coping response (Roozendaal et al. [40, 41]). Activation of the CeA may be linked with the augmentation of passive behavioral coping (Roozendaal et al. [42]) and potentiated startle reflex as well as post-stress freezing (Tinsley and Fanselow [43]).

While the cellular and molecular effects of PACAP have not been examined specifically at the level of CeA, several lines of evidence suggest that in general, PACAP is an excitatory neuropeptide. PACAP is known to colocalize with the major excitatory transmitter glutamate in the

retinohypothalamic nerve fibers (Hannibal et al. [44]). The presence of PACAP in primary afferent nerve fibers of the spinal and medullary dorsal horn as well as brainstem catecholamine neurons also suggests an association with excitatory neurotransmission (Legradi et al. [45]; Dun et al. [46]; Legradi et al. [47]; Das et al. [48]).

Interactions between PACAP and other neuropeptides/neurotransmitters, such as CRH, are quite likely to occur. Based on earlier reports, we hypothesize that the effects of PACAP on fear-related behaviors may be mediated through interaction between PACAP and CRH neurons at hypothalamic, as well as extrahypothalamic, sites (Kozicz et al. [20]; Agarwal et al. [8]). Psychological stress induces CRH gene expression in the amygdala (Makino et al. [49]), antagonism of CRH receptors in the CeA reduces freezing induced by foot shocks (Diamant et al. [50]) and icv CRH administration promotes freezing and reduces shock-probe burying (Swiergiel et al. [51]). Thus, the central action of CRH mediated in part at the level of the CeA is to enhance passive emotional coping. In this context, PACAP in the CeA appears to mimic actions of CRH. Perhaps CRH is an immediate downstream target of PACAP's action in the CeA. If this were the case, then coadministration of a CRH antagonist and PACAP should abolish or significantly blunt the effects of PACAP on fear-related behaviors.

The action of PACAP on the CeA and the resultant reorganization of behavior towards a passive, rather than an active, stress-coping mechanism, is perhaps responsible for shifting of the balance between competing active/passive-coping strategies, regulated by the interplay between various centers of the brain. It is possible that the normally occurring active shock-probe burying response is related to the function of the medial prefrontal cortex (mPFC), a key structure in the organization of goal-oriented behaviors (Haddon and Killcross [52]). The presumed PACAP-induced increase in the activity of the CeA may override the influence of the mPFC (decision-making) process, in favor of the more instinctual immobility responses to shock. In support of this speculation are the findings that mPFC stimulation inhibits CeA output neurons (Quirk et al. [53]), and that excitotoxic lesions of the mPFC or its pharmacologic inactivation with muscimol potently inhibit fear, specifically reducing active stress coping such as shock-probe burying (Shah and Treit [54]; Shah et al. [55]).

It is important to further note that PACAP injection alone, in the presence of an unelectrified probe, did not have an effect on measures of locomotion, immobility, frequency, and duration of probe exploration and zonal preference as compared to the corresponding aCSF- injected controls. Thus, the potentiation of fear-related behaviors by intra-CeA PACAP injection occurred only in shocked rats. This finding provides strong support for the notion that PACAP is active in modifying CeA functions only when the animal is challenged by an aversive stimulus.

In summary, the present study reveals substantial effects of PACAP microinjection into the CeA on the expression of behavioral coping strategies in response to a fear-provoking stimulus. In the shock-probe fear test (defensive burying paradigm), intra-CeA PACAP at 50 or 100 pmol doses induced a remarkable shift from active (burying) to passive (withdrawal) coping strategies. Infusion of PACAP into CeA resulted in no specific alterations in locomotion or probe exploration responses when animals were tested with an unelectrified probe, indicating that PACAP's effects were manifested only when the animal was challenged by aversive stimuli (shock). Thus, in addition to delineating the PACAPergic modulation of amygdala physiology and the neurobiology of fear, these studies may also have important implications toward understanding the role of PACAP in the neural basis of anxiety disorders.

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Research Article

Differential MR/GR Activation in Mice Results in Emotional States Beneficial or Impairing for Cognition

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Corticosteroids regulate stress response and influence emotion, learning, and memory via two receptors in the brain, the high-affinity mineralocorticoid (MR) and low-affinity glucocorticoid receptor (GR). We test the hypothesis that MR- and GR-mediated effects interact in emotion and cognition when a novel situation is encountered that is relevant for a learning process. By adrenalectomy and additional constant corticosterone supplement we obtained four groups of male C57BL/6J mice with differential chronic MR and GR activations. Using a hole board task, we found that mice with continuous predominant MR and moderate GR activations were fast learners that displayed low anxiety and arousal together with high directed explorative behavior. Progressive corticosterone concentrations with predominant action via GR induced strong emotional arousal at the expense of cognitive performance. These findings underline the importance of a balanced MR/GR system for emotional and cognitive functioning that is critical for mental health.

1. INTRODUCTION

Stress and emotions facilitate or impair learning and memory processes [1]. Glucocorticoids are the stress hormones secreted from the adrenals after activation of the hypothalamus-pituitary-adrenal (HPA) axis; that is, corticosterone in rats and mice, cortisol in humans. The effect on synaptic plasticity and memory formation is mediated by two types of nuclear receptors: MR (mineralocorticoid receptor) and GR (glucocorticoid receptor) which are located in areas involved in emotion, learning, and memory. While MR is present in the hippocampus and to lesser extent in the prefrontal cortex, amygdale, and paraventricular nucleus [2–5], GR can be found throughout the brain with high levels in the hippocampus and paraventricular nucleus [5]. Other characteristics are the differential affinities for corticosterone: MR has a tenfold higher affinity than GR, resulting in predominant MR occupation during low basal levels and additional GR activation during increased corticosterone concentration due to stress or circadian peak activity of the hypothalamic-pituitary-adrenal (HPA) axis [6]. The precise involvement of MR and GR in emotion and cognition is still debated.

Animal studies have shown that activation or blockade of either receptor influences behavior related to anxiety, exploration, and memory. These behaviors are linked to the limbic system and are part of the behavioral repertoire tested in spatial memory tasks and also in fear conditioning [7]. With respect to unconditioned fear-related behavior, Smythe et al. [8] have described that MR modulates anxiety-like behavior of rats in the light/dark box. Oitzl et al. have shown that intracerebroventricular injection of a rather selective MR antagonist in rats influenced corticosterone-induced behavioral reactivity to spatial novelty [9]. Recent findings in mutant mice with inactivated MR in the forebrain (Cre-loxP recombination [10]) support the pharmacologically detected role of MR on the modulation of behavioral strategies. Loss of the limbic MR impaired behavioral plasticity, evidenced by a differential performance during the first exposure to learning tasks, that is, their behavioral reactivity to novelty. In contrast, learning slopes in the water and radial arm maze were not affected. This increased behavioral reactivity to novel objects was observed in the face of normal anxiety-like behavior in the open field and elevated-O-maze [10]. Indeed, it should be clarified whether MR affects anxiety or appropriate context-dependent behavioral reactivity.

Others suggest that adaptive behavior is modulated by a combined MR/GR mediated action. An example is the inhibition of corticosterone production and thus prevention of

GR activation in the face of full MR activation: this led to decreased fear-induced immobility and fear-related anxiety in rats [11]. Complementary, exogenous corticosterone application or prior social defeat increased anxiogenic behavior in rats tested in the elevated plus maze 24 hours later. Antagonism of the GR in the lateral septum eliminated the anxiogenic effect [12]. Interesting in this study is the 24-hour delay, indicating involvement of memory. Indeed, GR is implicated in memory consolidation processes, demonstrated by using GR-agonists and GR-antagonists in rats, chickens, as well as GR mutant mice [13–18]. Calvo and Volosin have shown that corticosterone-induced effects on anxiety after restraint stress require both MR and GR [19]. Taken together, MR appears to be responsible for the immediate facilitative effects of corticosterone on memory acquisition, while the modulation of spatial and fear memory relies on the presence of a functional GR [20]. To disentangle the combined contribution of MR and GR to most adequate performance, we will study the functions of these receptors in a task that allows simultaneous registration of emotional and memory parameters.

How emotion and cognition affect each other is still relatively unknown. Forgas and George suggested that a stimulus first needs to be identified before the appropriate emotional response will follow [21]. Others focus more on the neurobiological process of emotion and cognition, which can be functionally, anatomically, and even pharmacologically separated [22]. We hypothesize that emotion and cognition are interdependent and both will be affected by differential MR and GR activations: we propose that the two corticosteroid receptors MR and GR contribute differentially but in a coordinated way to information processing.

The aim of this study was to examine how MR and GR interact in information processing presented by emotional and learning/memory elements of a task. Next to the well-known use of MR and GR antagonists, MR/GR activation ratios can be endocrinologically and pharmacologically adjusted by removal of the adrenals (adrenalectomy (ADX)) and additional subcutaneous corticosterone pellet implantation. In contrast to rats, mice that undergo adrenalectomy remain to produce low concentrations of corticosterone from scattered cell groups in the vicinity of the adrenals [23–25]. Therefore, ADXed mice provide an excellent model for predominant MR activation. Different degrees of continuous GR activation can be achieved via corticosterone released from implanted pellets. We used this approach and tested mice in the modified hole board [26] measuring behaviors that define general activity, emotions, motivation, and learning and memory. Subsequent principal component analysis will allow to determine the correlation between emotions and cognition.

2. MATERIAL AND METHODS

2.1. Animals

Forty eight 12-week-old male C57BL/6 mice were obtained from Charles River (Maastricht, The Netherlands). After arrival, the mice were housed individually in the experimental room with sawdust bedding, water and food *ad libitum*, at 20°C with controlled humidity under a 12 h : 12 h light/dark cycle (lights on at 08.00 am) for at least one week. To familiarize with the bait used in the modified hole board task, all mice received a few pieces of almonds daily in the week before surgery. All experiments were approved by the committee on Animal Health and Care from the Leiden University, The Netherlands, and were performed in strict compliance with the EEC recommendations for the care and use of laboratory animals.

2.2. Endocrine manipulation of MR/GR activation

Mice were randomly selected for one of the following groups and operated accordingly: (i) sham-operated (Sham), (ii) adrenalectomized mice (ADX), (iii) adrenalectomized mice with an additional low corticosterone pellet (ALC), or (iv) adrenalectomized mice with an additional high corticosterone pellet (AHC).

2.2.1. Surgery

Mice were gas anaesthetized with a mixture of isoflurane/nitrous oxide (4% isoflurane bolus followed by 2% isoflurane). Body temperature was kept constant at 37°C by a heating pad. Adrenals were removed (ADX) using the dorsal approach followed by subcutaneous pellet implantation on the flank of the animal. While in rats ADX removes the endogenous source of corticosterone, in mice it clamps corticosterone to low concentrations comparable to the circadian trough of adrenally intact mice. Accessory adrenocortical cells secrete stable amounts of corticosterone [23–25, 27] that maintain extensive occupation of MR. Stress or circadian rhythm does not lead to a rise in corticosterone in ADX mice. High circulating levels of ACTH indicate the lack of GR activation; that is, no negative feedback.

Sham operation involved the same procedures as adrenalectomy except for the removal of the adrenals. Surgery was performed between 10.00 and 12.00 am and lasted maximally 10 minutes per mouse. Adrenals were removed within 5 minutes. After surgery, all mice received an additional bottle containing 0.9% salt solution. Behavioral testing started 3 days after surgery. To confirm effectiveness of the adrenalectomy and pellet implantation, plasma corticosterone levels were measured 2 days after surgery, on day 0 of the experiment, and one day after the last behavioral test on day 11. Mice with abnormal corticosterone concentrations in the blood were excluded from further analysis. This resulted in seven mice per group.

2.2.2. Pellet preparation

Two types of pellets were made for subcutaneous implantation: (i) a 5% corticosterone (ICN Biomedicals, Inc., Calif, USA) 95% cholesterol pellet for moderate MR/GR activation and (ii) a 20% corticosterone 80% cholesterol pellet for strong MR/GR activation. All pellets weighed 100 mg, with

Table 1: Behavioral parameters measured in the modified hole board.

Total number	Sit
—	Rearing
—	Stretched attend
—	Grooming
—	Center board entries
—	Hole visits
—	Baited holes visited
—	Nonbaited holes visited
—	Repeated hole visits
—	Baits obtained
Latency	First center board entry
—	First hole visit
—	Eat bait
Time	Sit
—	Grooming
—	On center board
—	To finish task

a diameter of 7 mm and thickness of 2 mm and were homemade. Corticosterone dose was chosen following a pilot experiment in which plasma corticosterone concentrations of about 100 and 150 ng/mL for the 5% and 20% pellets, respectively, were measured two days after implantation.

2.3. Modified hole board testing

2.3.1. Setup

The modified hole board consisted of an opaque grey PVC box (50 × 50 × 50 cm) with a center board (37 × 20 cm) on which 10 grey cylinders (4 cm height) were staggered in two lines [26]. Always the same three cylinders were baited with a small piece of almond on top of a grid, and were marked with a white ring. Seven other cylinders contained a nonobtainable almond underneath the grid and were marked with a black ring. The mice were placed in the modified hole board for 3 trials per day with changing start positions. One trial lasted maximally 5 minutes, or until the mouse had found the three baits. All testings were performed between 9.00–12.00 am.

2.3.2. Behavioral observation

The behavior of the mice was observed, recorded, and analyzed with a semiautomatic scoring system (The Observer Mobile 4.1, Noldus Information Technology, Wageningen, The Netherlands). All measured behavioral parameters are represented in Table 1. As indication for (i) working memory, the number of repeated holevisits was calculated and (ii) reference memory, the number of visits to nonbaited holes was taken. In addition, a camera was installed above the setup to measure distance moved and velocity of the mice with an automatic tracking system (Ethovision 1.95, Noldus Information Technology, Wageningen, The Netherlands).

2.4. General experimental procedure

Mice were tested in the modified hole board over 10 days. On days 1 to 5 and 8, the three baited cylinders were marked with a white ring as visual cue while the remaining cylinders were marked with a black ring. This allowed visuospatial discrimination. On days 6 and 7, mice were not tested. On days 9 and 10, all rings were removed from the cylinders, but the bait remained in the same cylinders. This allowed to estimate if the mice used a spatial strategy or visual discrimination to solve the task.

A trial lasted maximally 5 minutes and was ended when the mouse had eaten all three baits.

On days 0 and 11, blood was collected via a tail incision or after decapitation. Blood plasma was used to measure corticosterone concentrations (ICN Biomedicals, Inc., Calif, USA). Because exposure to high concentrations of corticosterone results in shrinkage of the thymus, thymus weight was estimated as well.

2.5. Statistical analysis

Differences in corticosterone concentrations between groups and days were analyzed by two-way ANOVA (SPSS 11.5.0) with Tukey's post-hoc analysis. To analyze thymus and bodyweight differences, a one-way ANOVA was performed.

The behavioral data are presented as mean of 3 trials per day ± SEM. Data were subjected to general linear model (GLM-) repeated measures with Tukey as post-hoc test to analyze progression over days and group differences per day. Furthermore, factor analysis (principal component analysis (PCA)) was performed over groups and days to obtain a more comprehensive analysis of emotional and cognitive parameters. This analysis uses cross-mouse comparisons to distinguish the relation between behavioral parameters. It includes as much data as possible in each factor to minimize residual variance from the original dataset. The PCA was performed with a varimax rotation on variables with communalities over 0.7, that is, of which 70% of the variance is explained by the factors extracted. The number of extracted factors was not predefined; factors with an eigenvalue > 1 were accepted. Factor scores were subjected to a two-way ANOVA to determine differences between groups and days. $P < .05$ was accepted as level of significance.

3. RESULTS

3.1. Behavior

3.1.1. Emotion and exploration

Figure 1 shows the results for some of the emotional and explorative parameters during all days of testing in the modified hole board. Figure 1(a) illustrates that ADX followed by ALC mice have a high percentage of time spent on the center board, indicative of low anxiety [26, 28–30] during the

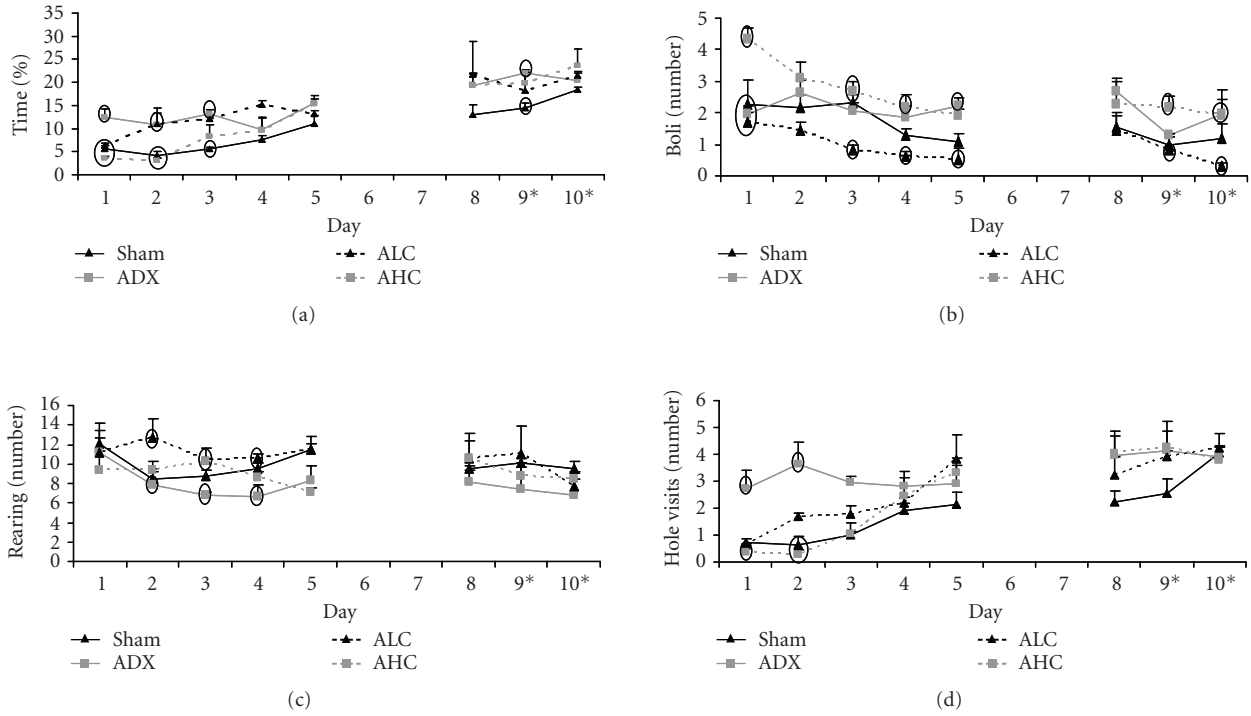

Figure 1: Behavior of mice in the modified hole board. (a) Percentage of time spent on center board, (b) number of defecations, (c) number of rearings, (D) number of hole visits, including revisits of sham (black line), ADX (grey line), ALC (striped black line), and AHC mice (striped grey line). Days 9 and 10 on the *x*-axis indicate removal of rings from all cylinders, while the bait remained in the same cylinders as before. Data present the mean of the three trials per day ± SEM. Ovals mark data points with significant differences $P < .05$ between groups within days.

first few days. In contrast, AHC and sham mice spent little time on the center board during this period. From day 4 on, few significant differences were found between groups. GLM from day 1 to 10 revealed a significant group/day interaction F(21,588) 2.355, $P = .001$.

Figure 1(b) shows that AHC mice display twofold more defecation compared to other groups, indicating high arousal. With repeated testing, ALC mice display less defecation compared to ADX and AHC mice. GLM revealed a significant progressive decrease over days F(21,588) 7.629, $P < .0001$, just passing statistical significance between groups (F(21,588) 1.524, $P = .063$).

The number of rearings was taken as measure for general exploration (Figure 1(c)). Comparing the first and the last days of testing, no differences were found between groups while on days 2, 3, and 4 ADX mice displayed the lowest number of rearings. GLM showed a significant change over days (F(21,588) 11.439, $P < .0001$) although not significant between groups (F(21,588) 1.25, $P = .203$).

ADX mice display highly directed exploration/behavioral reactivity on all days of testing, reaching statistical significance on days 1 and 2 as indicated by the number of hole visits (Figure 1(d)). Sham, AHC, and ALC mice start off with few hole visits which increase over time. GLM supported this by significant group/day interaction F(21,588) 1.983, $P = .006$.

Total distance moved and velocity were comparable between groups over all days of testing (data not shown).

3.1.2. Cognition

Figure 2 shows the results for three cognitive parameters on all days of testing in the modified hole board. Figure 2(a) illustrates increased repeated hole visits (working memory) in ADX mice on day 8 of testing compared to sham mice. We consider the low repeated hole visits on days 1 and 2 of sham, ALC, and AHC mice as not reliable, because the total number of hole visits is also very low on these days. Over time, sham, ALC, and AHC mice show increased repeats in parallel with increased total hole visits. GLM showed a significant group/day interaction (F(21,532) 2.029, $P = .005$).

Figure 2(b) shows no significant differences in nonbaited hole visits (reference memory) between sham, ADX, ALC, and AHC mice during all days of testing.

The time to finish the task is an additional learning parameter (Figure 2(c)). ADX and ALC mice were fast learners compared to sham and AHC mice. Removal of the rings on days 9 and 10 did not influence the time to finish the task, indicating the use of a spatial learning strategy at that time of training. At the last day of testing, performance of sham mice was still poor although progression over days proved to be significant (F(21,532) 18.327, $P = .000$).

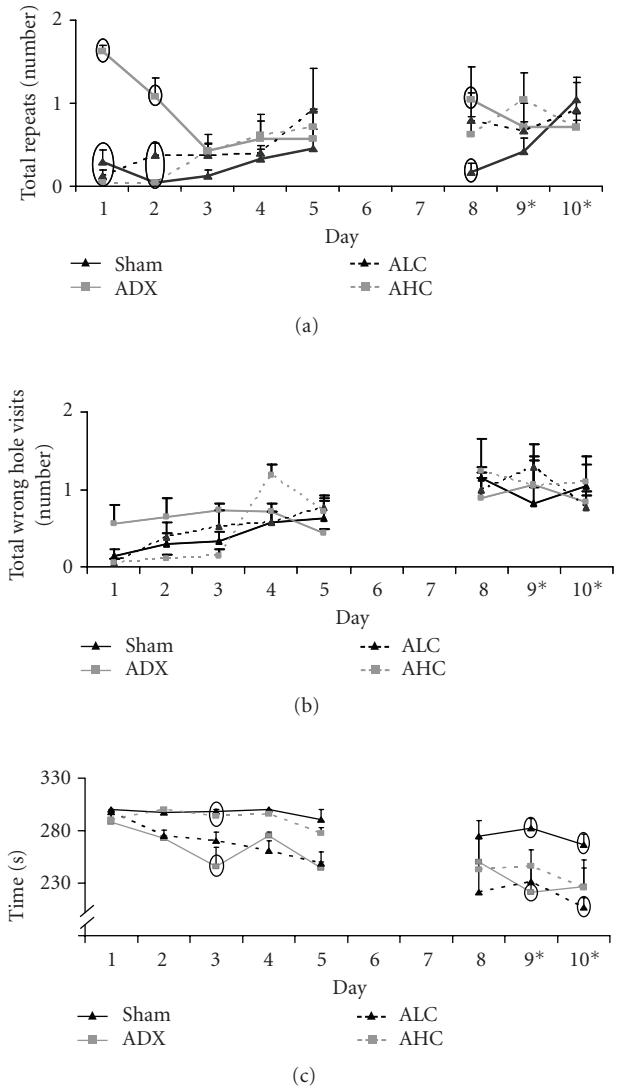

Figure 2: (a) Working memory expressed as number of holes revisited. (b) Reference memory expressed as visits to nonbaited holes. (c) Time to finish the task, that is, to obtain all three baits or 5 minutes, of sham (black line), ADX (grey line), ALC (striped black line), and AHC mice (striped grey line). Days 9 and 10 on the x-axis indicate removal of rings from all cylinders, while the bait remained in the same cylinders as before. Data present the mean of the three trials per day ± SEM. Ovals mark data points with significant differences $P < .05$ between groups within days.

3.1.3. Factor analysis

Principal component analysis (PCA) over all behavioral data resulted in the extraction of four factors (Table 2) which explain 81% of total variance. Factor 1 (41%) combines behavioral parameters that can be classified as anxiety, motivation, and good learning, Factor 2 (19%) represents directed exploration, behavioral reactivity, and working memory, Factor 3 (11%) represents general activity and Factor 4 (10%) includes behavioral parameters that can be classified as impaired learning.

One-way ANOVA between groups on factor loadings for Factor 1 (anxiety, motivation, good learning) revealed significant differences between sham mice compared to ADX, ALC, and AHC mice (F(3,279) 11.562, $P = .000$). Significant group differences were also found between ADX mice compared to sham, ALC, and AHC mice for Factor 3 (general activity; F(3,279) 8.362, $P = .000$).

Furthermore, when comparing the factor loadings over days, significant differences were found for Factor 1 between days 3 and 4 compared to days 9 and 10, (F(7,279) 4.460, $P = .000$). This indicates low anxiety, more motivation, and better learning at the end of testing in all groups. Factor 3 was significantly different between day 2 and days 1, 8, and 9 (F(7,279) 2.522, $P = .016$), which indicates that general activity was decreased at the end of testing.

3.2. Corticosterone and thymus weight

Plasma corticosterone and thymus weights are presented in Table 3. Both low and high corticosterone pellet groups, ALC and AHC, had higher plasma corticosterone concentrations on day 0 (F(3,31) 29.540, $P = .0001$) than the sham and ADX mice. On day 11 of the experiment, only AHC mice showed significantly increased corticosterone levels (F(3,31) 28.977, $P = .0001$), compared to sham, ADX, and ALC mice. Plasma corticosterone in sham and ADX mice remained at the same low basal morning level throughout the experiment, while corticosterone concentrations of ALC and AHC mice decreased in the course of the study (F(1,15) 7.835, $P = .014$ and F(1,15) 13.344, $P = .003$).

Thymus weights on day 11 supported the exposure to elevated corticosterone during the experiment with significantly lower thymus weights for ALC and AHC mice compared to sham and ADX mice (F(3,31) 22.332, $P = .000$). In fact, ADX mice had an enlarged thymus. ALC mice had a less shrunken thymus than AHC mice, indicating exposure to lower corticosterone concentrations than AHC. Body weight on day 11 was comparable between groups F(24,27) 1.731, $P = .187$.

4. DISCUSSION

Four groups of mice were generated by endocrine manipulation, resulting in different amounts of circulating corticosterone concentrations in the blood. Given the different affinities of the receptors for the hormone, we expect a differential MR/GR activation in these groups: (i) sham mice with an intact HPA axis, (ii) ADX mice with residual stable low corticosterone levels and thus continuous MR activation, (iii) ALC mice with moderate elevated circulating corticosterone concentrations allowing extensive MR and moderate GR activations, and (iv) AHC mice with a full MR and a substantial GR activation due to high circulating levels of corticosterone. We found emotional expressions and cognitive performance related to differential corticosteroid receptor activation. Continuous predominant MR activation directed emotional components indicative for less anxiety to the benefit of cognition, while continuous additional GR activation was associated with impaired learning.

Table 2: Principal component analysis over all data, with varimax rotation and Kaiser normalization. Behavioral parameters are represented as factor loading per factor. Factor loadings with equal value are positively correlated, while loadings with opposing values are negatively correlated. Loadings < 0.6 are not included in this table. Eleven of the seventeen measured parameters (Table 1) have communalities > 0.7 and are included in the factor analysis.

	Factor			
	1	2	3	4
	Anxiety, motivation, good learning	Directed exploration/behavioral reactivity, working memory	General activity	Impaired learning
Latency to eat bait	−0.887	—	—	—
Number of baits obtained	0.862	—	—	—
Latency to first hole visit	−0.792	—	—	—
Number of baited holes visited	0.781	—	—	—
Time on center board	0.678	—	—	—
Number of repeated hole visits	—	0.927	—	—
Number of hole visits	—	0.807	—	—
Time sitting	—	—	0.840	—
Number of rearings	—	—	−0.810	—
Number of nonbaited holes visited	—	—	—	0.911
Ratio of right hole visit/ % and wrong hole visits %	—	—	—	−0.723

Table 3: Plasma corticosterone, thymus, and body weight. Corticosterone was measured before the first day of testing (day 0) and 24 hours after the last testing day (day 11). Data are presented as mean ± SEM.

	Plasma corticosterone (ng/mL)		Thymus weight (mg)	Body weight (g)
Group	Day 0	Day 11	Day 11	Day 11
Sham	13.78 ± 2.37	17.96 ± 4.10	49.3 ± 0.9	25.1 ± 0.8
ADX	12.39 ± 1.50	15.24 ± 8.81	64.2 ± 2.5*	27.4 ± 0.7
ALC	88.67 ± 19.26*	33.18 ± 4.87	38.9 ± 0.5*	24.7 ± 0.7
AHC	168.00 ± 19.23*	88.63 ± 10.58*	21.2 ± 1.2*	25.3 ± 1.2

*$P < .05$ compared to all other groups.

4.1. Continuous predominant MR activation results in emotions that can be beneficial for learning

Mice with stable predominant MR activation (ADX) show increased directed exploration/ behavioral reactivity towards the cylinders (hole visits) and low anxiety during the first days of testing, that is, when the setting is novel. This corresponds to the observation that transgenic mice with low GR, and rats with ICV injection of GR antagonist express low-anxiety-related behavior [31, 32]. However, it contrasts previous findings that GR blockade by single infusion of RU38486 into the hippocampus has no anxiolytic effect in rats in the light/dark box [33]. Of course, the methods to achieve predominant MR activation differ in the history of inactivated GR, species, stressed state of the animals, and behavioral task. Also a differentiation between context-related behavioral reactivity and anxiety is not possible. However, the design of the present study allows to make this distinction. Factor analysis reveals that the variables time on center board (anxiety, motivation, good learning; Factor 1) and hole visits (directed exploration and behavioral reactivity; Factor 2) are not correlated. Thus, the general idea that mice which are more prone to go to the unprotected center area are likely to display more cylinder directed behavior is not supported. In contrast, anxiety is correlated with motivation (latency to first hole visit, latency eat bait): mice with a low anxiety approach the unprotected area faster.

Overall, low anxiety and high directed exploration/behavioral reactivity could be beneficial for the onset of learning, especially during the first days of testing. We observed an apparent fast onset of learning in these predominantly MR mice. High directed exploration towards the cylinders will

eventually result in finding all baits, without any necessary learning of the task. Indeed, mice of this group show an increase in working memory errors (revisits) after the two-day break without testing. GR is expected to promote the consolidation of MR-related adaptive behavior, leaving the lack of GR activation as the most likely explanation for the memory deficit. The results of the Berger study [34] can be interpreted the other way round: the lack of forebrain MR resulted in working memory deficits in the water maze task because a functional GR facilitated the consolidation of nonadaptive behavior. We conclude that the observed behavior of animals with differential MR and GR conditions will only be understood in relation to the contribution of both receptors.

4.2. For optimal cognitive performance, not only MR but also moderate GR activation is necessary

ALC mice with MR and moderate GR activations display low anxiety during the first days of testing, general low arousal, and fast learning. Corticosterone levels in the ALC mice were continuously elevated in the range of the circadian rise, thus it would not be expected to cause damage to neurons, downregulation of MR and GR, or alterations in neurotransmitters implied in cognitive impairments [35]. In fact, ALC mice with MR and moderate GR activations showed the best cognitive performance.

Part of this improved learning and memory ability could be explained by the emotional state of the mice. Like ADX mice, ALC mice have low anxiety (and arousal) during the first days of learning which is correlated with increased motivation and good learning. Supporting our argument is the most recent finding of Herrero, that rats with low anxiety showed faster spatial learning together with increased hippocampal MR; opposite results were found in high-anxiety rats [36]. Stronger MR availability and activation might underlie the fast onset of learning, while GR are responsible for the consolidation of this context-related information. [7, 17, 37, 38]. Therefore, it is not surprising that ALC mice with a moderately activated GR display improved or normal cognitive performance compared to ADX mice with little or no GR activation throughout testing. For optimal coordination of cognition and emotion, both MR and a moderate activation of GR are necessary [39, 40].

4.3. Substantial continuous GR activation in addition to MR activation are associated with high emotional arousal and impaired learning

As described by many others, chronic strong GR activation caused by, for example, severe stressors or pharmacological modulation of the HPA axis results in impaired learning and memory [41–43], reduced synaptic plasticity in the hippocampus [44], increased anxiety [45], and even depression-like symptomatology [38]. In patients suffering form depression or Cushing's disease, elevated levels of cortisol have been associated with poorer cognitive performance in verbal memory, working memory, and post-encoding tasks [46–48]. Furthermore, an association between cortisol level and increased fear perception has been found in patients suffering from recurring depression [49], which also indicates a modulatory role of glucocorticoids in emotional processes.

We find similar results for emotions and cognition: AHC mice with MR and continuous high GR activation have a slow onset of learning together with increased arousal and anxiety-like behaviors and suppression of directed exploration. It is not surprising that these mice display a slower onset of learning (opposite to low anxiety and fast learning as described above). At first glance, it seems surprising that when learning starts to occur, the magnitude of learning (Figure 2(c): time to finish task, slope of the learning curve) is the same in ALC and AHC mice. The change in corticosterone availability, due to the encapsulation of the pellet, is most likely responsible for the altered behavior. Corticosterone levels decreased over the days to concentrations in the "normal" range, that is, comparable to circadian peak secretion and the amount of corticosterone measured in ALC mice at the beginning of testing. Thus, in AHC mice we deal with memory impairments and high emotional arousal only during specific stages of learning, namely during the first days of testing that coincide with really high exposure to corticosterone.

4.4. The highly anxious sham-operated control group

We used sham-operated mice that have an intact HPA axis as control group. Unexpectedly, these mice were characterized as highly anxious and with little motivation, with high arousal and a slow onset and little progress of learning. Factor 1 was significantly different over time between sham and all other groups tested: low motivation and high anxiety throughout testing days. We got the impression that the behavioral setting remained anxiogenic to these mice. Lack of exploration of the centre board might also prevent learning basic rules, for example, that cylinders are baited with almonds. This and the possible role of a prolonged effect of surgery on the HPA system resulted in a follow-up experiment. We used three groups of mice ($n = 6$ per group): (1) sham-operated mice and (2) naïve, nonoperated mice received almonds in the homecage to familiarize with the bait, like the experimental groups, (3) naïve mice received almonds in the cylinders four times in the week before the modified hole board task. Sham and naïve mice without preexposure to the cylinders displayed similar high anxiety and slow learning as we saw before. However, after pretraining with baited cylinders anxiety decreased, motivation increased and learning improved (Figure 3).

Since surgery did not influence behavior on the modified hole board, incomplete recovery from the surgery is unlikely to affect performance. Using a somewhat different experimental design, comparably long times to finish the task have been reported for C57BL/6 mice (Ohl 2003; still 280 to 300 seconds after eight days of training). In contrast, prior familiarization to items of the test condition reduced anxiety-like behavior and increased motivation, which could (in part) increase cognitive performance like it was observed in ADX and ALC mice.

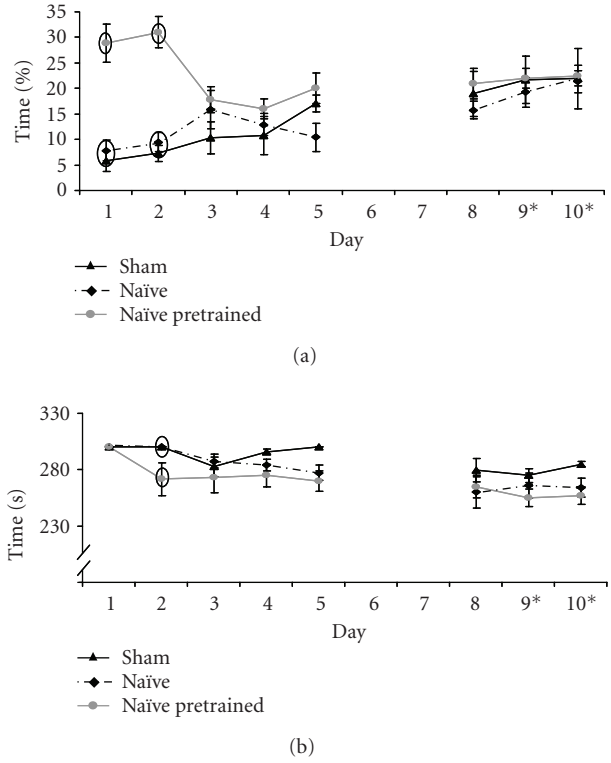

Figure 3: Examples of behavior of the mice during the followup experiment. (A) Percentage of time spent on center board. (B) Time to finish the task (5 minutes or finding all three baits) of sham (black line), naïve (striped black line), and naïve mice preexposed to a bait-containing cylinder in the homecage (grey line). Days 9 and 10 on the x-axis indicate removal of rings from all cylinders, while the bait remained in the same cylinders. Data present the mean of the three trials per day ± SEM. Ovals mark data points with significant differences: $P < .05$ between groups within days.

It is remarkable that mice without adrenals dysregulated HPA-axis activity and additional pellet implantation "did better" compared to the relative intact sham and naïve control groups. These findings even more underscore that (i) high anxiety and arousal have negative consequences for cognition while (ii) less anxiety, increased motivation, and goal-directed exploration have a positive influence on behavior (see also [36]). We consider the role of MR in the integration of sensory information and behavioral strategies central for reduced anxiety-related behavior.

4.5. Adrenalectomy: other hormones and anxiety

The adrenalectomy-induced deficit in corticosterone secretion results in the disinhibition of HPA activity, and thus enhanced release of corticotrophin-releasing hormone (CRH) and vasopressin (AVP) from the hypothalamus. Also the adrenal medulla as source of adrenaline is eliminated. CRH, AVP, and adrenaline, all might play a role in emotional expressions and cognitive performance [50] of ADX mice, with and without supplementary corticosterone.

Considering the function of the GR in the negative feedback, we may expect that ADX mice (predominant MR activation) and ALC mice (MR and moderate GR) have a deficient suppression of CRH and AVP activities [51, 52]. Mice with elevated levels of CRH that acts predominantly via CRH receptor 1 are expected to display increased anxiety. Mutant mice with a deficient CRH receptor 1 either by genetic deletion or pharmacological blockade are less anxious [53]. Clearly, CRH is involved in anxiety-related behavior. However in the present study, ADX and ALC mice show low anxiety-related behavior, while AHC mice (predominant GR activation) are highly anxious. These findings do not support a role of hypothalamus-related CRH activity in anxiety behavior in the present study. The same argument holds true for AVP.

In response to stress, noradrenalin release increases. This is thought to contribute to the anxiogenic effects of stress [50, 54], in which the amygdala plays an important role [55]. AHC and sham mice showed the strongest arousal (defecation) and were characterized as most anxious: a participation of catecholamines in these responses cannot be excluded. Furthermore, changes in metabolism and food intake have to be considered. Although food was present *ad libitum* throughout the experiment and body weight did not differ between the groups, motivation to go for the almond-bait might have been increased in ADX and ALC mice. Factor analysis also underlines the role of motivation in relation to anxiety for the performance.

4.6. Less directed exploration: is this anxiety?

Anxiety-related behavior in rodents is generally deduced from the avoidance of an open, bright, and unprotected area. However, tasks characteristics largely influence behavior. For example, rats that are specifically selected for their avoidance of open arms of the elevated plus maze, and thus classified as high anxiety rats, do not avoid the center (open) area of a hole board task [56]. Complexity and duration of the task, as well as motivational aspects might overcome state anxiety. Directed exploration or behavioral reactivity is expressed by approach to certain stimuli, for example, the number of visits to a specific location in the testing area. These opposing behaviors are both related to locomotor activity. Does directed exploration rely on reduced anxiety? In the present study, animals with low directed exploration would spend little time near the cylinders on the centre board. The interpretation of this behavior could be high anxiety. Although it is likely that anxiety interacts with directed exploration, this does not necessarily has to be the case. It could be that our interpretation of high anxiety is characteristic for a more passive exploration strategy [57, 58] without a dominant role for anxiety-related behavior. The setting of our task and subsequent factorial analysis allowed us to differentiate anxiety-like behavior from directed exploration: they did not coincide into one factor, indicating no correlation between the two.

5. CONCLUSION

Anxiety and motivation are important factors for the onset of learning, a process in which MR and GR and their coordinated activation play a crucial role. Continuous predominant

MR activation appears to be beneficial for the emotional state, resulting in low anxiety, high motivation, and high directed exploration and behavioral reactivity, but does not result in better learning and memory. Additional moderate GR activation also results in low anxiety and high motivation, with the advantage of improved cognition expressed as a decrease in working memory errors. In contrast, MR with additional substantial GR activation results in a slow onset of learning together with high anxiety, showing similarities with patients suffering from depression and Cushing's disease. We conclude that optimal performance is bound to continuous MR activation together with moderate GR activation. Further increase in corticosterone, and therefore substantial GR activation, will increase emotional arousal with impairing effects for learning and memory.

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Research Article

Anxiety in Mice: A Principal Component Analysis Study

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Two principal component analyses of anxiety were undertaken investigating two strains of mice (ABP/Le and C57BL/6ByJ) in two different experiments, both classical tests for assessing anxiety in rodents. The elevated plus-maze and staircase were used for the first experiment, and a free exploratory paradigm and light-dark discrimination were used for the second. The components in the analyses produced definitions of four fundamental behavior patterns: novelty-induced anxiety, general activity, exploratory behavior, and decision making. We also noted that the anxious phenotype was determined by both strain and experimental procedure. The relationship between behavior patterns and the use of specific tests plus links with the genetic background are discussed.

1. INTRODUCTION

Most behavioral procedures for studying the pharmacology of anxiety use models involving nonconditioned behavioral responses that are usually based on novelty-induced variations in exploratory activity. Ethological observations show that while rodents naturally tend to explore a novel environment, open fields are aversive and counter normal behavioral responses [1–3]. The light-dark discrimination and elevated plus-maze tasks are used for the same purpose. In these tasks, pharmacological studies have shown that benzodiazepines (BZ) or 5-HT_{1A} agonists ligands have anxiolytic-like effects on mice, increasing time spent in the lit box and exploring the open arms in the elevated plus-maze [2, 4, 5], while BZ antagonists or inverse agonists and anxiogenic 5-HT drugs decreased both of these behavioral measurements [6–9]. In humans, two main types of anxiety which are well identified have been reported: "state" and "trait" anxieties [10]. "State anxiety" is anxiety that a subject experiences at a particular moment in time and which is increased in the presence of an anxiogenic stimulus. In contrast, "trait anxiety" does not vary from moment to moment and is considered to be an "enduring" feature in an individual [11–13]. In rodents, "state anxiety" has been extensively studied but "trait anxiety" is less well known. Belzung and Griebel proposed the light-dark task and the elevated plus-maze device as the most appropriate for assessing "state anxiety," while the free-exploratory paradigm can be used for "trait anxiety" [4, 14]. Unlike most behavioral models using spontaneous aversion (unconditioned fear) to a new environment, the free-exploratory paradigm does not force the animal to explore. After 24-hour exposure to the two compartments (familiar/novel) of the apparatus, the animal can choose to explore familiar or novel areas. Thus, "trait anxiety" is associated with approach responses to the unfamiliar (novel) compartment being followed by avoidance reactions, while "state anxiety" is associated with neophobia to the new environment and/or avoidance reactions to an unprotected compartment when animals are forced to explore it.

To gain a better understanding as to whether specific behavioral variables can be related to "trait" or "state" anxiety, the aim of the present study was first to record behavioral patterns in four specific behavioral tests assessing "trait anxiety" (free-exploratory paradigm) and "state anxiety" (staircase, elevated plus-maze, and light-dark discrimination) in mice, and to carry out principal component analyses of the data, this being a commonly used method [15–21]. Second, many animal studies using inbred strains have reported

strain differences in anxiety-related behavior, suggesting that genetic factors could be associated with anxious phenotypes [22–27]. We recently reported behavioral differences in the open-field and in the light-dark devices studying two inbred strains of mice: C57BL/6ByJ (B6) and ABP/Le (ABP), observing that ABP was anxious compared to B6 [28, 29]. B6 mice have often been used by scientists in behavioral and pharmacological studies, but there is insufficient knowledge of the ABP strain [30]. A study of anxiety-related behavior by principal component analysis was therefore undertaken on the two strains to provide a more accurate definition of the differential components and to test the hypothesis of genetic determinism for anxiety.

2. MATERIALS AND METHODS

2.1. Animals

The animals were reared in groups of 5 or 6 male and female mice from ABP/Le and C57BL/6ByJ parent strains bred in the laboratory in Paris. They were reared under standard conditions: temperature 23.5 ± 0.5°C, photoperiod 12 h/12 h with lights on between 8 am and 8 pm; food (IU UAR), tap water ad libitum, and dust-free sawdust bedding. The animals were given a two-week recovery period after being transported from Paris to Strasbourg.

2.2. Behavioral testing

At the beginning of the experiment, the animals were 10-week old ±2 weeks when tested and were test-naïve. They were first tested in the Paris laboratory, and two weeks later in the Strasbourg laboratory. In the first experiment (Paris), 94 mice were tested: 50 ABP mice (24 males and 26 females) and 44 B6/ByJ (29 males and 15 females). In the second experiment (Strasbourg), 81 mice (from a total of 94 sent to Strasbourg) were tested: 47 ABP (21 males and 26 females) and 34 B6/ByJ (24 males and 10 females). The experiments took place in a room outside the housing room between 1 pm and 5 pm. Data were recorded using a handheld computer (Psion Organiser). Animals were kept on a 12 h/12 h light/dark cycle with lights on at 1 am so that we could observe the animals under dim red light during their active period between 2 pm and 5 pm. There was a minimum interval of one week between experiments.

All experiments complied with the ethical guidelines laid down by the French Ministry of Agriculture and with the European Community Council Directive of November 24, 1986 (86/609/EEC).

3. EXPERIMENT 1

3.1. Elevated plus-maze

The apparatus was a polyvinylchloride plus-maze with two lit open arms (27 × 5 cm) and two closed arms (27 × 5 × 15 cm). The two closed arms were darkened with cardboard to block out the light. The arms radiated from a central platform (5 × 5 cm) [31]. The apparatus was mounted on a base which raised the arms to a height of 38.5 cm above the floor. To initiate the test session, the mouse was placed on the central platform, facing an open arm, and its behavior was videotaped for 5 minutes. The mouse was considered to be on the central platform whenever two paws were on it, and in one of the arms when all four paws were inside.

Parameters recorded were time spent on open arms (TOA) for anxiety-related behavior, the number of entries into open arms (OAE) and closed arms (CAE) for locomotor activity, the time spent in the central area (TCA) and stretched-attend posture (SAP) for avoidance behavior, and unprotected head dipping (HD) (i.e., the animal extending its head below the open arm) for exploration activity [32, 33].

3.2. Staircase

The device consisted of a white wooden staircase similar to the one used by Simiand et al. [34]. The staircase was enclosed between vertical walls and had 5 identical steps 2.5 cm high, 10 cm wide, and 7.5 cm deep. The height of the walls remained constant along the length of the staircase. Each mouse was placed individually at the bottom of the staircase for a 5-minute observation period. The number of steps climbed (STEPS) and the number of rearings (R) were recorded as anxiety indexes [35].

4. EXPERIMENT 2

4.1. Light-dark discrimination

The apparatus consisted of two polyvinylchloride boxes (20 × 20 × 14 cm) covered with Plexiglas [36]. One box was dark and covered with cardboard and the second box had a 100-watt bulb suspended 25 cm above it as the only source of light. An opaque tunnel (5 × 7 × 10 cm) ran between the two boxes. The apparatus was placed on a stand in the mouse room. The observer always sat in the same position, next to the apparatus. Each mouse was placed individually in the darkened box and recordings were made over a 5-minute period, counting the time spent in the lit box (TLB) and the number of transitions (TRANS) across the tunnel. A mouse with all four paws in the destination box was said to have made a transition.

4.2. Free-exploratory paradigm (Hughes Box)

The apparatus consisted of a polyvinylchloride box (30 × 20 × 20 cm) covered with Plexiglas and subdivided into six identical square exploration units, all interconnected by small doors [4]. A removable partition could be used to divide the apparatus in half lengthwise. Approximately 24 hours before testing, each subject was placed in one half of the apparatus, with the temporary partition in place, to be familiarized with it. The floor in this half was covered with sawdust and the animal was given unlimited access to food and water. The next day, the mouse was exposed to both the familiar and unfamiliar compartments when the temporary partition was

Table 1: Rotated component patterns for experiment 1 (plus-maze and staircase). TOA = time spent in open arms; OAE = number of entries to open arms; CAE = number of entries to closed arms; TCA = time spent on the central area; SAP = stretched-attend posture; HD = unprotected head dipping (HD); steps = number of steps climbed; rearing = number of rearings. Only component patterns above 0.40 were recorded.

Variables	C 1	C 2	C 3
TOA	−0.45	—	—
OAE	—	—	0.86
CAE	—	—	0.83
TCA	0.80	—	—
SAP	0.67	—	—
HD	−0.75	—	—
Steps	—	0.91	—
Rearing	—	0.62	—

removed, without removing the animal itself from the box. The subject was then observed under red light for 10 minutes. The parameters recorded were the number of units entered (locomotion) in the novel area (LOCN), the time spent in the novel side (TIME), the number of units entered in the familiar environment (LOCF), the number of rearings in the novel area (RN), the number of rearings in the familiar environment (RF), and the number of approach responses to the unfamiliar compartment followed by avoidance reactions (attempts, AT).

4.3. Component analysis

Principal component analysis and varimax rotation were conducted for each of the two experiments. An eigenvalue greater than 1 was set as the criterion for selecting components.

4.4. Statistical methods

The procedure used to compare the groups of mice was a multivariate analysis of variance with "strain" and "gender" as the main components, plus their interactions, followed by two-way ANOVAs for each component identified in the factorial analyses. Partial comparisons were done using the adjusted means. SAS was used for all the statistical analyses (factor and GLM).

5. RESULTS

5.1. Experiment 1 ($N = 94$)

The principal component analysis produced three factors with eigenvalues greater than 1. These three factors explain 67.9% of the variance in the correlation matrix and varimax rotation was performed on them. The rotated factor patterns are presented in Table 1. Calculations were made giving each mouse a score for each component.

Component 1 (27.6% of variance) was mainly loaded by time spent in the center (TCA = 0.80), stretched-attend posture (SAP = 0.67), head dipping (HD = −0.75) and time spent in open arms (TOA = −0.45).

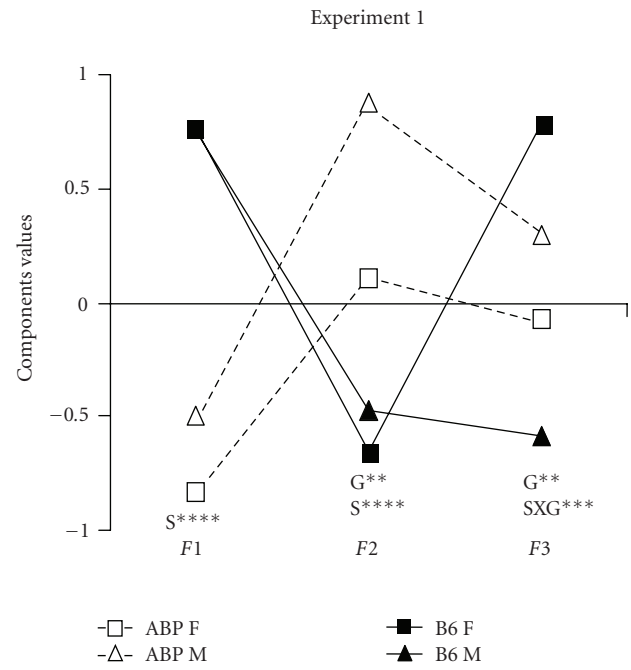

Figure 1: Mean scores by strain and gender of component values, S = strain effect; G = gender effect; SXG = strain-gender interaction, ** = $P < .01$; **** = $P < .0001$.

Component 2 (21.6% of variance) was explained by steps climbed (STEPS = 0.91) and rearings ($R = 0.62$) in the staircase test.

Component 3 (18.7% of variance) was loaded by the number of entries to open arms (OAE = 0.86) and closed arms (CAE = 0.83) in the elevated plus-maze.

MANOVA analysis of the scores for components 1, 2, and 3 from the principal component analysis, considered as dependent variables, showed significant effects for strain, gender, and Strain X Gender ($F_{(3,88)} = 102.9$, $P < .0001$; $F = 4.31$, $P < .007$; $F = 8.4$, $P < .0001$, resp.).

Profile analysis showed a level effect (Figure 1) for strain X gender ($F = 13.3$, $P < .0002$). The parallelism effect was significant for strain, gender, and strain X gender (Wilk's lambdas = 0.22, $P < .0001$; $\Lambda = 0.87$, $P < .002$; $\Lambda = 0.90$, $P < .01$, resp.).

ANOVA procedures revealed a strain effect for components 1 and 2 ($F_{(1,90)} = 90.92$, $P < .0001$; $F = 36.54$, $P < .0001$). Gender was significant for components 2 and 3 ($F = 7.46$, $P < .008$; $F = 6.98$, $P < 0.01$). Strain X gender was significant only for component 3 ($F = 20.72$, $P < .0001$).

5.2. Experiment 2 ($N = 81$)

The principal component analysis produced 4 components with eigenvalues greater than 1. These four components explain 76.9% of the variance in the correlation matrix and varimax rotation was performed on them. The rotated factor

Table 2: Rotated component patterns for experiment 2 (light-dark discrimination and free-exploratory paradigm). TLB = time spent in lit box; Trans = number of transitions; LOCN = number of units entered (locomotion) in the novel area; time = time spent in the novel side; LOCF = number of units entered in the familiar environment; RN = number of rearings in the novel area; RF = the number of rearings in the familiar environment; AT = attempts, taht is, number of approach responses towards the unfamiliar compartment followed by avoidance reactions. Only component patterns above 0.40 were recorded.

Variables	C 1 21.2%	C 2 19.0%	C 3 18.8%	C 4 17.9%
TLB	—	—	—	0.81
Trans	—	—	—	0.84
LOCN	0.82	—	—	—
TIME	0.45	−0.70	−0.41	—
LOCF	—	—	0.91	—
RN	0.88	—	—	—
RF	—	—	0.62	—
AT	—	0.83	—	—

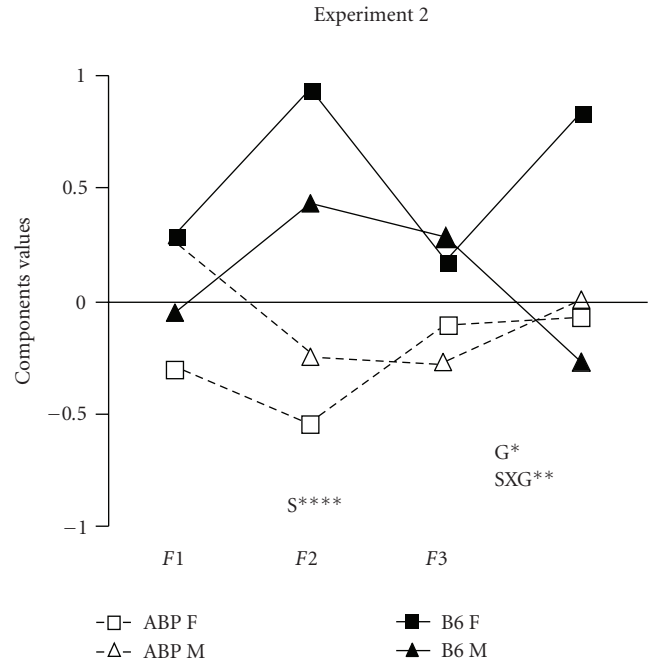

Figure 2: Mean scores by strain and gender of component values, S = strain effect; G = gender effect; SXG = strain-gender interaction, * = $P < .04$; ** = $P < .01$; **** = $P < .0001$.

patterns are presented in Table 2. Calculations were made giving each mouse a score for each component.

Component 1 explained 21.2% of variance. The number of locomotion events (LOCN = 0.82) and rearings (RN = 0.88) in the novel side mainly loaded this factor; time spent in the novel side (TIME = 0.45) also loaded the factor.

Component 2 explained 19.0% of variance and was loaded by the number of avoidance reactions to unfamiliarity (AT = 0.83) and by time spent in the novel area (TIME = −0.70).

Component 3 explained 18.8% of variance and was mainly loaded by rearings (RF = 0.62), locomotion in the familiar area (LOCF = 0.91), and time spent in the novel area (TIME = −0.41).

Component 4 explained 17.9% of total variance and was loaded by the number of transitions (TRANS = 0.84) and time spent in the lit box of the light-dark apparatus (TLB = 0.81).

MANOVA analysis of the scores from the principal component analysis (components 1 to 4), considered as dependent variables, showed a significant strain effect ($F_{(4,74)} = 9.38$, $P < .0001$). The strain X gender effect was also significant ($F = 4.03$, $P < 0.005$).

A profile analysis (Figure 2) showed a level effect for strain ($F_{(1,77)} = 22.10$, $P < .001$) and for strain X gender ($F = 9.87$, $P < .002$). The parallelism effect was significant for strain (Wilk's lambda = 0.088, $P < .02$).

ANOVA procedures showed only a strain effect for component 2 ($F_{(1,77)} = 28.19$, $P < .0001$) and tended towards significance for component 3 ($P < .06$). Gender was significant for component 4 ($F = 4.92$, $P < .03$). Strain X gender was mainly significant for component 4 ($F = 6.72$, $P < .01$). For components 1 and 2, strain X gender tended towards significance, ($F = 3.84$, $P < .06$; $F = 3.74$, $P < .06$).

6. DISCUSSION

It is commonly known that rodents, when confronted with a novel environment, either explore it or try to escape; many behavioral procedures therefore use unconditioned responses to measure anxiety [30]. As several authors have proposed the distinction between "trait anxiety" and "state anxiety" [4, 13], a principal component analysis was performed on the data to set behavioral parameters related to each of the two forms of anxiety, and specifically to distinguish anxious responses from exploratory and locomotor activities. The elevated plus-maze, the light-dark choice procedure, and the staircase test were assumed to measure "state anxiety," while the free-exploratory paradigm was used to assess "trait anxiety."

Analyzing data from the staircase and elevated plus-maze procedures (experiment 1), a 3-component structure explained 70% of total variation. After rotation, time spent in the center (TCA) and stretched-attend posture (SAP) were positively loaded on component 1, while time in the open arms (TOA) and head dips (HD) negatively loaded on this factor. Component 2 was defined by rearings (R) and climbed steps (STEPS) in the staircase test. The number of entries into the open (OAE) and closed arms (CAE) of the plus-maze defined component 3.

In the second experiment, a four-component model explained approximately 80% of total variation for the data observed in the light-dark choice and in the free-exploratory paradigm. The variables contributing to components 1 to 3 were all recorded in the free-exploratory paradigm, while the variables of component 4 were all in the light-dark situation.

The factors that mainly loaded on components 1, 2, and 3 were, respectively, locomotion (LOCN) and rearings (RN) in the unfamiliar compartment, then the number of attempts (AT) and the time spent in the unfamiliar compartment (TIME), and last, locomotion (LOCF) and rearings (RF) in the familiar area. The number of transitions between the lit and dark boxes (TRANS) and the time spent in the lit box (TLB) defined component 4.

Overall, the component analyses suggest the following.

(1) The light-dark procedure and the staircase produce a different set of responses as behavioral variables measured in these procedures specifically loaded on their own component (component 4, experiment 2; and component 2, experiment 1). It may be deduced that TRANS and TLB in the light-dark task and STEPS and rearings in the staircase task can be considered as behavioral indexes that are independent from the other parameters.

(2) Since the number of entries into both open (OAE) and closed (CAE) arms of the plus-maze model coincided in the same component (component 3, experiment 1), these variables may be related to locomotion and may provide a general activity index.

(3) Exploratory behavior was estimated in different ways. It was noted that the exploratory response loaded on two separate components depending on whether the exploration was in familiar or unfamiliar compartments of the free-exploratory paradigm. LOCN and RN (component 1, experiment 2) expressed exploration in the novel area, while LOCF and RF (component 3, experiment 2) expressed exploration in the familiar area. Both correlated negatively to time spent in the unfamiliar environment (TIME).

(4) TCA and SAP, which were inversely associated with TOA and HD (component 1, experiment 1), may reflect "decision-making behavior" when deciding to enter the open arms of the plus-maze. The more time the animal spent in the centre, the less it explored the open arms. We hypothesized that avoidance to explore may indicate anxiogenic-like effects in the plus-maze paradigm, as with AT and TIME behavior parameters (component 2, experiment 2) in the free-exploratory paradigm. As these behavior patterns loaded on different components, they can be used to define different kinds of anxiety.

The time spent in the lit box and the number of transitions between the two boxes in the light-dark model, the time spent in the open arms in the elevated plus-maze, and the time spent in the novel side of the free exploration model are usually considered as a measurement of anxiety-related behavior: the more time an animal spends in the lit box and in the open arms, the less anxious it is [4, 30, 37]. Very few studies have reported data on the staircase test as a measurement of anxiety [34, 38, 39]. The authors of such papers, on rats, have suggested that the number of steps climbed may be a locomotor component index, and that rearings relate to anxiety. A recent ethopharmacological study reported an increase in both steps climbed and rearings by BALB/cBy mice given diazepam, suggesting that mice climbing the greatest number of steps and recording that the most rearings are less anxious [35].

Overall, our data tally with the literature and show that the number of transitions between lit and dark boxes is not linked to other locomotion variables, confirming that the parameter is not related to motor activation, but rather to a particular emotional state [2, 40, 41]. Although the light-dark choice situation measures "state anxiety," our data suggest that the test also reveals a type of anxiety different from that measured by the plus-maze or the staircase procedures.

Previous plus-maze studies have suggested that open-arm entries and unprotected head dippings are the best indicators of anxiety. Total entries into closed and open arms were associated with locomotion, while total head dippings were associated with exploration, and the percentage of time spent in the center and stretched-attempt posture were associated with avoidance to explore [2, 32, 42]. Our results concur with the findings of these authors and confirm that exploration-related behaviors and locomotion loaded on separate components [31, 43]. Our study also suggests that exploration/novelty avoidance behavior can be a relevant index to measure anxiety. The time spent in open arms was a function of the time spent in the center, and the animals appeared to use the central area to "make decisions," confirming the link between the central area and novelty avoidance. Finally, these data show the four behavioral procedures used in the study to be a means of identifying different responses for coping with novelty-induced anxiety. In the staircase and light-dark choice procedures, we can distinguish specific behavioral phenomena which may be defined as parameters for "state anxiety," while general locomotion and exploration are defined in the plus-maze apparatus and the free-exploratory paradigm, respectively. Two other anxiety-related behavior patterns can be identified with these two procedures: "state anxiety" may be assessed through so-called "decision-making variables" in the plus-maze, and "trait anxiety" can be seen through "avoidance variables" in the free-exploratory paradigm. These data confirm previous studies showing that animal behavior recorded in these tests did not reflect the same emotional *status* [4, 11, 41, 44, 45]. The response patterns in both the free-exploration and plus-maze models offer potential for studying the effects of anxiogenic/anxiolytic drugs and could be included in pharmacological studies.

Many studies have pointed to great genetic variability in anxiety in different strains of mice [41, 46–49], suggesting that genetic background may modulate the biological processes involved in the physiopathology of disease etiology. We previously reported strain differences in the open-field and light-dark tests observing two strains of inbred mice, ABP/Le and C57BL/6ByJ: the ABP strain being described as more reactive than B6 [28, 29]. To further characterize and compare the behavior patterns of the two strains, and after a factorial analysis applied to data from the four experimental behavioral environments, we compared them, performing a profile analysis by a two-way ANOVA (Figures 1 and 2). We found a significant strain X gender interaction in both experiments for components 3 (experiment 1) and 4 (experiment 2), but since B6 females were different from all the others ($P < .0001$, for both experiments), the assumption was that the effect

was only found with this population. The gender effect observed in components 3 (experiment 1) and 4 (experiment 2) may also be solely due to the female B6 group. However, the strain and gender effects observed in component 2 (experiment 1) specifically discriminated both strain and sex influences, and could be associated with differential behavioral patterns in the staircase test. Strain differences were also observed for components 1 (experiment 1) and 2 (experiment 2) and it was argued that they could be used to distinguish "state anxiety" from "trait anxiety." Moreover, we noted differential profiles in strains for behavior and procedure (Figures 1 and 2). ABP was "higher" than B6 in the staircase test, but "lower" than B6 in the plus-maze and free-exploratory paradigm, suggesting different strain strategies in response to novelty.

To sum up, strain-related behavior patterns were found to be dependent on the behavioral situation and the genetic background. ABP strain could generally be described as more reactive than B6 in the staircase, and less reactive in both the free-exploration paradigm and the plus-maze test. The differences observed in "avoidance behavior" in free-exploration and "decision making" in the plus-maze models might reflect differential adaptive strategies when the animals are confronted with a conflict procedure, that is, having to choose between exploring a novel environment or staying in a protected area. The relationship therefore between these two behavioral profiles in the two experimental procedures could be further investigated by pharmacological and ethological studies with a view to gaining a better understanding of these behavioral "markers" for anxiety.

Many behavioral and pharmacological studies have used the B6 strain to measure anxiety and/or differential sensitivity to anxiolytic/anxiogenic drugs [50–53]. The B6 strain has been reported as not being "anxious" [48, 54, 55] and is more suitable for investigating the actions of anxiogenic drugs [36, 56, 57]. Very few authors have published data on ABP, the strain identified as being more "anxious" and more sensitive to convulsant drugs when compared to B6 [58, 59]. We can confirm that ABP mice explored less in the elevated plus-maze and more in the staircase device (experiment 1). They also recorded less "avoidance" behavior (experiment 2) than B6, suggesting that anxiogenic or anxiolytic *status* was dependent on the environment. The data are complex but tally with other data recorded in our and other laboratories and would suggest that the genetic basis for complex behavior is modulated by the genetic background, with the genotype being expressed in quite different ways according to the environment [60–63]. When testing drugs used to treat anxiety, the ABP strain may be more appropriate with experiments in the plus-maze, while B6 might be used in the staircase test for the same purpose. These variations also suggest that the anxious phenotype mainly depends on the interaction between genetic background and the experimental environment. It can be deduced that the choice of both the behavioral procedure and the strain is of crucial importance when testing anxiolytic and/or anxiogenic drugs. The present data could thus provide a useful guide for the pharmacological study of anxiety-related behavioral phenomena.

7. CONCLUSION

The present report is a principal component analysis study applied to two different genetic backgrounds and four behavioral paradigms known to evaluate novelty-induced anxiety in mice. We found that anxiety could be seen as four components: novelty-induced anxiety, general activity, exploratory behavior, and decision making. Of the different procedures available to assess anxiety-related behaviour, the staircase and light-dark test provide specific behavioral models for specific emotional states. Our data obtained studying two selected strains support the hypothesis that an anxious phenotype is mainly determined by the interaction between the genetic background and the experimental environment. The choice of the strain to investigate will depend on the environmental/experimental situation best suited to the requirements of the pharmacological study of anxiety-related behavior.

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Clinical Study

A Spiderless Arachnophobia Therapy: Comparison between Placebo and Treatment Groups and Six-Month Follow-Up Study

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We describe a new arachnophobia therapy that is specially suited for those individuals with severe arachnophobia who are reluctant to undergo direct or even virtual exposure treatments. In this therapy, patients attend a computer presentation of images that, while not being spiders, have a subset of the characteristics of spiders. The Atomium of Brussels is an example of such an image. The treatment group ($n = 13$) exhibited a significant improvement (time × group interaction: $P = .0026$) when compared to the placebo group ($n = 12$) in a repeated measures multivariate ANOVA. A k-means clustering algorithm revealed that, after 4 weeks of treatment, 42% of the patients moved from the arachnophobic to the nonarachnophobic cluster. Six months after concluding the treatment, a follow-up study showed a substantial consolidation of the recovery process where 92% of the arachnophobic patients moved to the nonarachnophobic cluster.

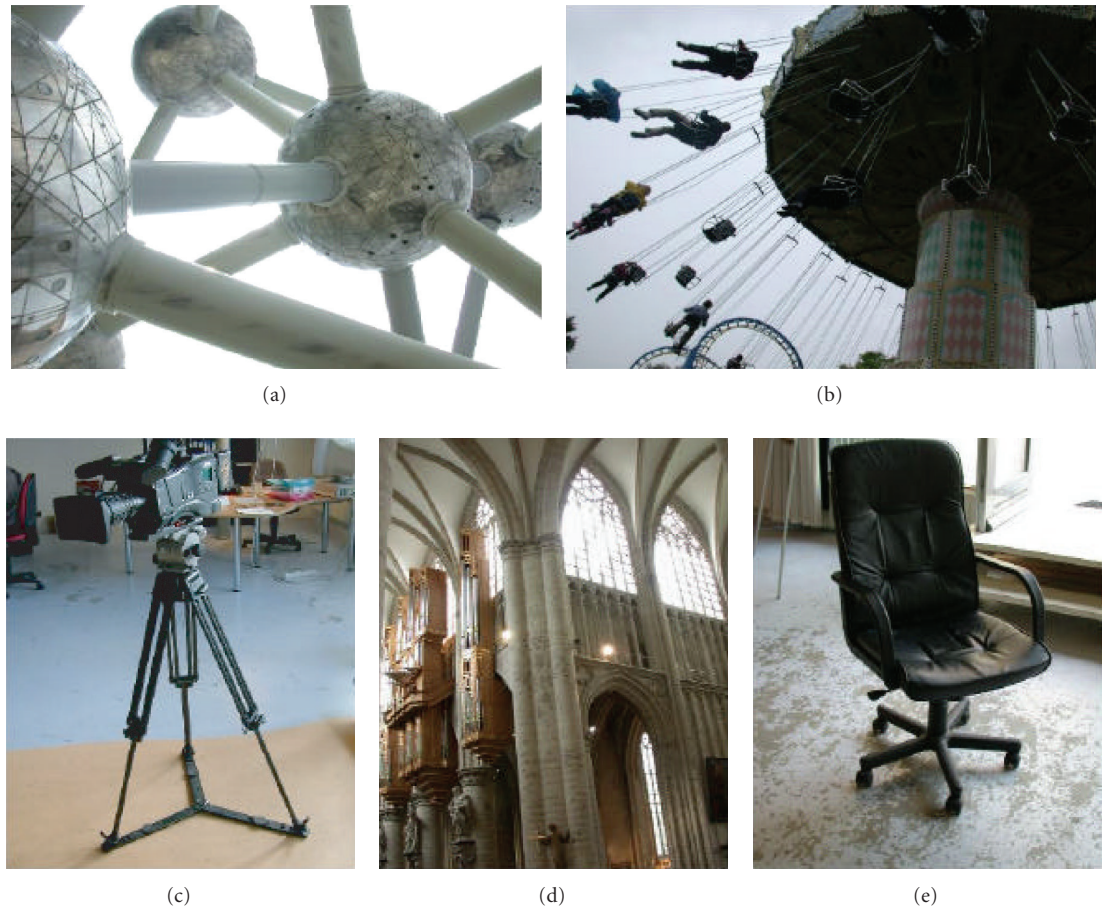
1. INTRODUCTION

According to the DSM-IV manual (American Psychiatric Association [1]), specific phobias are anxiety disorders that are characterized by an excessive, unreasonable, and persistent fear that is manifested by the presence or expectation of an object or feared situation (phobic situation). The manual states that 9% of the population suffers from specific phobias.

Spider phobia is one of the most common specific phobias (Bourdon et al. [2]). Arachnophobic individuals develop an avoidance behavior for all contexts related to the animal (APA [1]). Many patients are so afraid of being confronted by the phobic object that they refuse to undergo any kind of therapy (Marks [3]).

Existing therapies range from those that confront the patient with the real spider, such as "in vivo" exposure therapy (Ost [4]), to those that avoid this confrontation by requiring the patient to imagine situations involving spiders (Hecker [5]). In between, several therapies try to minimize the anxiety of the direct exposure by using computer simulations in which either the patient himself (Garcia-Palacios et al. [6, 7]) or a "virtual" person guided by the patient (Gilroy et al. [8, 9]) interacts with a "virtual" spider.

The treatment proposed here (SLAT: spiderless arachnophobia therapy) does not use any spider, neither real nor virtual or imaginary. It is specifically oriented to those patients with severe arachnophobia that would not undergo any kind of therapy involving a spider. This treatment makes use of the idea that aversive information does not need to be perceived consciously to trigger an emotional response. Nonconscious processing mechanisms of emotionally relevant stimuli are sufficient to activate the autonomic components of a phobic reaction (Öhman and Soares [10, 11]). From the neural point of view, fearful information does not need to reach cortical levels to generate the typical fear response. Individuals with bilateral destruction of the visual cortices exhibit amygdala responses to emotional faces even when brain damage is recent so that cortical networks have had too short time to reorganize (Pegna et al. [12]). In this case, the amygdala



(a) (b) (c) (d) (e)

Figure 1: Some "SLAT" images used in the treatment.

activation requires mediation by thalamic (pulvinar nucleus) or tectal (superior colliculus) areas (Morris et al. [13]; Pegna et al. [12]).

The thalamus and amygdala are, according to LeDoux et al., responsible for recognizing fearful stimuli and triggering subsequent autonomic responses such as increased heart rate, respiration, and sweating (LeDoux [14]; Doyére et al. [15]). According to these authors, when an aversive stimulus arrives at the thalamus, it passes rough, almost archetypal information, directly to the amygdala, producing a rapid response to the possible danger.

The therapy proposed in this paper makes use of these ideas by presenting to the patient a collection of images that contain a reduced subset of the features of a spider. Figure 1 shows some of these images: the Atomium of Brussels in which the spheres resembles the spider's body, a carousel in which the seats hang like the preys of a spider, a tripod whose legs are articulated like spider's legs, and so forth. These images, sharing a limited subset of features of a spider, were called SLAT images. After a preliminary presentation, only the images in which the features of the spider appear in a subtler way are kept in the final presentation. The images that evoke spider-related feelings above a certain degree are discarded from the final therapeutic set (see Section 2.3.2). To avoid the patient's thoughts related to spiders while seeing the treatment presentation, the patient is given a question that should be answered at the end of the run, like "In how many images there is a rounded object?"

2. METHODS

2.1. Participants

Patients were recruited by means of advertisements in several newspapers and on television. Of the 160 volunteers that made contact with us, 36 with symptoms of severe arachnophobia that were reluctant to undergo other types of treatments were personally interviewed. They were then included in the study if they (1) met DSM-IV criteria of specific phobia (APA [1]) assessed by Structured Clinical Interview for DSM-IV Axis I Disorders (SCID), (2) had been phobic for at least ten years,[1] (3) did not have any neurological or psychiatric problems, and (4) were classified as arachnophobes according to a k-means multivariate analysis.

[1] We have arbitrarily chosen this duration as an additional criterion to recruit only severe arachnophobic subjects.

Four volunteers were excluded because of the three first criteria. A further 6 were excluded because they had difficulty in coming on a regular basis to the university to participate in the experiments.

Regarding the last criterion, the k-means multivariate analysis was conducted using as inputs the five measurements obtained from a behavioral avoidance test (BAT) and from the fear of spider questionaire (FSQ); see Section 3. These instruments were applied to the remaining 26 volunteers, and to 29 nonphobic control subjects recruited among the personnel and students of São Paulo University, so that the algorithm could establish two well-defined clusters: the arachnophobic and the nonarachnophobic cluster. After applying the k-means multivariate analysis, the 29 control subjects were classified as nonphobic. One of the 26 volunteers was characterized as nonphobic by the k-means analysis and was eliminated from the study leaving 25 arachnophobic patients. The mean age and standard deviation of the arachnophobic patients and controls were 31.3 ± 7.4 and 32.6 ± 8.2 years, respectively. The duration of phobia among the patients was 23.0 ± 8.6 years. The five measurements (see the following section) that were used as inputs in the k-means algorithm were (a) the distance tolerated to a real tarantula in a BAT; (b) the distance tolerated to a photo of a tarantula in a BAT; (c) the subjective percentage of anxiety according to the subjective units of disconfort scale (SUDS), using a real tarantula; (d) the percentage of anxiety with a photo of a spider; (e) the numerical result of the FSQ test.

The chief advantage of the k-means algorithm is that it uses a multivariate approach (here, 5 measurements) in order to separate phobic from nonphobic subjects. This procedure is more robust than adopting only one measurement, such as the BAT or the result of the FSQ, as conventionally used for separating phobic from nonphobic subjects. It is also important to remark that the k-means algorithm does not use any arbitrary parameter that can bias the results.

2.2. Spider phobia assessment techniques

To assess the degree of spider phobia, three different instruments were used. As described, the SCID (First et al. [16]) was used to produce a preliminary selection of participants. Afterwards, the BAT and the FSQ provided the 5 measurements used to evaluate if participants showed improvement.

2.2.1. Structured Clinical Interview for DSM IV Axis I Disoders (SCID)

To verify that patients met DSM-IV criteria for specific phobias (300.29), all of them underwent an SCID (First et al. [16]).

2.2.2. Behavioral assessment test (BAT)

The BAT is a widely used measurement of clinical improvement in specific phobias (Lang and Lazovick [17]; Lang et al. [18]). It consists of an artificial situation in which the subject approaches the phobic object until discomfort sets in. The experimenter measures the distance from the subject to the object and assesses the subject's anxiety level using, in our case, the SUDS scale (Wolpe [19]). These tests usually start at 5 meters from the real spider, but in this study the initial distance was established as 25 meters because of the severity of arachnophobia in our patients.

The BAT was performed in two stages: first with a photo of a tarantula (Grammostola acteon, 20 cm) and afterwards with a real tarantula. In both cases the phobic object was placed at the end of a 25-meter long corridor. Before beginning the test, an assistant read the instructions to the subject: "This is a behavioral assessment test and is not part of the therapy. You are free to refuse my suggestions. Walk the farthest you are able to approximate to the spider at the end of the corridor without forcing yourself. I will remain at this point until you stop." When the subject stops less than one meter from the object, the assistant says: "Touch the photo" or "Touch the cage" in the case of the real tarantula.

Note that instead of asking the patient to approach as much as possible to the spider, the patient is asked to approach to the spider as much as possible without forcing himself. This kind of suggestion guaranteed complying with the desire of patients of not confronting in any way the phobic object.

The BAT was rated by measuring the distance from the subject to the phobic object, starting at 25 meters. The BAT score ranged from 26 if the subject refused to do the test, to -1, if the subject opened the lid of the cage. When subjects stopped, the assistant applies the SUDS by saying: "Please, rate you anxiety from 0% to 100%, 100% being the greatest fear you have had in your life."

2.2.3. Fear of spider questionnaire (FSQ)

The fear of spiders questionnaire (FSQ) assesses the subjective perception of spider fear (Szymanski and O'Donohue [20]). It is composed of 18 questions rated on a 1–7 Likert scale (1 = I strongly disagree, 7 = I strongly agree). The FSQ was able to discriminate between phobics and nonphobics, $F(1.111) = 5.99$, $P < .01$, $F(1.76) = 13.28$, $P < .01$, respectively (Szymanski and O'Donohue [20]). It also provided evidence for the improvement of phobic patients following a cognitive restructuring treatment (comparing pretest to posttest: $t(37) = 4.38$, $P < .01$, $t(79) = 5.09$, $P < .01$, resp.). When applied to nontreated subjects, the instrument did not show improvement from pretest to posttest. This instrument has an internal consistency of 0.92 with a split half reliability of 0.89.

2.3. Presentation of "SLAT" figures

The presentation used in the SLAT consists of an initial set of 165 images, 124 of them having some features that resemble any of the characteristics (color, shape, texture, etc.) of a spider and were selected as explained in Section 2.3.1. Examples include the image of a person with a Rastafarian hair style, the Atomium of Brussels, a carousel, and so forth.

The remaining 41 images were neutral and were selected with the purpose of making it more difficult for the subject to realize there were SLAT images in the presentation.

The placebo group presentation consisted of a sequence of images without arachniform features. Among the selected figures, there were abstract or surreal paintings that might induce placebo subjects to think there was something hidden in the figures.

2.3.1. Selection of figures

The images were selected from the Internet. We chose 132 images with spider features and 44 neutral images. The features that were selected in the images were related, for example, to the radial symmetry of spiders, the design of their webs, their texture, the way they articulate their legs, the hook-like shape of their extremities, or the fact that they hang from a string.

For validating our selection, 43 nonarachnophobic persons were asked to rate, on a 0 to 10 scale, the content of spider features in all the images. Not to bias the process of rating the images, no instructions related to what features to consider in rating the images were given to these persons.

It was necessary to establish a threshold in this scale for separating SLAT images from neutral images. This threshold was obtained by means of the Bayes decision rule that yields a threshold of 0.92. Images with a greater rate were classified as SLAT images, and images with a lower rate were classified as neutral. According to this rule, 8 of the figures initially classified as SLAT images were neutral, and 3 neutral figures were SLAT images. Therefore, a total of 11 images were excluded from the final therapeutic repertoire. To apply the Bayes decision rule, a histogram was created giving the probability of finding a SLAT image inside intervals of 0.6 unit length in the 0 to 10 "arachniform scale." The same was done with neutral images. We replaced both histograms by two curves after smoothing the histograms by using interpolation by splines. The intersection of the two curves yielded the value of 0.92 that served to discriminate between SLAT and neutral images.

2.3.2. Adjustment of presentation intervals

One of the assumptions that served to delineate the SLAT (see assumption (a) in Section 4.1) deals with avoiding a high activation in the neural circuits involved in fear. For this reason, we elaborated a procedure to exclude from the final therapeutic presentation those images that might produce discomfort in the patients, keeping only the more comfortable images that would probably not produce a high degree of activation in these neural circuits.

We adopted the following procedure.

(a) Once the entire set of figures had been shown to the patient in a preparatory presentation, we asked the patient to see the figures once more and collaborate with us to determine the adjusted duration, T_{ad}, of each one of the images. The patient was instructed as follows: "Each one of the following images will be presented by default for 5 seconds. If you do not like the image, press the "Enter" button to pass to the following image sooner. The sooner you press the button, the more fearful we will understand the image to be for you."

(b) After seeing all images the subjects were asked:

(1) Which images, if any, are intolerable?
(2) Which images are tolerable?
(3) Which images are so nice that you might place them in your bedroom?

With all this information, nine rules were applied to obtain the final duration of each image, T_{ad}, in the presentation. As some patients were faster than others in pressing the "Enter" button, the average time T_m for each subject served as the patient's unit of time.

In the following rules, times T_0, T_1, and so on were set as arbitrary multiples of T_m. The adjusted duration of each image, T_{ad}, was obtained by multiplying the duration chosen by the subject in the preparatory presentation, T, by a coefficient calculated as follows.

We defined three thresholds: $T_0 = T_m/5$, $T_1 = T_m/2$, $T_2 = T_m/3$.

(1) If $T < T_0$, the image was eliminated from the presentation.
(2) Intolerable images with $T < T_1$ were also eliminated.
(3) $T_{ad} = 0.2 * T$ in intolerable images with $T > T_1$.
(4) $T_{ad} = T$ for tolerable images with $T < T_2$.
(5) $T_{ad} = 1.5 * T$ for tolerable images with $T_1 > T > T_2$.
(6) $T_{ad} = 1.8 * T$ for tolerable images with $T > T_1$.
(7) $T_{ad} = 2 * T$ in images deemed nice.
(8) Other images, not included in previous groups, maintained their time T.
(9) To make the total presentation time equal to 12 minutes, each T_{ad} was multiplied by 12 and divided by the total duration (in minutes) of the presentation.

All procedures were the same for the placebo group.

2.4. Procedure

This research was approved by the Ethics Committee on Research of the Institute of Psychology of the University São Paulo.

As mentioned in Section 2.1, of the 160 patients that contacted us, 36 were interviewed and 25 were included in the experiment. These patients signed forms, agreeing to participate in either the placebo or treatment group, and allow the use of collected data for research. Patients were randomly divided into two groups: treatment ($n = 13$) and placebo ($n = 12$).

After adjusting the timing of the presentation, a personalized CD was prepared for each patient. In the following session, this CD was given to the patient. The patient was then instructed to run the presentation twice a day at home preferably during moments in which she/he was not tired or under stress. Prior to each presentation run, the patient was given one question to answer at the end of the run. These questions were intended to distract the patient from arachniform features in the images. Examples include: "In how many images there is an animal?" or "In how many images there

is a rounded object?" When answering the question, the patient was instructed to write, beside the answer, the date and time she/he ran the presentation. Every week these data were checked out in order to verify the rate of cooperation of patients and to encourage noncooperative patients, if any. In all subjects, the cooperation was satisfactory and no statistics were deemed necessary to measure the rate of cooperation.

To assess progress during the treatment, placebo and treatment subjects underwent the BAT (including the SUDS) each week. In the last week, the FSQ was also applied. Experiments were carried out in three stages. In stage 1, data collected during these first four weeks were used to compare placebo and treatment groups. A period of four weeks was established prior to the experiment with the intention of minimizing the duration of the experiment in order to avoid drop out. In stage 2, the treatment group (but not the placebo) was asked (and luckily agreed) to continue for two more weeks to assess if this additional time might help the treated group to achieve a more substantial recovery. They were evaluated at the end of the 6th week.

In stage 3, after the fourth week, placebo subjects were invited to receive the SLAT. The ten subjects that were accepted were treated for 6 weeks and evaluated after the 4th and 6th weeks.

3. RESULTS

3.1. Comparison between placebo and control groups at the beginning of the study

There were no difference between the placebo ($n = 12$) and treatment ($n = 13$) groups at the beginning of the study in the following demographic and clinical variables: age, $F(1,23) = 0.3315$, $P = 0.5703$; duration of phobia, $F(1,23) = 3.8758$, $P = .0611$. No significant differences were found in behavioral variables during the initial BAT test with the real spider BAT: $F(1,23) = 0.0015$, $P = .9692$; SUDS, $F(1,23) = 0.0739$, $P = .7881$; or with the spider photo BAT, $F(1,23) = 1.6764$, $P = .2082$; SUDS, $F(1,23) = 0.0003$, $P = .9866$. No significant difference was found in the subjective measure of fear of spiders, FSQ: $F(1,23) = 0.020$, $P = .8895$.

Of the 13 treatment subjects, 3 refused to stay at any distance from the real spider if the spider was visible. They received an arbitrary score of 26, one meter more than the maximum score of 25 meters used in the BAT test. Regarding the test with the spider photo, one subject refused to stay at any distance in which he could see the photo. Analogously, we assigned a score of 26 meters in the BAT test to this subject. We emphasize that, different from previous studies in which the initial distance of the BAT test was standardized to 5 meters, this distance was augmented to 25 meters because of the desire of the patients not to confront the spider in anyway.

3.2. Comparative evolution of placebo and treated groups

Table 1 shows the mean and standard deviation (in parenthesis) of the various groups evaluated. The percentage improvement (Table 2) was calculated by dividing the absolute improvement in each measure by the initial measure. After 4 weeks, the percentage improvement in all measurements was higher in the treated than in the placebo group. During the presentation of the real spider, the percentage improvement in the BAT was more than twice as high (61.6% versus 28.8%) in the treated than in the placebo group (see Table 2). The SUDS was more than six-fold (40.3% versus 5.9%) higher. The same measurements made with the spider photo yielded a percentage improvement of 19.3% (66.6%–47.3%) in the BAT and 32% (53%–21%) in the SUDS. Differences between placebo and treated groups were consistent throughout the four weeks of the experimental procedure (see evolution of measures in Figure 2).

Improvement in the FSQ was 13.1% (28.8%–15.7%) higher in the treatment than in the placebo group.

3.2.1. Repeated measures multivariate ANOVA

A 2 (group) $\times 5$ (times) repeated measure multivariate ANOVA (Hair et al. [21]) was conducted to evaluate whether the differences between placebo and treated groups were significant. In this multivariate analysis, 4 simultaneous variables were used: BAT and SUDS for real spiders; and BAT and SUDS for spider photo. By analyzing the results of the multivariate ANOVA, we conclude that the significant time effect $F(4,92) = 14.5475$, $P < .0001$, and the significant group effect $F(1,23) = 4.5678$, $P = .04344$ show the effectiveness of the treatment. The significantly different time-course of the improvement in the two groups is also reflected in a significant group $\times$ time effect $F(4,92) = 4.4217$, $P = .0026$. In order to evaluate how the test with the real spider and the test with the spider photo contribute to these results, a 2-group, $\times 5$ times, multivariate ANOVA was performed, first with the BAT and SUDS of the real spider and then with the BAT and SUDS of the spider photo. The test with the real spider yielded a significant group $\times$ time interaction: $F(1,23) = 7.981610$, $P = .009598$, MS = 1369.772 while the test with the spider photo yielded a moderate group $\times$ time interaction $F(1,23) = 2.908077$, $P = .101608$, MS = 750.1708. The FSQ also yielded a nonsignificant 2 (groups) $\times 2$ (time = pretreatment versus post treatment) interaction $F(1,23) = 1.833$, $P = .188$. The difference between BAT and SUDS tests and the FSQ test results are analyzed in the discussion.

3.3. Results of prolonging treatment until the sixth week

After the four weeks in which placebo and treated subjects were compared, treated subjects continued receiving the SLAT for two more weeks, achieving 76.6% improvement in the BAT and 45.6% in the SUDS with the real spider. With the spider photo, there was an 88.5% improvement in the BAT; a 61.4% improvement in the SUDS, and a 40% improvement in the FSQ.

The results of the treated placebo were consistent with the results of the treatment group (see Tables 1 and 2).

Table 1: Means and standard deviations (in parenthesis) of the BAT, SUDS, and FSQ scores. Treatment ($n = 13$) and placebo ($n = 12$) group scores were gathered and compared at the end of the 4th week. Treatment group continued treatment until the 6th week. After 4 weeks, ten placebo subjects also underwent treatment, and their improvement was calculated at the 4th and 6th weeks of treatment. Six months later, a follow-up study was performed.

	Real spider		Spider photo		FSQ
	BAT	SUD	BAT	SUD	
Treatment					
Start	15.6 (7,7)	82.8 (17,9)	12.3 (8.6)	56.9 (24.3)	105.5 (11.2)
4 weeks	5.9 (4.4)	50 (22.4)	3.2 (2.7)	22.3 (17)	74.7 (23.2)
6 weeks	3.9 (5.4)	43.5 (32.5)	1.4 (2)	17.7 (19.3)	63 (30.2)
6 months (follow-up)	2.01 (3.9)	32.1 (27.5)	1.0 (1.53)	14.6 (19.1)	48.2 (27.0)
Placebo					
Start	15.7 (7.2)	80.8 (19.2)	8.7 (4.8)	57.1 (22.8)	107.7 (16.8)
4 weeks	10 (5.2)	73.8 (25.9)	4 (3.7)	44.2 (28.7)	90.8 (22.7)
Treated placebo					
Start	10.8 (5.3)	81 (20.9)	4.6 (3.6)	49 (28.8)	99.1 (15.5)
4 weeks	5.9 (5.2)	60.5 (26.5)	2.1 (2.5)	27.9 (31)	73.4 (23.1)
6 weeks	3.1 (4.9)	45.6 (33.9)	1.1 (1.7)	23.2 (29.2)	59.6 (26.4)
6 months (follow-up)	1.8 (3.00)	34.2 (27.2)	0.6 (1.1)	19.9 (21.0)	49.2 (28.4)
Treatment and treated placebo					
Start	13.5 (7.0)	82.0 (18.8)	9.0 (7.8)	53.5 (26.0)	102.7 (13.3)
4 weeks	5.9 (4.6)	54.6 (24.3)	2.8 (2.6)	24.7 (23.6)	74.1 (22.6)
6 weeks	3.6 (5.1)	44.4 (32.4)	1.3 (1.7)	20.1 (23.7)	61.5 (28.0)
6 months (follow-up)	1.91 (3.4)	33.1 (26.7)	0.8 (1.3)	17 (19.2)	48.6 (26.9)

3.4. Six-month follow-up study

A six-month follow-up study was also performed. It showed a substantial consolidation of previously obtained results. There was 90.2% improvement in the treatment group in the BAT test: patients were capable of approaching a live tarantula at 2(3.9) meters (on average), six patients opened the lid of the tarantula cage and, of these, three patients touched the tarantula (*Grammostola acteon*, 14 cm, the initial one died).

In the case of the follow-up study with the treated placebo patients, there was an improvement of 79.2% in the BAT test. Three of them opened the lid of the cage and two of them touched the tarantula.

Only one patient dropped out of the follow-up study.

3.5. k-means cluster analysis

A k-means multivariate cluster analysis was used to assess the number of patients that made the transition from arachnophobic to normal during treatment. Five variables were used to characterize each subject: BAT and SUDS with real spider, BAT and SUDS with photo of a spider, and FSQ. The algorithm was applied with these five variables gathered from the 25 arachnophobes at the beginning of treatment, and from 29 normal subjects recruited in the university. The k-means algorithm was initially used to eliminate nonphobic subjects from the group of volunteers, as explained in Section 2.1. To calculate the percentages of patients that migrated from arachnophobic to normal along the different stages of the experimental procedure (see Table 2), the k-means algorithm was fed with the scores of the participants in each one of the stages (BAT spider, BAT photo, SUDS spider, SUDS photo, and FSQ).

During the four weeks of treatment, 41.7% of individuals in the treatment group and 25% of the placebo group moved over to the normal condition. When the placebo group was treated, 50% fell in the normal group.

A more substantial improvement was evident in the follow-up, six months after the conclusion of treatment: 91.7% of individuals in the treatment group and 90% of the treated placebo group were classified as nonarachnophobes. These results are discussed below.

4. DISCUSSION

In this section, the following topics will be discussed:

(1) the hypothetical assumptions taken into consideration to elaborate the therapy;
(2) the neurocomputational background of the therapy;
(3) the influence of the BAT assessment test in the efficacy of SLAT;
(4) the delay of improvement in the FSQ;
(5) the therapeutical limitations of the procedure;
(6) suggestions for further studies.

Table 2: Improvement of the BAT, SUDS, and FSQ scores in Table 1 expressed in percentages. The percentage of improvement was calculated from Table 1 by dividing the measurement by the initial score. The last column exhibits the percentage of patients that migrated to the condition of normal subjects, according to the *k*-means algorithm. According to this, in six months, 91.7% of the treatment-group subjects became nonarachnophobes.

	Real spider		Spider photo		FSQ	Recovery (*k*-means) (%)
	BAT	SUD	BAT	SUD		
Treatment						
Improv. (%) 4 weeks	61.6 (19.4)	40.3 (22,9)	66.6 (31.2)	53 (51.7)	28.8 (20.5)	41.7
Improv. (%) 6 weeks	76.6 (27.9)	45.6 (46.1)	88.5 (17.1)	61.4 (53.6)	40 (27.1)	50
Improv. (%) (follow-up)	90.22 (25.74)	62.0 (2.7)	87.49 (17.52)	70.6 (37.4)	55.2 (23.4)	91.7
Placebo						
Improv. (%) 4 weeks	28.8 (31.8)	5.9 (40.9)	47.3 (37.3)	21 (36.9)	15.7 (18.3)	25
Treated placebo						
Improv. (%) 4 weeks	46.8 (31.5)	24.1 (28.4)	46.2 (37.2)	42.3 (42.1)	26.2 (19.1)	50
Improv. (%) 6 weeks	71.2 (38.7)	44.2 (35.1)	67 (40.7)	54 (39)	39.4 (25.8)	50
Improv. (%) (follow-up)	79.2 (33.6)	58.3 (31.1)	87.0 (21.4)	63.3 (40.6)	50.4 (26.7)	90
Treatment and treated placebo						
Improv. (%) 4 weeks	55.2 (25.8)	33.3 (26.2)	57.7 (34.7)	48.3 (47.0)	27.7 (19.5)	43
Improv. (%) 6 weeks	74.3 (32.3)	45 (40.8)	79.1 (30.9)	58.1 (46.9)	39.7 (26.0)	50
Improv. (%) (follow-up)	85.2 (29.4)	60.3 (31.3)	87.3–18.9	67.3 (38.1)	53.1 (24.4)	91

4.1. *Hypothetical assumptions for elaborating the SLAT*

Two hypothetical assumptions that are consistent with neurological findings served to delineate the methodology of SLAT. The results of the therapy, however, are not intended to assess the validity of these preliminary assumptions, which would require much further confirmation.

(a) The first assumption is that some connections from thalamus to amygdala are abnormally potentiated in phobic patients, possibly because of a process in which a conditioned stimulus (CS), the phobic object, is associated with an unconditioned stimulus (US) such as a loud sound or an acute pain. The possibility of plastic changes taking place in the thalamo-amygdala pathway is supported by the work of Doyére et al. [15], in which they were able to induce long-term potentiation (LTP) in thalamic and cortical inputs to the amygdala in freely moving rats, demonstrating that LTP in thalamic inputs is much more persistent and long-lasting than LTP in cortical inputs. LeDoux, Schafe et al. (Apergis-Schoute et al. [22]) have further shown that intralaminar thalamic neurons contribute to presynaptic plasticity in the thalamo-amigdaloid pathway during fear conditioning. Thalamic intralaminar neurons are also described as a locus of functional CS-US convergence for fear conditioning to acoustic stimuli (Cruikshank et al. [23]). The possibility of altering these circuits by means of either habituation to the spider or by cognitive-behavioral therapy is also mentioned, for example, by Veltman et al. [24] and Paquette et al. [25].

Regarding the degree to which plastic changes would take place in the thalamo-amygdaloid pathway, it is worth mentioning that postsynaptic voltage value is critical to determining whether a synapse is reinforced or depressed (Figure 3). According to Figure 3, postsynaptic depolarization determines the potentiation or depression of a given synapse. If the value of postsynaptic depolarization is greater than a threshold, called the LTP threshold, active synapses are potentiated (i.e., increment their synaptic connectivity or synaptic weight); below this threshold they are depressed (Artola and Singer [26]; Bear et al. [27]) (these synapses experiment a decrement of their synaptic connectivity or synaptic weight). If the postsynaptic depolarization is very low, synaptic depression is small or null.

We conjectured that the effectiveness of SLAT depends on activating neurons that project from thalamus to amygdala in such a way that they are inside the depression interval. Unfortunately, depression intervals vary for each synapse according to a synaptic property called metaplasticity. The same postsynaptic activity may produce potentiation in one synapse and depression in another while leaving a third unaltered. We were also unable to directly evaluate the postsynaptic activity that a given SLAT figure produced in these neurons.

Despite all these difficulties, we conjectured that the fear reaction produced by SLAT figures was correlated to the postsynaptic activity in neurons in the thalamo-amygdaloid pathway. To avoid potentiation and favor depression, fearful images were omitted from the presentation (see Section 2.3.2). The duration of the remaining images were adjusted so that comfortable images were exhibited during a longer time and less comfortable images during a shorter interval.

(b) The second hypothetical assumption that served to delineate SLAT is related to the nature of the archetypal information that, according to LeDoux, is relayed from the thalamus to the amygdala. Morris et al. [28] found that the amygdala appears to sum, in a nonlinear manner, individual

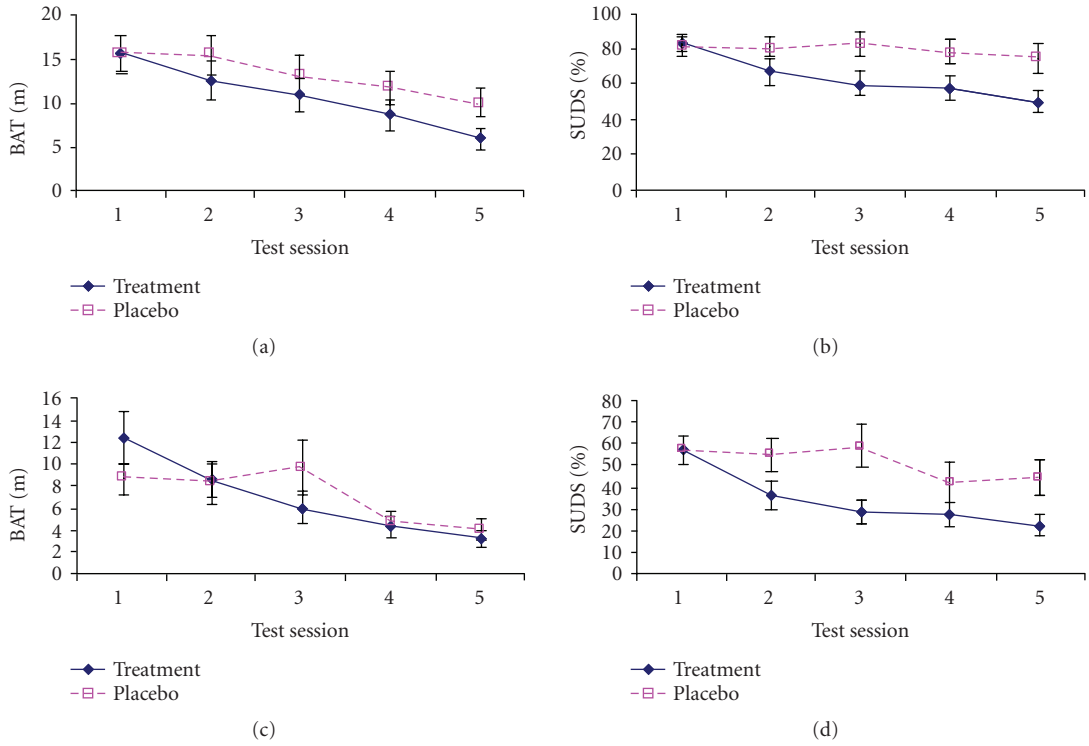


Figure 2: Time course of the BAT and SUDS means with a real spider, (a) and (b), and with a spider photo, (c) and (d), for placebo and treatment groups. Vertical segments indicate standard error.

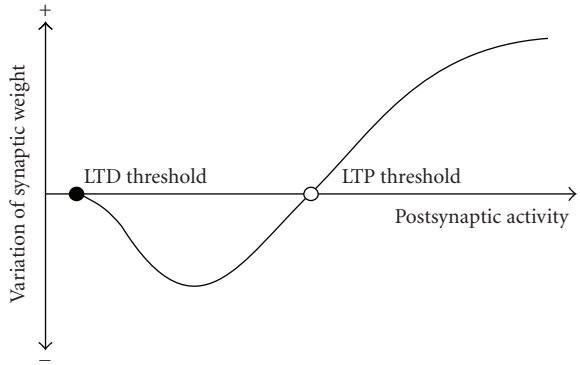


Figure 3: Variation of synaptic efficiency (synaptic weight) in terms of postsynaptic activity. For levels of postsynaptic activity above the LTP threshold, synaptic potentiation (positive variation of synaptic weight) takes place. Between the LTD and LTP thresholds, synaptic depression (a negative variation of synaptic weight) occurs. Below the LTD threshold there is no variation of synaptic efficiency.

responses to specific facial features. A two-stage theory for facial perception of emotions was proposed by De Bonis et al. [29] and tested by Morris et al. [28], who concluded that "the perception of emotional expressions depends on an initial processing of individual facial features followed by a nonlinear association of the different components." According to Weinberger and collaborators (Lennart and Weinberger [30]; Edeline and Weinberger [31]), the thalamus is able to recognize features, augmenting its response to a specific feature that was previously paired to a US.

4.2. Neurocomputational foundations

Neurocomputational models (Peláez [32, 33]) are consistent with the two-stage theory, conjecturing that the first stage of the process, the preliminary processing of individual features, is performed in the thalamus. According to these models, in the thalamus each sensory pattern is represented as a vector with components in a coordinate frame in which each axis corresponds to a specific feature of the pattern. Each one of these axes/features corresponds to the output of a thalamic reticular neuron. The output of these reticular neurons (Crabtree and Isaac [34]) is nonlinearly summed by intralaminar neurons (see Figure 4) and if this sum exceeds a threshold, the result is relayed to the amygdala. According to the computational model, the set of axes/features created by the firing of reticular neurons in the thalamus, constitute a code that identifies, in a rough way, each input pattern. This code would correspond to the rough, almost archetypal description of the aversive stimuli, that, according to LeDoux and colleagues (LeDoux [14]; Doyére et al. [15]), is passed from the thalamus to the amygdala.

According to the first assumption, a way of depressing thalamo-amygdaloid synapses would be by avoiding high post-synaptic potentials in thalamo-amygdaloid neurons by means of reducing the intensity of phobic stimuli (Figure 3). A possible way of reducing this intensity would

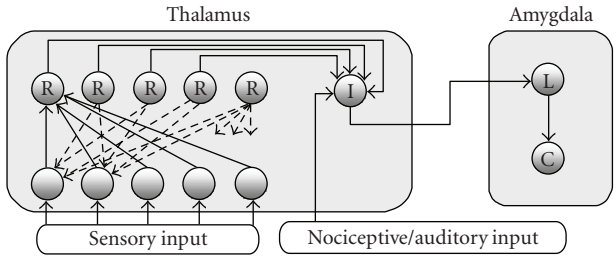


Figure 4: Hypothetical arrangement of thalamus and amygdala connections, used in the computational model that inspired the therapy here described (SLAT). R: thalamic reticular neurons; I: thalamic intralaminar neurons; L: lateral nucleus of the amygdale; C: central nucleus of the amygdala. Due to a competitive process performed between reticular neurons in the model, each one of them responds to a specific feature of a sensory pattern (Peláez [32, 33]). A similar competitive process takes place between intralaminar neurons, each one responding to a specific combination of features. Therefore, a certain number of features, that is, reticular neurons, are necessary for firing a specific intralaminar neuron. When this number is low, a low postsynaptic activity in intralaminar neuron favors synaptic depression, according to Figure 3, thereby reducing the possibility of future intralaminar neuron firing. In this way, the thalamic-amygdala pathway is depressed in the computational model.

be by masking or obscuring the phobic object. However, a masked or obscured phobic object is still intense enough to fire the amygdala (Whalen et al. [35]) and aversive for patients.

Instead of reducing the duration or intensity of spider images, we propose to reduce the number of arachnoid features present in each image. According to the second assumption, when the number of arachniform features in the input pattern is reduced, the activation of intralaminar neurons (computing the sum of these features) is also reduced. This lower activation of intralaminar neurons contributes to reduce the activation of the neurons in the thalamo-amigdaloid axis, so that their synapses would undergo depression instead of potentiation. Therefore, when, instead of the spider code, a code with a smaller repertoire of arachniform features is relayed, neurons in the thalamo-amigdaloid pathway are hypothetically less activated, their synapses more prompted to undergo depression rather than potentiation.

4.3. Influence of the BAT assessment test in the efficacy of the SLAT

Both treatment and placebo groups underwent BAT and SUDS assessment test weekly. Volunteers were told to approach the spider without forcing themselves. The purpose of this instruction was to adhere, during the BAT and SUDS tests, to the principles that inspired the therapy, that is, to avoid any stimuli that could contribute to enhance thalamo-amygdala connectivity.

It could be argued that the BAT assessment test could, by itself, have a therapeutical effect over arachnophobia. This effect might be thought to be responsible for the improvement observed in the placebo group. However, as shown in Section 3.2, improvement of patients in the treatment group was significantly better than that of patients in the placebo group.

4.4. The delay of improvement in the FSQ

Many patients reported that they did not realize that they had lost their fear of spiders until they were confronted to a real spider during their daily life. They had the strange sensation of not reacting with fear when, for the first time after treatment, they saw a real spider. Since during daily life, a real confrontation with a spider is an unpredictable event, the realization of having lost the fear varies from individual to individual. The BAT assessment test, independently of its possible placebo effect, could contribute to accelerate this process of realization.

Related to this, we observed that the improvement in the FSQ was delayed in comparison to the improvement in the automatic responses measured by the BAT and SUDS. This is consistent with the reasonable supposition that patients did not realize that they had lost their fear until they actually confronted a real spider during their daily life situations. Depending on the frequency with which they actually confronted a spider in their daily lives, the realization of recovery took a shorter or longer time in the different patients. This fact was reflected in the follow-up study that was carried out six months after the conclusion of the treatment.

4.5. Therapeutical limitations

Although the 25 subjects that took part in the experiment came from a very large sample of 160 arachnophobic volunteers, there were no volunteers above the age of 46. Taking into account that neural plasticity depends on age (Burke and Barnes [36]) and that our experiments were not able to assess the therapeutic effect of SLAT in elderly people, we suggest to apply the SLAT to patients below the age of 46, until performing an assessment with older volunteers in the future.

4.6. Suggestions for further studies

The 160 arachnophobic patients that contacted us were classified in terms of their degree of arachnophobia. Among the six with the highest scores, three of them suffered thyroid hormone impairment. We wondered whether this coincidence might be a possible psycho-somatic effect produced in the long run by arachnophobia. A similar case of thyroid hormone alteration was found in the literature (Friedman et al. [37]) among women with posttraumatic stress disorders. These considerations motivate a study to assess the relationship between thyroid hormone alteration and phobias.

According to our theoretical assumptions, the SLAT acts at subcortical levels. Neuroimaging studies could help to evaluate this assumption by comparing the brain activation before and after the SLAT. A similar comparison was done by Paquette et al. [25], in which arachnophobic patients were treated with cognitive behavioral therapy. This study concluded that the dorsolateral prefrontal cortex and

the parahippocampal gyrus diminished their activation significantly after treatment with cognitive behavioral therapy. In the case of the SLAT, we expect that reduction of activity in the dorsolateral prefrontal cortex and the parahippocampal gyrus will be preceded by reduced activity of amygdala and superior colliculus. This sequence would be consistent with the fact that during the SLAT, improvement in the BAT test (measuring automatic responses) proceeded the improvement in the FSQ tests (measuring cognitive variables related to fear of spiders).

5. CONCLUSION

A novel technique for treating spider phobia, that does not require any use of spiders, was described and tested. In the SLAT, here described, each patient is given a personalized presentation in a compact disk, containing a set of images that, although not containing spiders, present subsets of spider characteristics. The degree to which each image evokes a spider in different patients is different. The most evocative images are excluded from the personalized presentation whereas the less evocative images are presented to the patient during a longer interval (see Section 2.3.2). Regarding the subtlety of the images, two treatment group patients declared that they thought they were in the placebo group because their presentation caused no discomfort at all.

To compare the evolution of the placebo and treatment groups, a four-week experiment was designed. Treatment and placebo groups went through their corresponding presentation twice a day and came once a week to the university to apply the BAT and SUDS tests. To carry out these tests, instead of encouraging the subjects to approach as much as possible to a spider, they were told to approach the spider, but without forcing themselves. They could also refuse to do the test, which was the case of three treatment subjects in their initial evaluation (see Section 3.1). This kind of suggestion respects the desire of the subjects of not confronting the spider in any way, and is coherent with the main philosophy of the procedure, according to which the subtler the better. The improvement in every measure of phobia was higher for the treatment group than in the placebo group (see Tables 1 and 2). Moreover, the repeated measures multivariate ANOVA showed that the patients' improvement was not due to a placebo effect (group $\times$ time interaction: $F(1,23) = 7.98$, $P = .0096$).

In the follow-up study performed after six months, 91.7% of the patients in the treatment group were classified as nonarachnophobes by the k-means algorithm, six patients of this group opened the lid of the tarantula cage, and, of these, three touched the tarantula.

The therapy proposed here was aimed at subconscious, automatic responses, while behavioral or psychoanalytic therapies emphasize the rational control of fear reactions. According to LeDoux [38], the alteration of fear behavior can be produced by the cortical control of fear reactions without the actual deletion of what LeDoux calls "fear memories," that once established become relatively permanent. These "fear memories" were intentionally the targets of the therapy proposed in this paper.

SLAT is particularly appropriate for, but not exclusive to, those patients who, because of the severity of their arachnophobia or whatever other reason, are unwilling to undergo therapies that involve any real, imagined or virtual spider. The theoretical basis of the therapeutic strategy was aiming to produce plastic changes in the thalamo-amygdaloid circuit responsible for the subconscious, automatic reactions triggered when the subject sees a spider. The therapy might have been effective for other, fortuitous, reasons, but the consistency with the theoretical basis that motivated it (Sections 4.1 and 4.2) is very encouraging, both from a practical point of view, providing an additional strategy to deal with certain phobias, and from a theoretical point of view, motivating further studies to test these ideas.

ABBREVIATIONS

ANOVA: Analysis of variance
BAT: Behavioral avoidance test
CS: Conditioned stimulus
DSM IV: Diagnostic and Statistical Manual of Mental Disorderes (4th ed.)
FSQ: Fear of spider questionaire
LTD: Long-term depression
LTP: Long-term potentiation
SCID: Structured Clinical Interview for DSM IV
SLAT: Spiderless arachnophobia therapy
SUDS: Subjective units of disconfort scale
US: Unconditioned stimulus

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