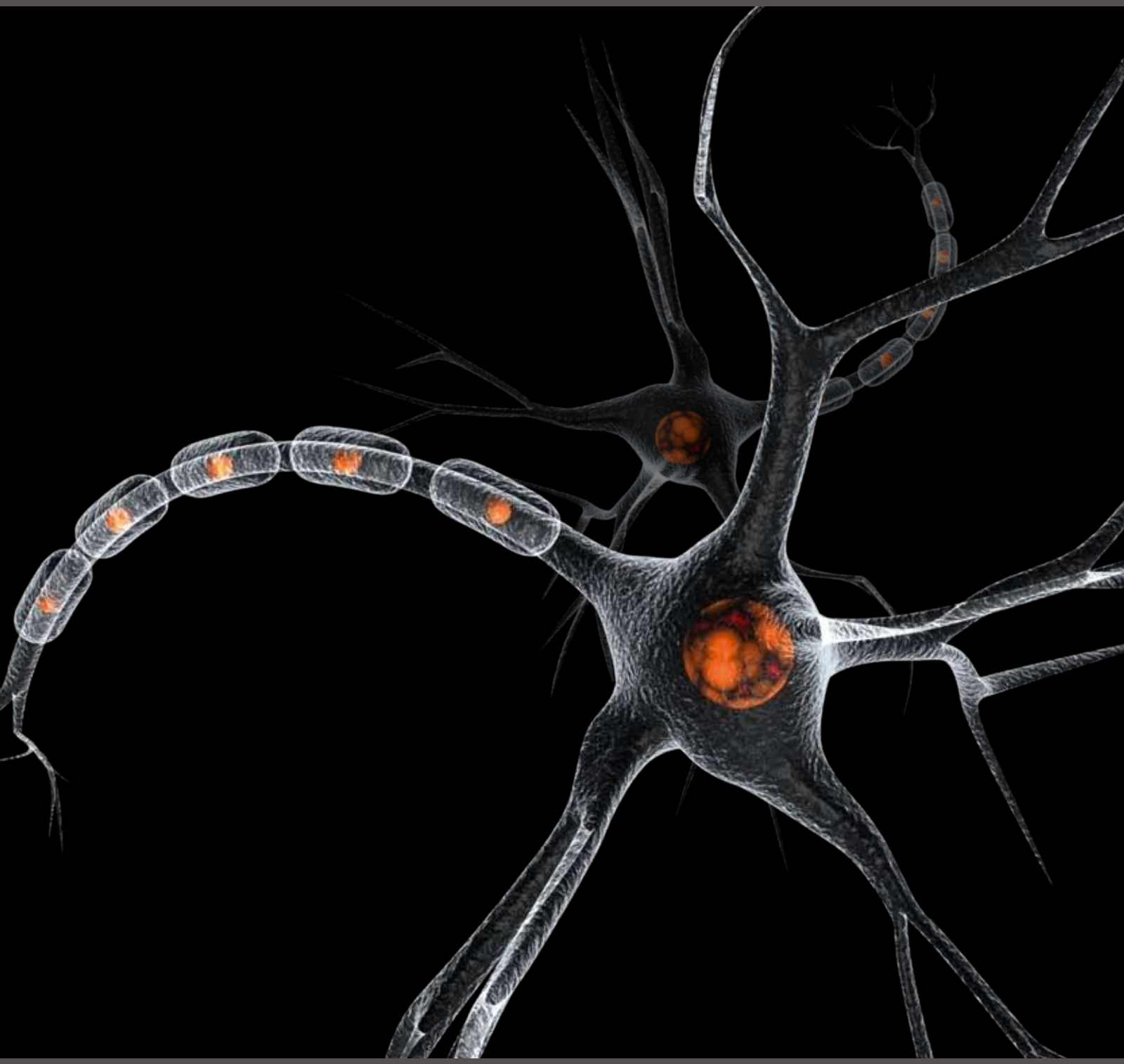
Neural Plasticity

The Periaqueductal Gray (PAG)

Guest Editors: Robert Adamec and Thelma A. Lovick



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Editorial Board

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Editorial
The Periaqueductal Gray (PAG)

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This issue covers a broad territory of PAG function: neuropharmacology, functional organization, and PAG plasticity in adaptive behavior, emotion, anxiety, and the less-studied plasticity of the PAG function in females.

Interest in the involvement of PAG in defensive behavior has a long history. Thinking about this area was radically changed by the seminal contributions of Bandler and DePaulis in defining functional columns arranged in both coronal and saggital planes of the PAG. Most papers in this section respect the importance of those functional columns while adding to our understanding how they might contribute to panic, anxiety, defensive response to natural threats, as well as aversive learning.

Del-Ben and Graeff in the paper entitled "Panic disorder: Is the PAG involved?" review preclinical as well as human imaging research in the contribution of PAG dysfunction to panic anxiety disorder. Others have shown how preclinical models of posttraumatic stress disorder (PTSD) and using brief exposure to predators (predator stress) have helped to advance our understanding of the neural substrates of stress-induced lasting sensitization of rodent anxiety including potentiation of startle response. Adamec et al. contribution in the paper entitled "Viral Vector Induction of CREB Expression in the Periaqueductal Gray Induces a Predator Stress-Like Pattern of Changes in pCREB Expression, Neuroplasticity and Anxiety in Rodents" extends initial findings implicating pCREB expression in the lateral column of the PAG in neuroplastic changes in amygdala-PAG communication as mediators of predator stress-enhanced startle and anxiety. Using viral vector enhancement of CREB expression in the PAG, the authors provide support for the idea that stress-precipitated increases in pCREB in the PAG are sufficient to potentiate central amygdala to PAG neural transmission, to increase anxiety in the elevated plus maze, and to potentiate startle response. In a related vein, exposure to stressful stimuli increases endocannabinoid (eCB) levels in the PAG and local administration of cannabinoid receptor 1 (CB1) agonists or drugs that facilitate eCB-mediated neurotransmission produce antinociceptive and antiaversive effects. Stimulated by these findings, Moreira et al. in "Anti-aversive effects of cannabinoids: is the periaqueductal gray involved?" explore the anxiolytic potential of PAG injection of CB1 agonists and cannabidiol in a variety of tests of anxiety as well as in contextual fear conditioning. They provide evidence that dorsal lateral PAG is a site of antiaversive actions of cannabinoids. Other PAG columnar function (ventrolateral PAG-VlPAG) in antinociception is explored by Morgan et al. in "Behavioral Consequences of Delta Opioid Receptor Activation in the Periaqueductal Gray of Morphine Tolerant Rats". It is known that chronic morphine administration shifts delta-opioid receptors (DOR) from the cytoplasm to the plasma membrane. Moreover, microinjection of morphine into the VlPAG produces antinociception. In light of these findings, it was hypothesized that movement of DORs to the membrane would induce antinociception to the DOR agonist deltorphin II as a way to compensate for morphine tolerance. Their findings suggest that chronic morphine administration alters DORs in the vlPAG with little evidence of compensation for the decrease in antinociception caused by morphine tolerance.

PAG Function in Females. Until very recently, research on the PAG along with most other brain areas was conducted almost exclusively in males with the implicit assumption that the anatomy and physiology were the same in females. Three articles in this issue show that this is not the case. Not only are there significant differences in the organization of the PAG between the sexes but the circuitry of the female PAG also exhibits considerable plasticity in response to changes in its hormonal milieu.

Lloyd and Murphy in the paper entitled "The Role of the Periaqueductal Gray in the Modulation of Pain in Males and Females: Is the Anatomy and Physiology Really That Different?" show that descending projections from the PAG, which are believed to modulate spinal processing of nociceptive input, are more numerous in female rats than in males. Paradoxically morphine, which exerts much of its antinociceptive effects by activating these projections, excited a much smaller proportion of the neurones in females compared to males, a finding that may explain the clinical finding of reduced efficacy of the analgesic effects of morphine in women compared to men. Lovick and Devall in the paper entitled "The Role of the Periaqueductal Gray in the Modulation of Pain in Males and Females: Is the Anatomy and Physiology Really That Different?" show how the fall in production of progesterone during the late dioestrus phase of the oestrous cycle leads to upregulation of certain subunits of the $GABA_A$ receptor in the PAG. One of the functional consequences is a decrease in ongoing GABAergic tone and an increase in neural excitability within PAG circuits. These changes may underlie the increased susceptibility to the development of stress-induced hyperalgesia that was shown to occur during late dioestrus and may also be relevant to Lloyd and Murphy's reports of a reduction in morphine's efficacy in female rats during dioestrus. Mota-Ortiz et al. in the paper entitled "Afferent Connections to the Rostrolateral Part of the Periaqueductal Gray: a Critical Region Influencing the Motivation Drive to Hunt and Forage" examine afferent inputs to the rostrolateral PAG (rlPAG) in female rats. In nursing females, morphine treatment induces a behavioral "switch" from maternal to foraging behavior which is mediated via the rostrolateral PAG (rlPAG). The authors report that the rlPAG receives inputs from medial prefrontal cortical areas involved in controlling attention-related and decision-making processes. Other afferents from different amygdalar, hypothalamic, and brainstem sites provide information to PAG related to feeding, drinking, or hunting behaviors. It is suggested that this unique combination of afferent connections positions the rlPAG to influence the decision whether hunting/foraging or other behaviors would be the most appropriate adaptive response for females, particularly in the presence of their young.

These papers are by no means exhaustive of the interest and breadth of work in the PAG field. Nevertheless, together they reflect the rich functional diversity of activities of the PAG. Moreover, they make apparent the importance of paying attention to sex and particular columnar areas when studying this fascinating structure, whose activity is of fundamental importance for survival of the individual in a challenging and constantly changing environment.

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Review Article

Panic Disorder: Is the PAG Involved?

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Data from studies with humans have suggested that abnormalities of midbrain structures, including the periaqueductal gray matter (PAG), could be involved in the neurobiology of panic disorder (PD). The electrical stimulation of the PAG in neurosurgical patients induces panic-like symptoms and the effect of drugs that are effective in the treatment of PD in the simulation of public speaking model of anxiety is in agreement with data from animal models of PD. Structural neuroimaging studies have shown increases in gray matter volume of midbrain and pons of PD patients. There is also evidence of lower serotonin transporter and receptor binding, and increases of metabolism in the midbrain of PD patients. Nevertheless, these midbrain abnormalities can not be considered as specific findings, since neuroimaging data indicate that PD patients have abnormalities in other brain structures that process fear and anxiety.

1. Introduction

Panic disorder (PD) is a common and incapacitating mental disorder characterized by the recurrence of spontaneous panic attacks, followed by a persistent concern about having additional attacks, worry about the implications of the attack or its consequences, and a significant change in the behavior related to the attacks. A panic attack is characterized as a discrete period of intense fear or discomfort, in which several symptoms, such as palpitations, pounding heart, or accelerated heart rate; sweating; trembling or shaking; sensations of shortness of breath or smothering; feeling of choking; chest pain or discomfort; nausea or abdominal distress; feeling dizzy, unsteady, lightheaded, or faint; derealization or depersonalization; fear of losing control or going crazy; fear of dying; paresthesias; chills or hot flushes, develop abruptly and reach a peak within 10 minutes. The symptoms are not related to substance abuse or general medical condition and are associated with a significant impairment of global functioning. Around 2/3 of patients with PD will also develop agoraphobia, which is defined as an anxiety about being in places or situations from which escape might be difficult, or embarrassing; or in which help may not be available in the event of an unexpected or situationally predisposed panic attack. Agoraphobic fears typically involve characteristic clusters of situations that include being outside the home alone, being in a crowd or standing in a line, being on a bridge, and traveling in a bus, train, or automobile [1].

Several brain structures that organize defensive reactions and represent the neural substrate of fear and anxiety have been implicated in the functional neuroanatomy of PD. Among those are prefrontal regions, amygdala, hippocampus, and parahippocampal area, hypothalamus, thalamus, and the periaqueductal grey matter (PAG) (for a recent review; see [2]). In regard to the latter region, animal studies have shown that electrical and chemical stimulations of the PAG cause urgent defensive reactions, such as freezing, fight, or flight. The same responses occur when the animal is faced by a clear and near threat, for instance, a predator [3]. Therefore, the PAG has been implicated in the defensive reaction to proximal threats, and drugs that increase the serotonergic function and are effective in the treatment of PD are able to reduce behaviors normally observed with the stimulation of the PAG (reviewed in [4]). Although other neurotransmitters, such as cholecystokinin [5] and glutamate [6], also appear to regulate fear/panic-related

defensive behavior, the main focus of this review will be on serotonin (5-HT) since this is the main neurotransmitter affected by the drugs clinically used for the treatment of PD.

Even though the evidence that supports the involvement of the neurocircuitry underlying defensive reactions in normal and pathological fear and anxiety has mainly been obtained with preclinical research, data from studies with humans also give support to the concept that structural and functional abnormalities in midbrain structures, such as the PAG, could be involved in the neurobiology of PD. Several reviews (e.g., [2]) have brought together animal findings showing the role of PAG in fear reactions and defensive behavior to proximal threats, but data coming from studies with human beings have not been completely explored. Therefore, the focus of this review is on results from human studies, including healthy volunteers and patients with PD, which provide evidence for a participation of the PAG in the pathophysiology of PD.

2. Symptomatic Homology

A pivotal evidence for the involvement of midbrain structures in PD came from the induction of panic-like symptoms by electrical stimulation of the PAG in neurosurgical patients. Awaked patients submitted to the stimulation of the PAG report feelings of terror or impending death, desire to flee, palpitation, and respiratory arrest or hyperventilation [7–9]. The remarkable similarities between the effects of PAG electrical stimulation in neurosurgical patients reported above and the symptoms that occur during a panic attack led the Brazilian psychiatrist Valentil Gentil to suggest a participation of the PAG in the neurobiology of panic attacks. Commenting on the changing in the behavior of rats due to the stimulation of the dorsal PAG, Gentil remarked, "I believe that (this animal) model is particularly useful for the understanding of the pathophysiology of panic attacks, especially the "spontaneous" attacks. ... (Bearing in mind that) the panic attack is a very primitive behavior ... the isomorphic validity of the central gray's (PAG) poorly organized responses to γ-aminobutyric acid (GABA-A) antagonists and electrical stimulation to the maladaptive flight behavior of full-blown panic seems high" [10]. Further, the phenomenological resemblance between panic attacks and the effects of the electrical stimulation of the PAG in both humans and animals has been systematically explored [11, 12], the main results being summarized in Table 1.

Although the similarity between the symptoms of a spontaneous panic attack and the effects of electrical stimulation of PAG is often cited as a face-validity criterion for implicating the PAG in PD, information obtained from awaked patients about the subjective and somatic responses provoked by stimulation of PAG is rare in the recent literature. An exception is the work carried out by Green et al. [13], using deep brain stimulation, who have obtained results similar to those reported by Nashold et al. [7], four decades ago. In the procedure of deep brain stimulation, electrodes are implanted permanently into specific areas of the brain. The electrodes are connected by wires under the skin to a generator allowing continuous electrical stimulation of specific brain areas. In this study, patients had electrodes implanted inside the PAG to control neuropathic pain. It has been observed that electrodes placed more dorsally in the PAG increased systolic and diastolic arterial blood pressure, what did not occur with electrodes placed more ventrally in the PAG. Moreover, two patients with dorsal electrodes reported nausea, sweating, and anxiety, symptoms commonly observed during a spontaneous panic attack. Although this issue has not been completely established, there is some evidence pointing to an association between PD and hypertension. In this regard, it has been proposed that both conditions would share a dysfunction of brainstem structures that regulate the autonomic nervous system and are inhibited by 5-HT [14].

3. Experimental Anxiety in Humans

Aiming to conciliate seemingly conflicting results derived from animal studies about the role of 5-HT in anxiety, it has been proposed that 5-HT projections from the dorsal raphe nucleus (DRN) facilitate inhibitory avoidance in limbic forebrain structures, predominantly amygdala and frontal cortex, while inhibit escape in the dorsal PAG [12]. This arrangement may have adaptive value, since it allows inhibition of fight/flight behavior in situations where threat is only potential or remote.

More recently, Lowry et al. [15] have shown that the 5-HT projections to cortical and limbic structures arise from a neuronal set located in a specific part of the caudal DRN, which is particularly sensitive to stressful stimuli. The rostral projections from these neurons seem to constitute a mesocorticolimbic 5-HT system that modulates defense. Based on correlations between the pharmacological efficacy of antidepressants and anxiolytic drugs in anxiety disorders and the results obtained in experimental models of anxiety in humans (discussed below), it has been further suggested that generalized anxiety disorder (GAD) would be related to the inhibitory avoidance and conditioned anxiety, whereas PD would be related to the escape response and innate fear [16]. A schematic representation of the hypothesis on the dual role of 5-HT in anxiety and defense is represented in Figure 1.

This theoretical model has been systematically tested using two experimental procedures that generate anxiety in human beings: the simulated public speaking (SPS) and the skin conductance response (CSCR) tests (for a review; see [17]). It is important to note that this experimental approach is different from that used in pharmacological challenges aimed at provoking a panic attack in vulnerable individuals. In this case, the most used are the infusion of sodium lactate and the inhalation of CO_2. Both challenges induce panic attacks in around 60 to 80% of panic patients, as compared to 0 to 20% of healthy controls. This seems to be a very specific response, since these challenges do not cause panic attacks in phobic or obsessive compulsive patients. Moreover, pharmacological studies have evidenced that antidepressant treatment decreases the vulnerability of

Table 1: Phenomenological similarities between panic attacks and effects of electrical stimulation of the periaqueductal gray matter (PAG) in humans and rats. Adapted from Jenck et al. [11] and Schenberg et al. [12].

Spontaneous panic attack	Stimulation of dorsal PAG in humans	Stimulation of dorsal PAG in rats
Intense fear or discomfort	Panic, terror	—
—	Intense distress	Aversion
Palpitations, pounding heart, or accelerated heart rate	Tachycardia	Tachycardia
Sweating	Sweating	—
Trembling or shaking	Sensation of vibration	—
Sensations of shortness of breath or smothering	—	Tachypnea
—	Hyperventilation	Hyperventilation
—	Apnea	—
Chest pain or discomfort	Chest and heart pain	—
Nausea or abdominal distress	Bladder voiding urge	Micturation
—	—	Defecation
Fear of dying	"Scared to death"	Escape responses
Chills or hot flushes	"Burn/cold" sensations	—

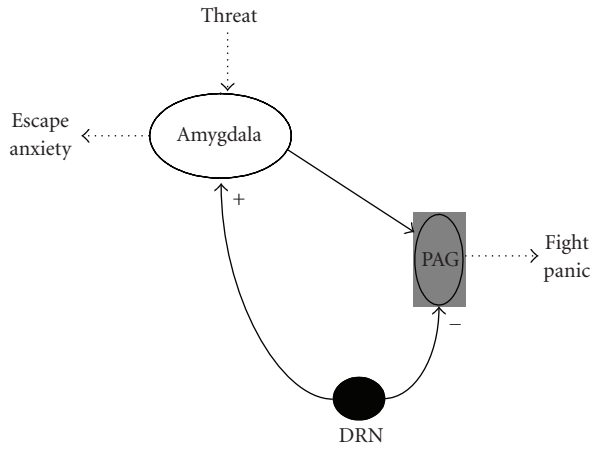

Figure 1: Schematic representation of the dual role of serotonin on fear and anxiety, according to Deakin and Graeff theory.

panic patients to lactate and/or CO_2 [18]. The similarities between the effects of lactate and CO_2 led to the hypothesis that both challenges have a common mechanism of action, causing an intraneuronal hypercapnia in brain areas that are stimulated by CO_2 during suffocation. The sensitivity of such suffocation alarm system would be abnormally heightened in PD patients [19].

Basically, the SPS test consists in the preparation and performance of a speech in front of a videocamera, with the participant seeing his/her own image on a TV screen. Subjective and physiologic measures of anxiety are taken before, during and after the speech. The emotional state induced by SPS is supposed to be species-specific fear, given that fear of speaking is highly prevalent in the general population [20] and occurs in healthy persons, irrespective of their personality trait to react with more or less anxiety to stressful situations [21]. Pharmacological studies have shown that drugs that facilitate 5-HT function decrease, whereas drugs that impair 5-HT function increase speaking fear [17]. On the other hand, the CSCR test is based on classical conditioning theory, consisting in the presentation of 10 neutral tones (habituation phase), followed by a neutral tone paired with a loud white noise (acquisition phase) and by the representation of 10 neutral tones (extinction phase). During the procedures, measures of skin conductance are taken. Drugs that increase 5-HT tend to facilitate conditioning [17].

Several 5-HT acting drugs have been assayed in these tests. For instance, a single dose of chlomipramine [22] and nefazodone [23] administered to healthy volunteers increased the fear provoked by the SPS, and this effect has been related to the clinical worsening observed at the beginning of the treatment with antidepressants [24–26]. While some animal studies have shown an increase in cortical extracellular level of 5-HT following acute administration of antidepressants [27–29], others have shown a greater increase of extracellular 5-HT in the raphe nuclei than in the neocortex [30]. If so, a single dose of an antidepressant would preferentially increase the concentration of 5-HT near the cell bodies of serotonergic neurons, which would activate somatodendritic 5-HT_{1A} autoreceptors, reducing neuronal firing [31] and, consequently, leading to a decrease in the release of 5-HT in the synaptic cleft. Therefore, the fear-enhancing effect of a single dose of antidepressants in SPS could be due to a lack of 5-HT inhibition of brain systems that generate panic attacks, likely to be localized in the dorsal PAG [4, 32].

In agreement with the hypothesis about the dual role of 5-HT in fear and anxiety, ritanserin, a 5-HT receptor antagonist, has shown opposite effects in the SPS and CSCR tests, prolonging the fear induced by SPS and decreasing conditioned skin conductance responses [33]. These results resemble reported clinical results with ritanserin, showing improvement of GAD [34], but a tendency to aggravate PD [35, 36]. To the opposite direction, the 5-HT releaser d-fenfluramine has been shown to reduce SPS-induced fear [37] and to improve PD [38, 39]. In contrast, d-fenfluramine tended to increase the amplitude of conditioned skin conductance responses, suggesting an anxiogenic-like effect [37].

Hence these pharmacological results with experimentally-induced fear and anxiety in humans are in agreement with the hypothesis that 5-HT enhances anxiety, which can be evaluated by the CSCR test, whereas inhibits fear, which can be assessed by the SPS test. The former effect would be related to the action of 5-HT on forebrain structures and the latter to its action on dorsal PAG. It has been well demonstrated that the chronic use of drugs that increase the availability of serotonin in the synaptic cleft is effective for the treatment of PD [40] and it has been proposed that the reduction in the occurrence of panic attacks with the use of antidepressants could be due to enhancement of the inhibitory action of serotonin on the PAG [4].

4. Panic Patients and Experimental Models of Anxiety

It is important to note that the SPS is not taken as a model of panic attack and it is not expected to provoke panic attacks in susceptible individuals. The possible association between the experimental model and the mental disorder is based on the rationale that public speaking would engage the neural substrates involved in the process of innate fear, which would be abnormal in PD.

Therefore, if the predictions derived from pharmacological studies with the human tests discussed above are correct, it would be expected that patients with the diagnosis of PD and healthy volunteers would perform differently in the SPS, but not in the CSCR test, given that the former would engage the brain mechanisms implicated in the neurobiology of PD, but the latter would not.

Aiming to test this hypothesis, we submitted panic patients free of treatment to both models of anxiety [41]. As predicted, controls and panic patients showed a similar response to CSCR. In contrast, during the SPS test, panic patients demonstrated higher levels of subjective anxiety than healthy volunteers from the beginning to the end of the experimental session but were less responsive to the speaking challenge. The profile of the subjective response of panic patients to the SPS test bears a resemblance to the effect of metergoline, a nonselective 5-HT-receptor blocker, given to healthy volunteers. Metergoline enhanced the subjective anxiety before and after the speech, but not during the preparation or the performance of the speech [42]. These results were in agreement with the suggestion that an impairment of the 5-HT function leading to a reduced of the inhibition of PAG may be present in the neurobiology of PD [16].

Using a similar protocol [43, 44], new groups of symptomatic panic patients and healthy controls were submitted to the SPS test. In addition, a third experimental group composed by panic patients who had become nonsymptomatic after long-term pharmacological treatment with antidepressant drugs was added. The aim was to verify whether the differences between healthy subjects and PD patients, if replicated, would remain after recovery, being thus related to a vulnerability trait, or otherwise decrease, and therefore being related to the clinical condition (state).

As can be seen in Figure 2, and in agreement with the former study, symptomatic drug free panic patients had more subjective anxiety during the experimental session than controls, despite the changes introduced in the procedures to minimize differences in expectancy and familiarity that might enhance or decrease initial anxiety, respectively. A more prolonged period of habituation decreased the anxiety in all groups, but the response to the SPS challenge was smaller in symptomatic patients than in normal controls. Moreover, nonsymptomatic patients stand between controls (below) and symptomatic panic patients (above) with regard to subjective anxiety, measured by the visual analogue mood scale (VAMS) and to bodily symptoms, measured by the total score of the bodily symptoms scale (BSS). Therefore, these measures seem to be related to the magnitude of clinical manifestations of PD rather than to a vulnerability trait, since they were affected by pharmacological treatment.

This study has also shown a significant decrease in the level of salivary cortisol from the initial to the pretest phases of the experimental session, in parallel with habituation of the anticipatory anxiety induced by the experimental setting. Additionally, a positive correlation between levels of subjective anxiety and of salivary cortisol has been found in control subjects at the initial phase of the experimental session. In contrast, salivary cortisol did not increase during the 60 minutes following the end of the speech, neither in patients, nor in controls, despite the levels of anxiety measured during speech preparation and performance being at least as high as those at the onset of the experimental session. Therefore, the SPS task does not seem to increase cortisol secretion. In agreement with these results, neither spontaneous panic attacks [45] nor the electrical stimulation of the dorsal PAG of the rat [46] activates the hypothalamic-pituitary-adrenal axis.

A final remark about the possible abnormal processing of innate fear in PD has come from a study carried out in our laboratory with patients with social anxiety disorder (SAD) submitted to the SPS test (MC Freitas, A Santos Filho, F Osório, SR Loureiro, CM Del-Ben, AW Zuardi, FG Graeff, JAS Crippa, unpublished results). SAD and PD are different anxiety disorders, but they keep some similarities, such as the response to the treatment with antidepressants that act on 5-HT function. However, in comparison to healthy controls, SAD patients have shown a larger enhancement of the fear induced by the SPS, what is different from the results obtained with PD patients. For that reason, we could speculate that the lower fear response induced by SPS could be specific to PD and related to abnormal functioning of brain structures involved in the process of innate fear.

5. Neuroimaging Data

As discussed earlier, evidence from preclinical studies suggests that the neural substrates involved in the defensive reactions to environmental threats of mammalian species could be implicated in the pathophysiology of PD. The main

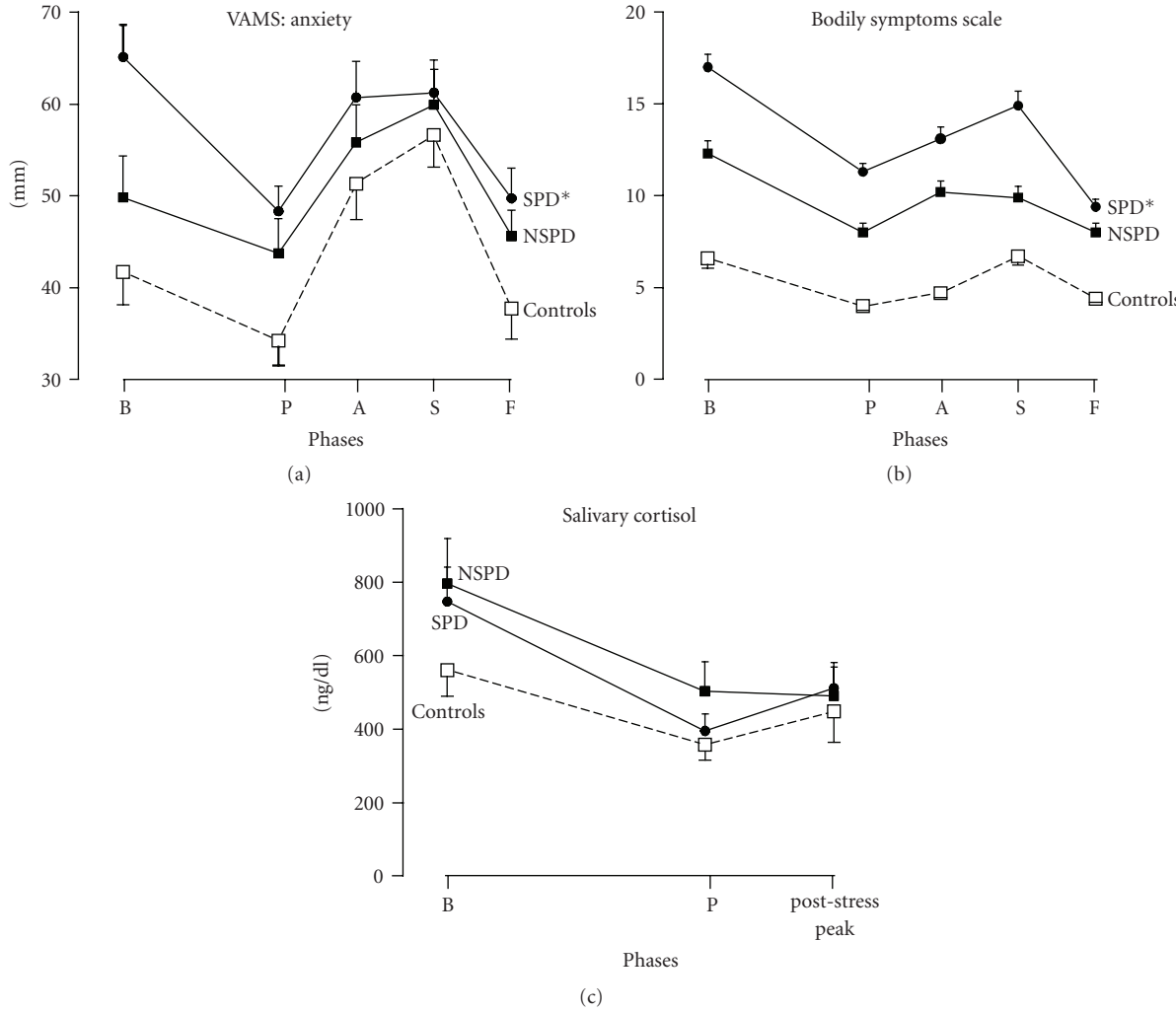

Figure 2: Changes in anxiety (VAMS, upper panel), bodily symptoms (BSS, mild panel), and salivary cortisol levels (lower panel) induced by simulated public speaking (SPS) in symptomatic panic patients (SPD), nonsymptomatic patients (NSPD), and healthy controls. The phases of the experimental session are beginning (B), pretest (P), anxiety during speech preparation (A), performance anxiety during the speech (S), and final (F). Points in the curves indicate mean values and vertical bars the S.E.M. Figure modified from Garcia-Leal et al. [43] and Parente et al. [44]. * = significant difference ($P < .05$) from controls.

brain structures possibly involved in the neurobiology of PD encompass the prefrontal cortex, anterior cingulated cortex, hypothalamus, amygdala, hippocampus, and the midbrain, including the periaqueductal grey matter [2].

Structural neuroimaging studies, using magnetic resonance imaging (MRI), have shown that anatomical brain abnormalities, particularly in the temporal lobes, are more frequently observed in panic patients than in controls [47–49]. A quantitative evaluation of specific brain structures has also demonstrated differences between PD patients and healthy volunteers, characterized by a reduction of the volume of temporal lobes, amygdala, and hippocampus (trend) in PD patients compared to controls [50–52].

Voxel-based morphometry (VBM) is a more sophisticated approach of structural neuroimaging that provides an automated method of segmentation into gray matter, white matter, and cerebrospinal fluid (CSF) compartments and allows the investigation of differences in regional volumes along the whole brain [53]. Using the VBM technique, Protopopescu et al. [54] have shown an increase in gray matter volume of the midbrain and rostral pons of the brainstem of panic patients compared with healthy controls. At a lower significance threshold, they have also reported increased ventral hippocampal and decreased regional prefrontal cortex volumes in PD.

In a recently published study [55], we have also found a relative increase in gray matter volume of midbrain and pons (on left) in panic patients. As it can be seen in the Figure 3, additional findings include increase in gray matter volume of the left insula and left superior temporal gyrus and a relative gray matter decrease in the right anterior cingulate cortex. The anterior insula has close connections to the amygdala and, together with the ventromedial prefrontal cortex, anterior cingulate cortex,hypothalamus, and periaqueductal gray

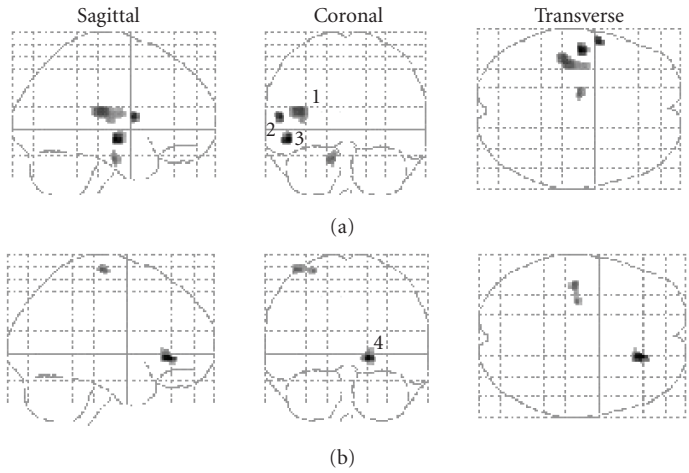

Figure 3: Statistical parametric maps displaying significant differences in gray matter volume of PD patients ($n = 19$) relative to healthy controls ($n = 20$). (a) Increased gray matter volume, (b) decreased gray matter volume. 1 = left insula; 2 = left superior temporal gyrus; 3 = midbrain; 4 = right anterior cingulate.

matter, is considered as part of a network that modulates the identification of, and the response to, aversive or threatening stimuli [56] and has been proposed as a key structure involved in the neurobiology of anxiety disorders [57]. In particular, the increase of gray matter volume of midbrain is in agreement with the proposition that periaqueductal gray matter would be implicated in the pathophysiology of PD as well in the antipanic action of antidepressant drugs [2, 4, 16].

Functional neuroimaging studies have also contributed to a deeper understanding of the neural substrates of PD. In a seminal work, using positron emission tomography (PET), Reiman et al. [58] have found abnormalities in the parahippocampal gyri, characterized by an abnormal asymmetry (left less than right) of the regional cerebral blood flow (rCBF), observed, during rest, in panic patients vulnerable to the lactate challenge. Further functional studies have also shown alterations in the metabolism or blood flow of hippocampus and parahippocampal areas of panic patients [59–65] and this seems to be the most consistent finding across the studies with functional neuroimaging in PD. Other areas implicated in the pathology of PD by functional studies are prefrontal cortex [59, 60, 65], anterior cingulate gyrus [62, 65], superior temporal cortex [61, 62], amygdala [63, 64], hypothalamus [62], and thalamus [63, 64].

Considering that the PAG is a small brain structure, the detection of dysfunctions of its metabolism is not straightforward, due to limitations of the neuroimaging technique itself. Even so, some studies have reported abnormalities in the midbrain of panic patients.

Just before being submitted to a pentagastrin challenge, panic patients, compared with healthy volunteers, have shown an increase of blood flow in parahippocampal gyrus, left hippocampus, right temporal lobe, orbitofrontal cortex, anterior cingulate gyrus, hypothalamus, thalamus, and midbrain, "probably" PAG [62]. Interestingly, bilateral insula, inferior frontal gyrus, and right amygdala have shown abnormalities in their metabolism in the opposite direction, with a decrease of blood flow, what suggests that the inhibitory function of forebrain structures over phylogenetically more primitive structures, such as the PAG, would be impaired in panic patients.

In the same direction, Sakai et al. [63, 64] have found higher levels of glucose uptake in the midbrain, caudal pons, and medulla in panic patients than controls. They also have shown an increase of the metabolism in bilateral amygdala, hippocampus, and thalamus. In a further study, the same group [65] has shown a decrease of glucose uptake in the right hippocampus, left anterior cingulate, left cerebellum, and pons and an increase of glucose uptake in bilateral medial prefrontal cortices in panic patients that had shown clinical improvement after a cognitive-behavioral therapy intervention. These changes in the brain metabolism with the treatment with antidepressants or cognitive-behavioral therapy have not been found in previous studies [60, 66]. More interestingly for this review is the fact that they have demonstrated a correlation between the percent changes in glucose utilization in the midbrain "around PAG" and those of the number of panic attacks during the 4-week period before each scan, which shows a direct relation between PAG metabolism and the occurrence of panic attacks.

As discussed above, serotonin has been largely implicated in the pathophysiology of panic disorder, and some evidence from neuroimaging studies suggests alterations in the 5-HT system of PD patients. The intravenous administration of d-fenfluramine, which induces the neuronal release of serotonin, has provoked a decrease of blood flow in the left posterior parietal-superior temporal cortex in panic patients [66]. A lower volume of distribution of a selective radioligand of serotonergic receptors 5-TH_{1A} type has been described in the anterior cingulate, posterior cingulate, and raphe of nonmedicated panic patients relative to controls [67]. A significant decrease in the serotonin transporter (5-HTT) binding in the midbrain, temporal lobes, and thalamus

of symptomatic panic patients free of medication has also been reported [68]. However, in comparison to patients with current symptoms, panic patients in remission and free of medication have normal 5-HTT binding properties in the midbrain and in the temporal regions but still show significantly lower thalamic 5-HTT binding. Considering all the patients' (current and in remission) significant negative correlations between the severity of panic symptoms and the midbrain, temporal lobe, but not thalamic 5-HTT binding, has also been demonstrated [68].

These abnormalities in the binding of 5-HT receptors and transporter in midbrain areas are in agreement with the hypothesis that the occurrence of panic attacks would be caused by spontaneous activations of the fight/flight response organized by the PAG and inhibited by 5-HT [16].

Although few studies have applied functional magnetic resonance imaging (fMRI) in panic patients so far, most of them confirmed alterations in the brain areas previously supposed to be involved in the neurocircuitary of PD. In a paradigm of mental imagery of neutral, moderate, and high anxiety situations, panic patients have shown increased neuronal activation in the inferior frontal cortex, hippocampus, and anterior and posterior cingulate, extending into the orbitofrontal cortex bilaterally, during the anxious blocks compared to neutral blocks [69]. Panic patients have also shown significantly higher activation in left posterior cingulate and left middle frontal cortices and a more pronounced asymmetry (right > left) in parahippocampal regions in response to a threat-related stimuli, in comparison to healthy volunteers [70]. Compared to healthy controls, panic patients have demonstrated significantly less activation to fearful faces in the cingulate cortex and the amygdala, bilaterally [71].

For our knowledge, none of these fMRI studies have reported functional alterations in the midbrain. This not only can be due to limitations of the technique itself that do not allow the analysis of changes of fMRI signal in such a small area but also can be related to the hypotheses underlying the studies, which drive the choice of the paradigm of psychological activation and determine the regions of interest where the possible alterations will be looked for. For instance, in the light of the comprehensive view of the neurobiology of anxiety and fear proposed by Deakin in Graeff [16] a suitable paradigm to provoke enough haemodinamic response of midbrain areas would be related to the process of innate fear in humans.

In this regard, a very interesting work carried out with healthy volunteers has brought some light to this discussion. Mobbs et al. [72] have evaluated the effects on brain activation of the distance of a virtual predator. In this paradigm, participants could control the movements of a virtual prey (represented as a dot) in a labyrinth presented on a video-screen, using a keyboard, aiming to avoid a virtual predator (represented by a triangle) with the ability to chase, capture, and inflict pain. In the case of the predator caught the prey, two levels of pain represented by either one or three electric shocks administered to one finger of the participant. When the predator was far from the prey, the haemodinamic responses observed in the prefrontal cortex and lateral amygdala were more pronounced, particularly when the expected shock intensity was low. In contrast, when the predator was closer, the haemodinamic response shifted to the central amygdala and the PAG, reaching the maximum of activation when the highest level of pain was anticipated. Even more interesting, there was a positive correlation between PAG activation and the reported subjective degree of dread and decreased confidence of escape. These results give strong support to the role of the midbrain PAG in proximal defense, and possibly panic, as early proposed [2, 14].

6. Conclusions

In accordance to results coming from animal research, data from experimental models of anxiety, pharmacological challenges, and neuroimaging studies carried out with healthy volunteers and patients with PD point to the involvement of the PAG in the neurobiology of PD. Nevertheless, these midbrain abnormalities cannot be considered as specific findings, since neuroimaging data have also shown that PD patients have changes in other brain structures that participate in the regulation of fear and anxiety. In a more integrative approach, it is reasonable to suppose that a dysfunction of PAG could be part of a global dysfunction that affects a network of related brain structures, or even a consequence of other dysfunctions, such as low 5-HT function, impairment of inhibitory efferent pathways from rostral brain areas, or both. Further studies conciliating biological vulnerability, environmental influences and, mainly, the connectivity among different brain structures with a clear hypothesis-driven approach are needed.

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Research Article

Viral Vector Induction of CREB Expression in the Periaqueductal Gray Induces a Predator Stress-Like Pattern of Changes in pCREB Expression, Neuroplasticity, and Anxiety in Rodents

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Predator stress is lastingly anxiogenic. Phosphorylation of CREB to pCREB (phosphorylated cyclic AMP response element binding protein) is increased after predator stress in fear circuitry, including in the right lateral column of the PAG (periaqueductal gray). Predator stress also potentiates right but not left CeA-PAG (central amygdala-PAG) transmission up to 12 days after stress. The present study explored the functional significance of pCREB changes by increasing CREB expression in non-predator stressed rats through viral vectoring, and assessing the behavioral, electrophysiological and pCREB expression changes in comparison with handled and predator stressed controls. Increasing CREB expression in right PAG was anxiogenic in the elevated plus maze, had no effect on risk assessment, and increased acoustic startle response while delaying startle habituation. Potentiation of the right but not left CeA-PAG pathway was also observed. pCREB expression was slightly elevated in the right lateral column of the PAG, while the dorsal and ventral columns were not affected. The findings of this study suggest that by increasing CREB and pCREB in the right lateral PAG, it is possible to produce rats that exhibit behavioral, brain, and molecular changes that closely resemble those seen in predator stressed rats.

1. Introduction

Study of the neurobiology of long-lasting changes in affect occurring after stressful events is of interest, an interest heightened by the fact that fearful events may precipitate affective psychopathologies [1, 2]. In extreme cases, a single aversive experience may induce posttraumatic stress disorder (PTSD) [3, 4]. Animal models are useful to enhance understanding of the impact of stress on brain and behavior, permitting simulation of a human condition in a controlled setting allowing study of disorder development. Conditioned fear paradigms, behavior in unfamiliar situations that are fear or anxiety provoking, and more recently, predator stress, are all models used to understand the neurobiology of the impact of fearful events on affect.

Predator stress in our hands involves the unprotected exposure of a rat to a cat [5]. Predator stress may model aspects of PTSD for several reasons. First, predator stress has ecological validity due to the natural threat posed by the predatory nature of the stressor. Second, duration of anxiety-like effects in rats after predator stress, as a ratio of life span, is comparable to the DSM IV duration of psychopathology required for a diagnosis of chronic PTSD in humans. Third, predator stress has neurobiological face validity in that right amygdala and hippocampal circuitry are implicated in behavioral changes produced by predator stress, and these areas are consistent with brain areas thought to be involved in PTSD [6–9]. For example, brain imaging implicates hyperexcitability of the right amygdala in response to script-driven trauma reminders in the etiology of PTSD [10–14]. Fourth, parallel path analytic studies using data from Vietnam veterans suffering from PTSD and predator stressed rodents find that in both humans and rodents, features of the stressor predict the level of anxiety [6]. For example, in predator stressed animals, the more cat bites received, the higher the level of anxiety measured a

week later. Finally, similar lasting changes in startle and habituation of startle are seen in both predator stressed rats and humans with PTSD [6, 15–18].

Predator stress is fear provoking and stressful [19–22]. Moreover, cat exposure produces long-lasting increases in rat anxiety-like behavior (ALB) [5, 23], with some behavioral changes lasting three weeks or longer [5, 6, 24]. Behavioral effects of predator stress have been evaluated in a number of tests including hole board, elevated plus maze (EPM), unconditioned acoustic startle, light/dark box, and social interaction. Anxiogenic effects of predator stress are NMDA receptor-dependent. Systemic administration of both competitive and non-competitive NMDA receptor antagonists 30 minutes before, but not 30 minutes after, predator stress prevents lasting changes in ALB [16, 25]. Moreover, local NMDA receptor block in the amygdala prevents predator stress-induced increases in ALB [26].

In addition to the behavioral changes, amygdala efferent and afferent neural transmission is altered after predator stress. Specifically, predator stress causes a long-lasting potentiation in neural transmission from the right amygdala (central nucleus-CeA) to the right lateral column of the periaqueductal gray (PAG), and from the hippocampus via the right ventral angular bundle (VAB) to the right basolateral amygdala (BLA) [9, 23, 27]. Moreover, potentiation in these pathways is NMDA receptor-dependent [7]. In addition, NMDA receptor antagonists produce anxiolytic-like effects when microinjected into the dorsolateral PAG [28, 29]. The PAG is also implicated in rodent ALB [30], and is activated by predator stress [31]. Together, these data suggest NMDA receptor-dependent long-term potentiation (LTP)-like change in amygdala afferent and efferent transmission following predator stress contribute to the lasting anxiogenic effects of cat exposure [7, 9, 16]. In support of this conclusion are the findings that amygdala afferent and efferent LTP-like changes are highly predictive of severity of change in ALB following predator stress [9, 23, 27].

Predator stress induced changes in ALB and amygdala neural transmission are accompanied by changes in phosphorylated cAMP response element binding protein (pCREB). Specifically, pCREB-like-immunoreactivity (lir) is elevated in the basomedial (BM), BLA, CeA, and lateral (La) amygdala after predator stress compared to control rats [32]. This is consistent with the elevation of pCREB-lir in the amygdala after forced swimming stress [33, 34], fear-conditioning in mice [35], retrieval of a cued-fear memory [36], and electric shock [37]. In addition to the amygdala, predator stress increases pCREB-lir in the right lateral column of the PAG (lPAG) [23].

As mentioned, NMDA receptor antagonism prior to predator stress blocks increases in ALB and potentiation of amygdala afferent and efferent neural transmission. Since phosphorylation of CREB may be regulated by NMDA receptors [38, 39] and pCREB-lir is increased after predator stress [23, 32], the question of whether NMDA receptor antagonism can block predator stress induced enhancement of pCREB-lir was recently tested. Blocking NMDA receptors with the competitive blocker, CPP, 30 minutes prior to predator stress, prevented stress induced increases in pCREB expression in amygdala, and right lPAG [40]. Of importance, the same dosing regime also blocks predator stress effects on affect and amygdala afferent and efferent transmission [7, 16, 25, 26].

Together these findings provide compelling evidence that predator stress induced increase in pCREB is an important contributor to the changes in brain and behavior of predator stressed rodents. The purpose of the present study was to directly manipulate CREB and pCREB expression to confirm this notion.

Local changes in gene expression in the brain can be achieved with viral vectoring as a method of delivering recombinant genes directly into neurons [41]. There are a variety of viral vectors available but several characteristics of the herpes simplex virus (HSV) make it an ideal candidate for this study. The non-toxic replication defective HSV vector is capable of infecting most mammalian differentiated cell types, it accepts very large inserts and has high efficiency in infecting neurons, being naturally neurotrophic [41, 42]. One of the earliest studies to utilize this method and apply it to rodent anxiety tests found that HSV vectored expression of CREB in the BLA increased behavioral measures of anxiety in both the open field test and the EPM, and enhanced cued fear conditioning [43].

The present study was designed to test the functional significance of pCREB changes within the right lateral column of the PAG. To do this we genetically induced increased expression of CREB in the right lPAG with HSV vectors and determined the effects of these manipulations on behavior and amygdala efferent transmission (CeA-lPAG). We transfected the neurons of the right PAG in an area where pCREB levels and CeA-PAG transmission are elevated after predator stress (see Adamec et al. [8, 23]).

2. Methods

2.1. Ethical Approval. The procedures involving animals reported in this paper were reviewed by the Institutional Animal Care Committee of Memorial University and found to be in compliance with the guidelines of the Canadian Council on Animal Care. Every effort was made to minimize pain and stress to the test subjects while using as few animals as possible.

2.2. Animals. Subjects were male hooded Long Evans rats (Charles River Canada). Rats were housed singly in clear polycarbonate cages measuring 46 cm × 24 cm × 20 cm for one week prior to any testing. During this week, rats were acclimatized to their cage, and handled. Handling involved picking up the rat and gently holding it on the forearm. Minimal pressure was used if the rat attempted to escape, and grip was released as soon as the rat became still. Rats were handled in the same room as their home cage for one minute each day during the week long adaptation period. Rats were given food and water *ad lib* and were exposed to a 12-hour light/dark cycle with lights on at seven a.m. The rats weighed approximately 200 g on arrival and between 230 and 280 g on the day of testing.

2.3. Groups. After lab adaptation and handling, the 12 subjects were randomly assigned to one of three groups of four. One group served as a handled control (Handled GFP) while another was predator stressed (Predator Stressed GFP). Both these groups were injected in the right lateral PAG (described in what follows) with the HSV-GFP vector before further treatment. This vector consisted of an HSV virus carrying a green fluorescent protein gene (GFP), a reporter used to visualize vector placement and virus induced gene expression. This injection also served to control for any effects that GFP per se might have. The third group was also handled (Handled CREB) and before further treatment received an injection in the right lateral PAG with an HSV-CREB vector. This vector included genes for both CREB and GFP. The GFP served as a reporter of gene expression, and the CREB gene elevated CREB levels in the target area.

It is recognized that a group size of four is small for behavioral studies of this nature. The small numbers were necessitated by the availability of the virus. The implications of the small group size are addressed further at the end of Section.

2.4. Surgical Microinfusion of Viral Vectors. Virus injections were done in the lateral column of the right PAG, where pCREB increases in predator stressed rats have been observed [23, 32, 40]. The injections involved lowering a sterile 25 gauge needle attached to a microliter syringe into the brain using a sterotaxically mounted microliter syringe holder. The coordinates for the microinfusion according to the atlas of Paxinos and Watson [44] were, 6.3 mm posterior to bregma, 0.5 mm lateral from the midline, and 5.5 mm below the skull. The injection of 0.5 μL (in a concentration of 4.0×10^7 infectious units/mL supplied from University of Texas South West Medical School) was given at a rate of 0.5 μL per five minutes with the needle left in place for five minutes post injection. This dose and rate were derived from the experience with the vector of one of us (Berton). Moreover, in pilot studies with HSV-GFP, a 0.5 μl injection at this rate produced GFP expression localized to the right lateral column of the PAG over an AP plane range of.7 mm at three days post injection, the time of maximal protein expression induced by this vector [43, 45].

Injections were performed under chloral hydrate anesthesia (400 mg/kg, IP) using aseptic technique. Preanaesthetic doses of atropine were given (1.2 mg/kg). Local anesthesia of wound edges was achieved with marcaine and epinephrine (2%) infusion and supplemented as needed. Holes in the skull were closed with sterile gel foam and sealed with sterile bone wax and scalp wounds sutured. Rats were kept warm under a lamp post surgery until they began to walk and groom, at which time they were returned to their home cage. Surgery took approximately one hour for each subject.

2.5. Cat Exposure and Handling Procedures. Three days after virus (HSV) injection, when viral expression is peaking [43, 45], rats were either handled or predator stressed. On the day of testing, predator stressed rats were exposed to the same adult cat as described elsewhere [5]. The cat exposure lasted 10 minutes and was videotaped to capture the activities of both the cat and the rat. The cat generally observed the rat at a distance with the intermittent approach and sniffing. On occasion, the cat would mildly attack the rat but no injuries were ever observed. At the end of the test, the rat was placed back into its home cage and left undisturbed. Rats in the other two groups whose treatment included only handling did not come into contact with the cat, cat odors or rats that had previously been exposed to cats. On the day of testing, rats in these groups were weighed and handled as previously mentioned for 1 min. After this handling period the rats were returned to their home cage and left undisturbed. Handled and predator stressed rats home cages were kept in separate rooms.

2.6. Behavioral Testing and Behavioral Measures. Four days after HSV injection and one day after treatment, ALB was measured in the hole board, EPM and startle tests. The hole board test took place just before the EPM as an independent test of activity and exploratory tendency [46].

2.6.1. Hole Board and Elevated Plus Maze Testing and Measures. The hole board and EPM were constructed and used as described elsewhere [5]. The behavior of the rats in the hole board and EPM was videotaped remotely for later analysis. Rats were first placed in the hold board for 5 minutes. At the end of this time period they were transferred by gloved hand to the EPM for a further 5 minutes of testing. At the end of this testing period the rats were returned to their home cages.

Several measures of activity and exploration were taken while the rat was in the hole board. They included frequency of rearing (activity), and head dips, a measure of exploratory tendency scored when the rats placed its snout or head into a hole in the floor. Fecal boli deposited were also counted. A measure of thigmotaxis was time spent near the wall of the hole board. This measure was quantified as the rat having all four feet in the space between the holes for head dipping and the wall. Time spent in the center of the hold board was also recorded. A rat was considered to be in the middle when all four feet were in the center space defined by a square drawn through the four holes in the floor of the box.

In the EPM, exploration and activity were scored as the number of entries into the closed arms of the maze (closed arm entries). An entry was only recorded when the rat had all four feet inside one arm of the maze. Other measures of exploration included head dips, scored when a rat placed its snout or head over the side of an open arm, and rearing as a measure of activity. These behaviors were divided into three types: protected (rat had all four feet in closed arm for rearing or hindquarters in the closed arm for head dips), center (rat has all four feet in center of maze), and unprotected (rat has all four feet in an open arm). Time spent grooming was also recorded using the same three subdivisions.

Measures of anxiety-like behavior were also taken. Two measures assessed open arm exploration: ratio time and ratio entry. Ratio time was the time spent in the open arms of the maze divided by the total time spent in any arm of the maze. The smaller the ratio the less open arm exploration

indicating a more "anxious" rat. Ratio entry was the number of entries into the open arms of the maze divided by the total entries into any arm of the maze. Again, the smaller the ratio, the less the open arm exploration experienced, the more "anxious" the rat.

Adamec and Shallow [5] were the first to adapt the concept of risk assessment to the EPM. This measure was scored when the rat poked its head and forepaws into an open arm of the maze while keeping its hindquarters in a closed arm. The frequency of risk assessment was measured and converted to relative risk assessment by dividing these frequencies by the time spent in the closed arms. Fecal boli deposited in the EPM were also counted.

2.6.2. Startle Testing and Measures. Startle testing was conducted on the same day as the hole board and EPM. The startle response was determined using a standard startle chamber (San Diego Instruments). The apparatus was fitted with a 20.32 cm Plexiglass cylinder used to hold the animal during the test, as well as a speaker for producing the sound bursts. A piezoelectric transducer positioned below the cylinder detected motion of the animal in the cylinder. The output from this transducer was fed to a computer for sampling.

Prior to startle testing, animals were adapted to the apparatus for 10 minutes with a background white noise level of 60 dB. Then rats were subject to 40 trials (1/30 seconds) of 50 milliseconds bursts of 120 dB of white noise rising out of a background of 60 dB. Half the trials were delivered while the chamber was dark while the other half were delivered with an accompanying light (light intensity of 28 foot candles or 300 lux). The light trials were randomly interspersed among the dark trials. During the light trials, the lights would come on 2.95 seconds prior to the sound burst and remain on for the duration of the sound burst, terminating at sound offset (lights on for a total of 3 seconds). The chamber was in darkness between trials. A computer attached to the transducer recorded 40 samples of output. Samples included a 20 milliseconds baseline and 250 milliseconds sample after onset of the noise burst. Average transducer output just prior to noise burst was saved as a baseline (V_{start}). The computer then found the maximal startle amplitude within each of the samples (V_{max}). Both these measures were saved for later analysis. Peak startle amplitude was expressed as V_{max}-V_{start} for analysis. At the end of the startle session the rats were returned to their home cages. The apparatus was washed between rats.

2.7. Electrophysiological Recording Procedure. Five days after HSV injection and two days after treatment, all rats were anaesthetized with urethane (1.5 g/kg) given in three divided doses separated by 10 minutes. Then the rats were placed in a sterotaxic instrument and injected under the scalp with marcaine (2% epinephrine) to locally anesthetize and reduce bleeding. The skull was exposed and holes drilled to permit stereotaxically guided insertion of stimulating electrodes into the central amygdala (CeA). Recording microelectrodes were placed into the PAG. Stimulating and recording electrode pairs were placed in both hemispheres. In addition, skull screws were placed over the olfactory bulb to serve as a ground and references. Stimulation electrodes were twisted bipolar stainless steel (0.125 mm in diameter, Plastics One) aimed at the CeA. Recording electrodes were stainless steel microelectrodes (1 μm tip diameter, 0.6–1 MΩ, Frederick Haer) aimed at the PAG (verified coordinates appear in Table 1). Rats were placed in a shielded box for stimulating and recording experiments. Temperature was maintained between 36-37°C by a rectal thermistor connected to a digital thermometer and feedback control to a DC heating pad (Frederick Haer) under the rat. CeA was stimulated using a single biphasic constant current pulse (width .2 milliseconds) at 1/5 seconds over a range of intensities (.025–2.5 mA), 10 stimulations per intensity. Evoked potentials were sampled by computer and later analyzed from data stored on computer using DataWave software (see Adamec et al. [27] for further method details).

Table 1: Mean (and SEM) of electrode coordinates averaged over group and hemisphere.

Brain area	Mean	SEM
CeA AP	2.37	0.036
CeA Lateral	3.98	0.037
CeA Vertical	7.92	0.027
PAG AP	6.1	0.061
PAG Lateral	0.33	0.02
PAG Vertical	5.62	0.034

AP: Anterior-posterior plane (mm posterior to Bregma); Lateral: lateral plane (mm lateral to mid line); Vertical: vertical plane (mm below Bregma); CeA: central amygdala; PAG: periaqueductal gray

At the end of recording, rats were overdosed with Chloral Hydrate (1000 mg/mL, 1 mL, IP) and perfused with cold phosphate buffered saline and 4% Para-formaldehyde. Brains were extracted, sunk in 20% sucrose overnight at −4°C and then stored at −70°C. Subsequently brains were examined histologically for electrode locations, under green fluorescence microscopy to visualize GFP production and immunohistochemically to study pCREB expression.

2.7.1. Electrophysiology Analysis Methods. The main measure of the size of the evoked potential was peak height (PH). The peak height at each intensity was taken by computer from field potential averages as illustrated in Figure 4. The raw PH at each intensity was expressed as a ratio of PH observed at threshold (see [23, 27]).

2.8. Immunocytochemistry. Thick frozen coronal sections (40 μm) were cut from 5.8 to 6.8 mm posterior to bregma [44] to capture the same areas of the PAG studied in past predator stress experiments, and to capture the targets of virus injection and electrophysiological recording . Anterior-posterior (AP) plane location was determined by counting sections from the decussation of the anterior commissure (AP −0.26 from bregma, [44]) to the desired AP plane. This counting of sections allowed for an estimation of the AP plane position to the nearest 40 μm during cutting. Every second section was saved, which provided 12 sections from

each brain for processing. To ensure even distribution a multiple of three brains (one brain from each group) was cut and processed at the same time.

After sectioning, one section from each group was placed in a plastic tube with nylon covering at one end and then immersed in a plastic well containing phosphate-buffered saline (PBS). Each tube contained three sections, which were processed at the same time. The tubes were removed, blotted, submerged in a solution of normal goat serum and Triton X-100 and placed on a rocker for 1 hour. The sections were washed with PBS, blotted and incubated at −4°C for either 24 or 48 hours (reused antibody) in the primary phospho CREB antibody (Upstate/Chemicon). Consistent with past work [23, 32], a dilution of 1/500 for the primary antibody was used. After incubation, sections were washed again with PBS, blotted, and then immersed in the secondary biotinylated antibody (goat antirabbit) for 1 h. Sections were washed, blotted, and placed in the ABC (Vector Stain kit) solution for 1 h on a rocker. Finally, sections were washed with PBS for a third time, blotted and submerged in diaminobenzadine (DAB) solution for 5–25 min, monitoring for staining. Sections were then washed with PBS again, before mounting onto slides, dehydrated and cover slipped.

2.8.1. Image Analysis (Densitometry). Stained sections were analyzed blind to group assignment using image analysis software (MOKA software, Jandel). Hemispheres were measured separately. The PAG was divided into ventral, dorsal, and lateral areas to reflect the functional columnar organization described by Bandler and Depaulis [47]. This was done using the aqueduct of Sylvius as a guide. Horizontal lines were drawn from the top of the aqueduct to the outside edge of the PAG and from the bottom of the aqueduct to the outside edge of the PAG for both left and right hemispheres (see also [23]). The top sections were considered dorsal PAG, the middle sections were lateral PAG and the bottom sections were ventral PAG.

Raw pCREB lir densitometry data of each column in each hemisphere were converted to optical density (OD) units relative to the whole section. This was done by converting the raw PAG and raw whole section densitometry data to OD units via a calibrated step wedge. An image of the calibrated step wedge was taken at the same time as section images for each rat. Exponential fits of raw transmission values (x) to calibrated OD values were done by computer (Table Curve program, Jandel). All fits were good (all df adjusted $r^2 > .9$, $P < .01$). The exponential was then used to interpolate and convert raw transmission values to OD units. Analysis was performed on the ratio of average OD values in particular PAG areas to average OD values for the entire section.

3. Results

3.1. Anxiety-Like Behavior in the EPM. Groups differed in the measures of open arm exploration-ratio time and ratio entry (all P (2,9) ≥ 14.78, P <.002). Predator stress reduced ratio time and ratio entry in the EPM, consistent with many past studies (Figure 1, upper right panel, ratio time only is shown, ratio entry findings were very similar). Predator stress

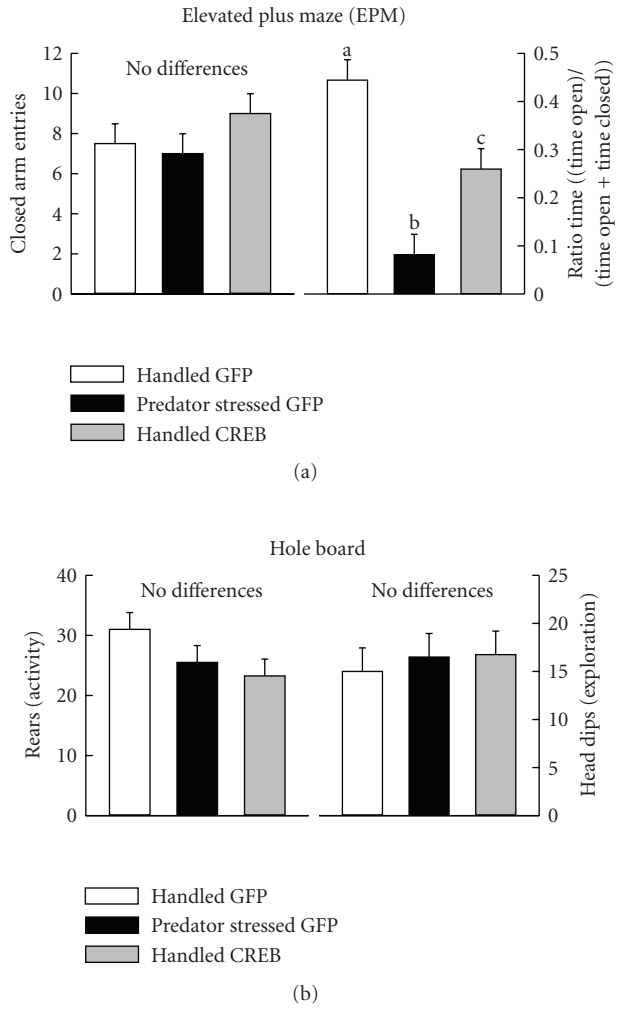

Figure 1: Plotted over groups are mean ± SEM of EPM and hole board behaviors. Means marked with a different letter differ from each other ($P < .05$). (a), shows frequency of closed arm entries (left) and ratio time (right) in the EPM. (b), shows the frequency of rears (left) and head dips (right) in the hole board test.

reduced open arm exploration (increased anxiety) the most relative to controls (Handled GFP). Injection of HSV-CREB in the right PAG alone was also anxiogenic in the EPM, reducing ratio time and entries in Handled-CREB rat to a level between Handled GFP and Predator Stressed GFP rats (Figure 1, Tukey Kramer test, $P < .05$).

With regard to ratio frequency of risk assessment, though there was no group effect (F (2,9) = 2.58, P <.13), a planned t-test contrasting the predator stressed group with the two handled groups combined (which did not differ) revealed that predator stress reduced risk assessment relative to both Handled groups (Figure 2; t (9) = 2.19, $P < .029$, 1 tailed). This finding of reduced risk assessment following predator stress is consistent with many previous studies.

3.2. Exploration and Activity in EPM and Hole Board. There were no differences between groups in closed arm entries (activity) in the EPM (Figure 1, (a) left panel). Similarly,

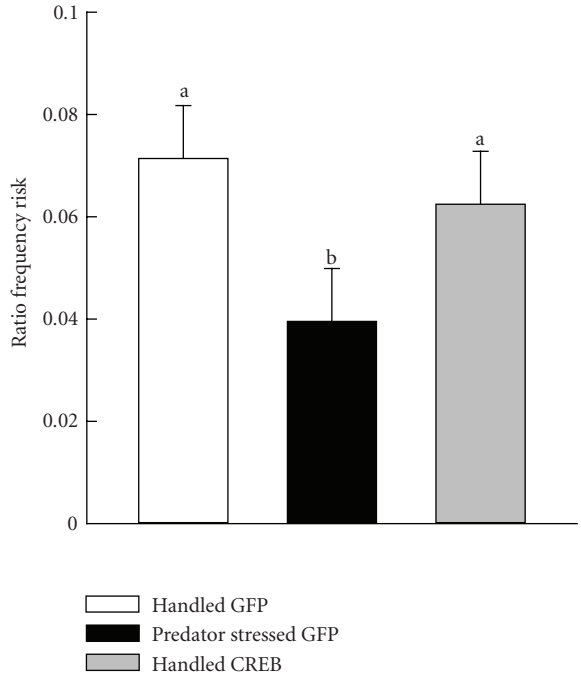

Figure 2: Plotted over groups are mean ± SEM of risk assessment behavior in the EPM. Means marked with a different letter differ from each other ($P < .05$).

groups did not differ in rears (activity) and head dips (exploration) in the hole board (Figure 1, (b) two panels). These data indicate that group differences in open arm exploration seen in the EPM are not the result of changes in activity or exploration.

3.3. Acoustic Startle Response. Startle in the light and dark trials did not differ so analyses across light and dark trials were combined.

3.3.1. Startle Amplitude. Between groups startle data were not normally distributed (Omnibus Normality Test = 148.07, $P < .0001$). Therefore, Kruskal-Wallis one way nonparametric ANOVA on medians of peak startle amplitude over trials was used. Groups differed (χ^2 (2) = 119.90, $P < .001$). Planned comparisons (Kruskal-Wallis multiple comparison z-test $z > 3.98$, $P < .01$) revealed that predator stress increased startle over both handled groups (Figure 3, bottom left panel). Nevertheless startle amplitude of Handled CREB rats was also higher than Handled GFP, but lower than predator stressed animals (Figure 3, (b) left panel).

3.3.2. Habituation of Acoustic Startle Response. Predator stress prolongs habituation to startle [6, 15, 16, 48]. Therefore, habituation to startle in the three groups was determined and compared. Exponential decline functions of the form

$$y = y_0 + ae^{-t/\tau} \quad (1)$$

were fit to the peak startle amplitude mean data from each group across 20 trials (combined light and dark startle trials)

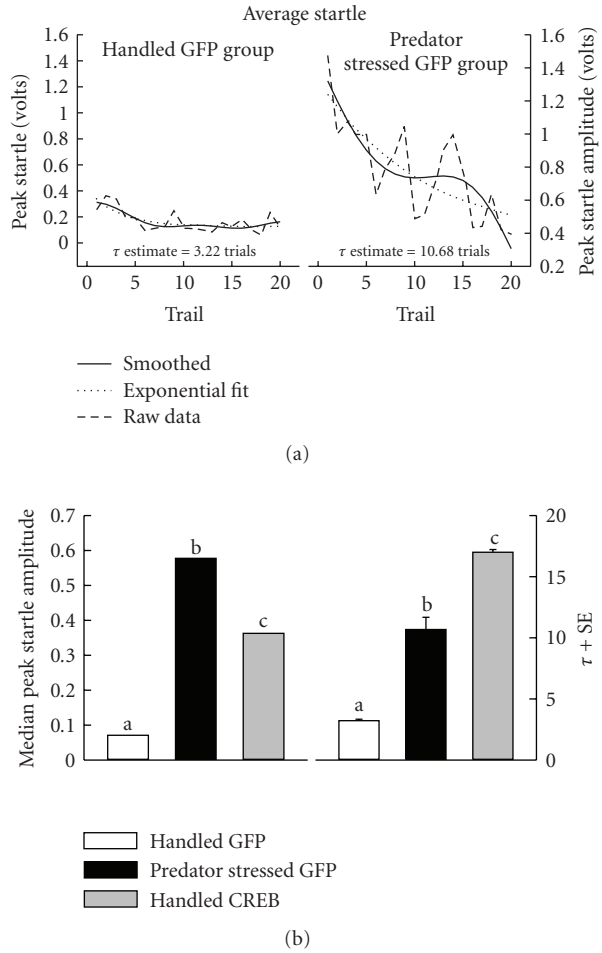

Figure 3: (a) shows example fits (solid line) to an FFT smoothed (20%) function (dashed line) of the means of peak startle amplitude (dotted line) over 20 trials for Handled GFP control (left) and predator stressed (right) rats. Plotted in (b) are median peak startle amplitudes (left) and $\tau \pm$ SE (right), estimated from declining exponential functions, for rats in each experimental group. Medians and Tau values marked with a different letter differ from each other ($P < .05$).

using Jandel table curve V 4.0. In (1), y and y_0 are peak startle amplitude, a is a constant, e is the base of the natural logarithm, t is the trial number and τ is the trial constant, or the number of trials to decline to 37% of the maximal peak startle amplitude. To improve the fit, an FFT smoothing function provided by the program (20% FFT smooth) was applied. Care was taken to ensure the smoothing did not distort the data (Figure 3(a)). All fits were good (degrees of freedom adjusted $r^2 > .84$; all fits F (2,17) > 58.3, $P < .001$; t (38) ≥ 6.18, $P < .01$ for all t-tests of differences from zero of τ). The estimate of τ included a standard error of estimate. These standard errors were used to perform planned two tailed t-tests between groups using the different τ values (Figure 3(b), right panel). The pattern of the findings from this analysis was surprising. Both the Handled CREB and predator stressed group took significantly longer to habituate than Handled GFP controls. While this result was expected for the predator stressed group given previous work, the

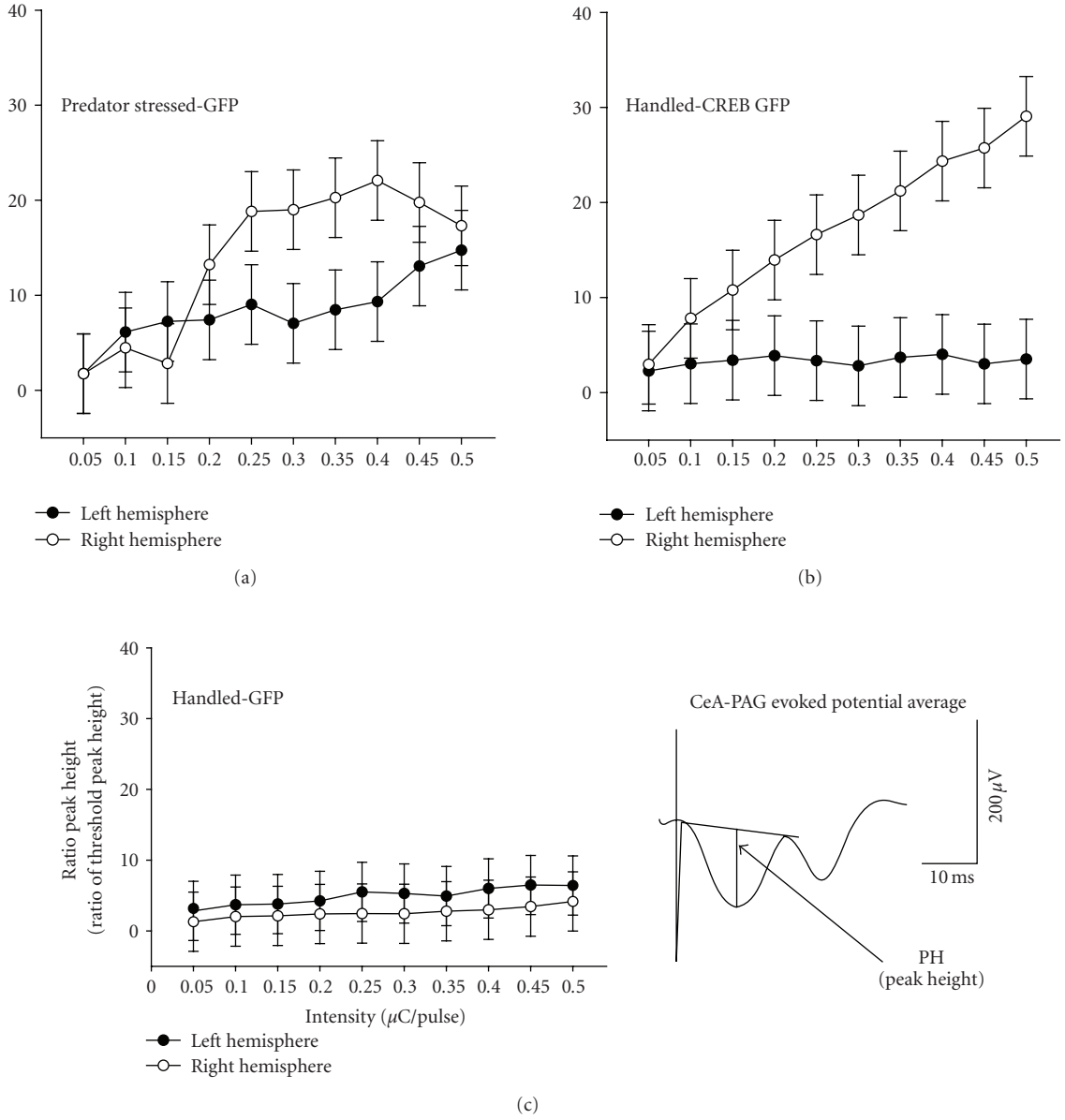

Figure 4: In the lower right is a computer average of a CeA-PAG evoked potential illustrating how peak height (PH) was measured by computer. Plotted in the graphs are means ± SEM of PH of CeA-PAG evoked potentials expressed as a ratio of threshold PH versus intensity of stimulation in μC/pulse (calculated as intensity in μA times pulse width in microsecond to take pulse width into the intensity measure). Means are plotted separately by group and within a group separately by hemisphere.

fact that the Handled CREB group took longer to habituate than the predator stressed group was uncharacteristic of the amplitude findings.

3.4. Electrophysiology. A three way ANOVA was done on ratio PH of the CeA-PAG evoked potential data. The factors examined were Group (Handled GFP control, predator stressed, and Handled CREB), Hemisphere (right and left) and Intensity of stimulation. There was a significant Group x Hemisphere x Intensity interaction (F (12,54) = 2.24, P <.04). The interaction is displayed in Figure 4. Intensity of stimulation was expressed in μC (micro-coulombs) per pulse. All groups were stimulated using the same intensity series, so group differences cannot be attributed to difference in the intensity of stimulation.

Planned comparisons t-test mean contrasts were used to examine the interaction by comparing the three groups at each intensity in each hemisphere. All groups showed the same ratio PH values at intensity 1 in both hemispheres. Moreover, ratio PH in Handled GFP controls were equal in both hemispheres and unchanged over intensity of CeA stimulation (Figure 4(c)). Similarly, left hemisphere ratio PH of Handled CREB rats did not change over intensity and did not differ from ratio PH in right or left hemisphere of Handled GFP controls. In contrast right hemisphere ratio PH of Handled CREB rats rose over intensity (t(54) = 5.61,

Table 2: Mean (and SEM) of PAG cannula coordinates and in relation to PAG electrodes.

Right cannula	Mean	SEM	Mean absolute distance (mm) from the right PAG electrode	SEM
PAG AP	6.13	0.068	0.28	0.034
PAG Lateral	0.36	0.032	0.09	0.026
PAG Vertical	5.62	0.04	0.11	0.028

AP: Anterior-Posterior Plane (mm posterior to Bregma)
Lateral: Lateral Plane (mm lateral to mid line)
Vertical: Vertical Plane (mm below Bregma)
PAG: Periaqueductal Gray

$P < .01$, comparing intensity 1 and 10) and differed from their own left hemisphere ratio PH over intensities 2–10 (all $t(54) > 2.80$, $P < .05$; Figure 4(b), top right panel). Therefore, CREB injection per se selectively potentiated right hemisphere CeA-PAG evoked potentials relative to the left hemisphere and relative to Handled GFP controls, which did not differ from Handled CREB rats in the left hemisphere.

As might be expected from previous work, predator stress potentiated right and left hemisphere CeA-PAG evoked potentials (Figure 4(a), upper left panel). Ratio PH in left and right hemispheres rose over intensity (all $t(54) > 4.68$, $P < .01$, comparing intensity 1 and 10). However, right hemisphere response exceeded the left at intensities 4–9 (all $t(54) > 2.09$, $P < .05$). This suggests that left hemisphere potentiation in predator stressed rats was fading relative to right hemisphere potentiation two days after treatment. Nevertheless, predator stress potentiated left CeA-PAG ratio PH over that seen in the left hemisphere of Handled CREB rats or in the left or right hemispheres of Handled GFP control rats, in that left ratio PH of predator stressed rats exceeded left ratio PH of Handled CREB rats (and left and right ratio PH of Handled GFP control) rats at intensities 3, 5, 9-10 (all $t(54) > 2.04$, $P < .05$).

Comparing right hemisphere ratio PH of Handled CREB and predator stress rats suggests nearly equal potentiation. Groups did not differ at intensities 1-2 and 4–8, but Handled CREB ratio PH did exceed that of predator stressed at intensities 3, 9 and 10 (all $t(54) > 2.15$, $P < .05$). Therefore right PAG CREB injection per se is as effective, or even more effective, than predator stress in potentiating right CeA-PAG evoked potentials.

3.5. Histological Verification of Electrode and Cannula Placements. Tips of stimulating and recording electrodes were visualized microscopically from tissue sections and plotted onto rat atlas sections [44]. Rats from all three groups had correctly placed electrodes, allowing the use of each subject for data analysis. Two way ANOVAs were done examining group and hemisphere factors with separate analyses for the coordinates of each plane (AP, lateral and vertical) for each electrode target. Lateral and vertical coordinates were taken from the atlas sections while AP plane was calculated from section number. No group, hemisphere, or groups x hemisphere interactions were observed. The CeA stimulating electrodes were correctly placed in the medial central nucleus while the recording electrodes were in the lateral columns of the right and left PAG. Average location of tips for both the stimulating and recording electrodes appear in Table 1. Verification of cannula placement was completed in much the same way, average coordinates appear in Table 2. Furthermore, the absolute distance from the recording electrode was very small (Table 2) indicating that electrophysiological recordings were taken from a position close to viral injection.

3.6. pCREB lir Immunohistochemistry Densitometry Analysis. Relative OD data were analyzed separately for each of the three columns in the PAG. The lateral column was of primary interest since this was the area where CREB protein expression was enhanced (Figure 5(a), top left panel). A one way ANOVA of right hemisphere data revealed a significant difference between the groups (F (2,41) = 3.30, $P < .05$). In contrast, groups did not differ in the left hemisphere (F (2,41) = 1.88, $P < .17$). Predator stressed rats had significantly more pCREB lir than Handled GFP controls with the Handled CREB rats falling in between these two groups, differing from neither (Tukey-Kramer Test, $P < .05$). The mean of pCREB lir in Handled CREB rats measured here at 5 days post HSV injection is likely an underestimate of its value at peak expression of CREB, which occurs at three days after HSV injection, when treatments occurred (stress or handling), and which fades thereafter [43].

One tailed t-tests were used to compare within groups across hemispheres based on the prediction that right column pCREB-lir would be increased in predator stressed rats based on previous findings, and on the prediction that increased CREB expression in Handled CREB rats would increase pCREB-lir. Both the predator stressed and Handled CREB rats exhibited more pCREB lir in the right over the left (all t, $P < .04$, 1 tailed), whereas there were no hemisphere differences in the Handled GFP control group.

Data from the dorsal column of the PAG were analyzed in the same way with somewhat differing results (Figure 5(b), top right panel). A one way ANOVA of right hemisphere data revealed a significant difference between groups (F (2,41) = 3.66, $P < .04$) while the left hemisphere again showed no group difference (F (2,41) = 0.74, $P < .49$). Comparison of the groups in the right hemisphere revealed that the predator stressed rats showed elevated pCREB lir which was greater than the Handled groups which did not differ (Tukey-Kramer tests, $P < .05$). Furthermore, comparison of groups across the two hemispheres revealed that, like the lateral

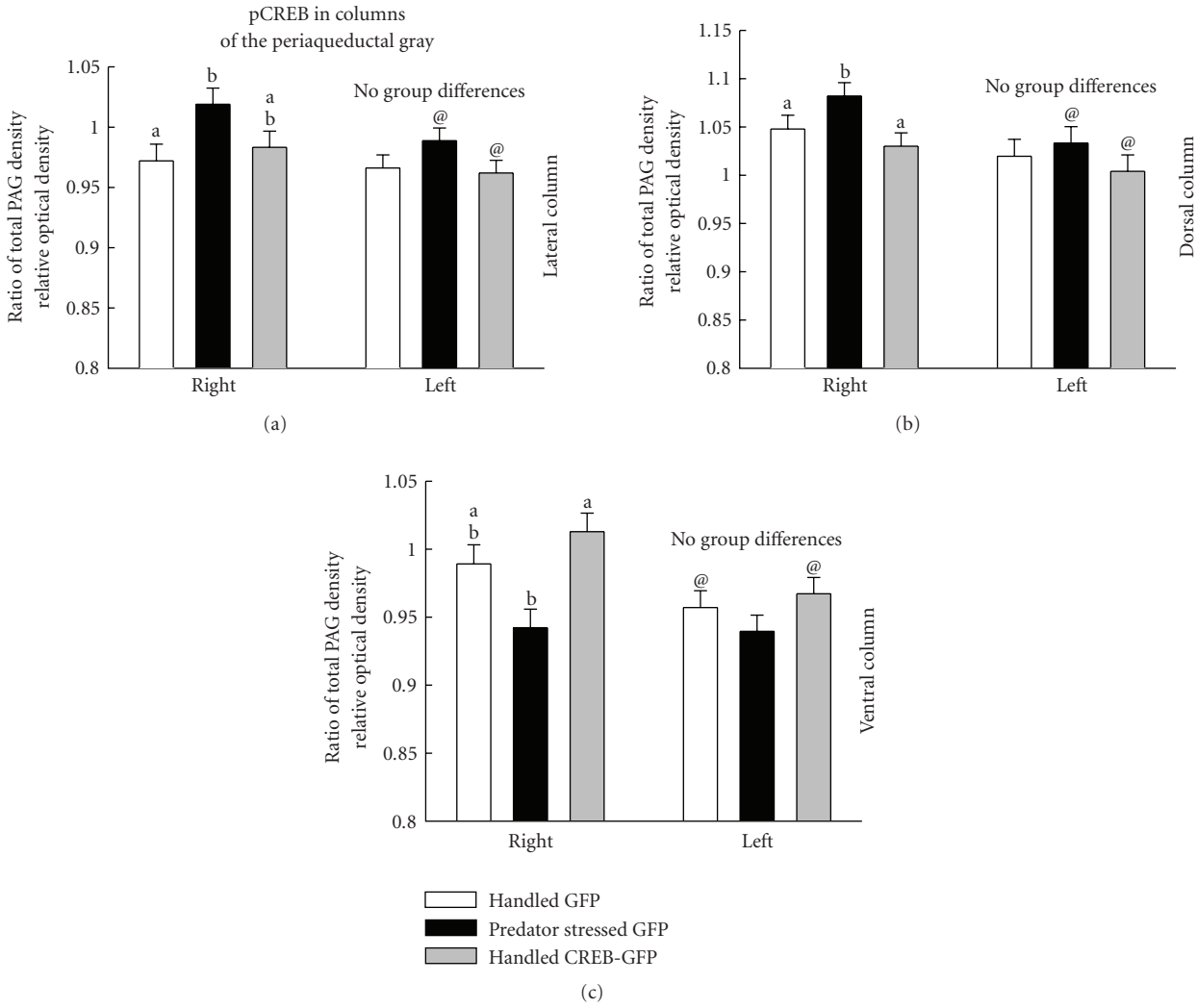

Figure 5: Mean ± SEM relative optical density units (PAG optical density units divided by total PAG section optical density units) in all three columns for all experimental groups are plotted. The left side of each panel displays data from the right hemisphere while the right side of the panel illustrates left hemisphere data. For a given column, means marked with the same letter do not differ, but differ from those with different letters, while means marked with two letters do not differ from means marked with either of the letters (Tukey-Kramer tests, $P < .05$). Means marked with "@" show a within group difference between hemispheres ($P < .05$ 1 tailed test).

column, both the stressed and Handled CREB group had elevated pCREB lir in the right hemisphere as compared to the left (all t, $P < .04$ t tailed) with the Handled GFP control group again showing no difference between hemispheres.

Expression of pCREB in the ventral column of the PAG presented another pattern of results (Figure 5(c), bottom panel). A one way ANOVA in the right hemisphere revealed a significant difference between groups (F (2,41) = 6.93, $P < .003$). In this case however, the stressed rats had significantly lower pCREB expression than Handled CREB rats with the Handled GFP control group falling in between, differing from neither (Tukey-Kramer tests, $P < .05$). Much like the other two columns, no difference was seen between groups in the left hemisphere (F (2,41) = 1.36, $P < .28$). Comparisons within groups across hemispheres showed that both the Handled GFP control and Handled CREB rats had increased pCREB in the right over the left hemisphere (all t, $P < .01$), while there was no hemisphere difference in predator stressed rats. These ventral column results mirror previous findings with the exception of the hemisphere differences [40]. The fact that the Handled CREB group did not differ from the Handled GFP controls indicates that CREB may not be having an effect in this column. This also suggests that regional differences in the pathways controlling phosphorylation of CREB may be dependent on predator stress.

3.7. Visualization of GFP. Verification of gene expression was achieved by examining all PAG sections taken for green flourescence as evidence of expression of the reporter GFP. Green flourescence in the right PAG verified gene expression of GFP occurred after HSV injection in the vicinity of the injection cannulas and PAG recording electrodes (Figure 6, e.g., five days after HSV injection). Since flourescence ranges from cannula to PAG electrodes, one can derive a sense of

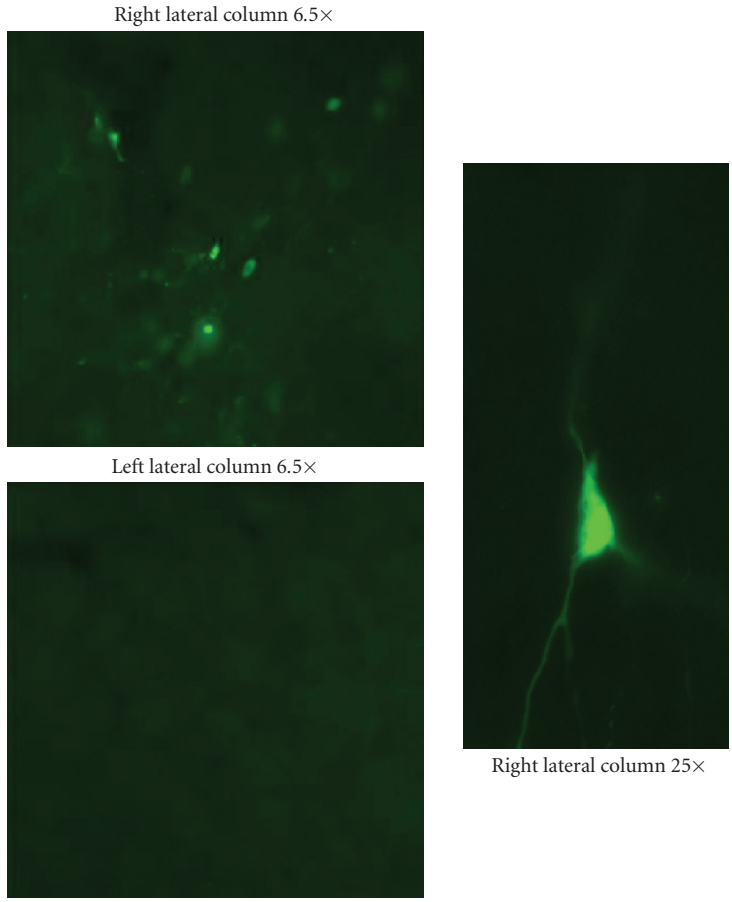

Figure 6: Depicted in the figure are fluorescence photomicrographs illustrating green florescent protein (GFP) localized in the right lateral column of the periaqueductal gray (PAG) 5 days after HSV injection in the right lateral PAG. Magnifications are 6.5 and 25 times (×).

the AP plane range of gene expression from PAG electrode position relative to cannulas. Referring to Table 2, evidence of gene expression five days post HSV injection appears over a range of ±.28 ±.034 mm (mean ± SEM) from the cannula in the AP plane. This represents a range nearly as extensive in previous pilot work which found that at the time of peak gene expression (three days post HSV injection), GFP expression was localized to the right lateral column of the PAG over an AP plane range of ±.35 mm from the cannula.

3.8. Power Associated with Significant Results. Given the small n of groups, power (α =.05) of all significant findings was calculated. Significant behavioral and electrophysiological findings all had power values in excess of.90. Power associated with pCREB expression analyses varied with column of the PAG, ranging from .82 to .91 in the dorsal and ventral columns to a reduced power for the lateral column results of .60.

The power of a test depends on the value of the type I error (here α = .05), the sample size, the standard deviation, and the magnitude of the effect being tested reflected here in magnitude of mean differences. Most findings appear quite robust with power values in excess of .80, suggesting robust effects of predator stress and virally induced CREB expression on brain and behavior. The reduced power for the lateral column pCREB findings suggests a fading effect in this column.

4. Discussion

The primary purpose of this study was to examine the functional significance of pCREB changes within the right lateral column of PAG. This was accomplished by genetically inducing an increased expression of CREB, through viral vectoring, and determining the behavioral, electrophysiological and pCREB expression changes in comparison to predator stressed and Handled GFP control rats.

4.1. Behavioral Effects of Viral Vectoring CREB . Viral vectoring to induce CREB expression in the right lateral column of the PAG produced behavioral effects resembling those seen in predator stressed rats. Handled CREB rats showed increased open arm avoidance in the EPM (decreased ratio time and entry) as compared to Handled GFP controls. However, predator stress was even more effective, increasing open arm avoidance over that seen in Handled CREB rats. Despite, this graded change in anxiety between groups, measures of activity and exploration in the plus maze or hole board did

not differ (Figure 1). This pattern of results suggests changes in open arm exploration (anxiety) in EPM are not due to changes in activity or exploratory tendencies, consistent with previous findings using predator stressed rats in similar testing situations [5, 9, 15, 23, 25, 32].

The ability of CREB per se to increase open arm avoidance in the absence of any predator stress is a remarkable finding. It suggests a direct role for CREB and possibly pCREB expression [32] in behavioral changes produced by stress. Predator stress likely induces CREB signaling change, and then behavioral changes via NMDA receptor activation in the PAG [7, 23, 25, 40]. In the present study, stress effects were mimicked by bypassing the NMDA receptor activation and directly activating CREB mediated processes.

Not all effects of predator stress were mimicked by PAG CREB induction, however. Normally predator stress reduces ratio frequency risk assessment in an NMDA receptor-dependent manner [5, 7, 16, 25, 26]. While predator stress in the present study also reduced risk assessment, the risk assessment of Handled CREB rats was unaffected and did not differ from Handled GFP controls (Figure 2). The lack of a predator stress type response in the Handled CREB rats suggests that increasing CREB expression in PAG may not be the only factor that mediates suppression of risk assessment, or alternatively may only affect some EPM behaviors. Other necessary factors at play could include changes in amygdala pCREB expression and potentiation of ventral hippocampal to BLA transmission, both of which follow predator stress [32]. In addition, risk assessment changes produced by predator stress are highly predicted by right hemisphere changes in transmission in both CeA-PAG and hippocampal to BLA pathways [9]. Since only PAG was manipulated in Handled CREB rats, it is likely these other factors were not engaged, but were engaged in predator stressed rats. Perhaps changes in risk assessment require all changes to occur. A change in hippocampal spatial information transfer to BLA might make sense, since risk assessment is described as a form of sampling the immediate environment for potential threats [49]. Other possible reasons include the following. Handled CREB rats were more anxious than Handled GFP controls in EPM, but their level of anxiety was not as great as predator stressed rats. Greater levels of anxiety may be associated with less risk assessment [49], and so the more anxious predator stressed rats displayed reduced risk assessment. Further testing will be required to decide between these possibilities.

Handled CREB rats also had elevated median peak startle amplitude in comparison to the Handled GFP control group. Moreover, the predator stressed group showed startle amplitudes that surpassed those of the Handled CREB rats. This graded response of enhanced startle over groups is reminiscent of open arm avoidance in the EPM, and supports the notion that inducing CREB expression per se induces an anxious state which is milder than that produced by predator stress. Reasons for the milder effects of direct PAG manipulation in comparison to predator stress may parallel those raised above to explain risk assessment discrepancies. Finally, the enhancement of startle amplitude in predator stressed rats is consistent with past studies [6, 23, 26, 27].

Predator stress also reliably decreases rate of habituation of the acoustic startle response [15, 16, 48]. Present data are consistent with these findings in that predator stressed rats took significantly longer to habituate than Handled GFP controls (Figure 3). This replication furthers the validity of predator stress as a model of hyperarousal aspects of PTSD, since delayed habituation to startle is also observed in PTSD patients [50–53].

Surprisingly, the Handled CREB group took even longer than the predator stressed rats to habituate to startle. This finding implicates CREB dependent mechanisms in delay of startle habituation, which are likely NMDA receptor-dependent, given that CPP administered 30 minutes prior to predator stress blocks delay of startle habituation as well as increased right lateral PAG pCREB expression [16, 40]. However, this finding also suggests some difference in mechanisms of induction of neural changes by CREB in PAG underlying enhanced startle amplitude and delay of habituation. Delay in startle habituation has been observed in the absence of increased startle amplitude making it likely that different neural circuits/mechanisms mediate changes in these two responses to acoustic startle [7, 16]. Additionally, recent studies suggest that separate portions of the CeA-PAG pathway mediate the stress induced changes in startle amplitude and startle habituation [7]. Another possible explanation could be the following. Though NMDA receptor-dependent potentiation of efferent transmission from amygdala to PAG mediates increases in startle amplitude [9, 23, 26], it is homosynaptic depression in brain stem startle pathways that underlies habituation [54], and direct CREB expression in PAG more powerfully engaged such depression than predator stress per second.

4.2. Effects of Viral Vectoring CREB on CeA-PAG Transmission. A fascinating finding was that viral vectoring of CREB induced a potentiation of the CeA-PAG pathway in the right hemisphere (Figure 4) analogous to that seen after predator stress. Moreover, potentiation in this group was restricted to the same hemisphere as injection. In fact the evoked potentials in the left hemisphere of the Handled CREB rats did not differ from those observed in Handled GFP controls. This implies that any behavioral changes observed in this group can be attributed to the change in transmission due to CREB induction in the right hemisphere.

In past studies, CeA-PAG potentiation by predator stress has been shown to be NMDA receptor-dependent. CPP administration prior to predator stress blocks both anxiogenic effects and and CeA-PAG potentiation [7, 16]. Moreover, given that predator stress induces NMDA receptor-dependent right PAG pCREB expression, it has also been suggested that long lasting right CeA-PAG pathway potentiation is dependent on pCREB expression [7, 40]. Present findings in Handled CREB rats support this hypothesis.

The present study also adds new data on the time course of CeA-PAG pathway potentiation in predator stressed rats. Current results show that, as expected, predator stressed rats exhibited potentiation in the right CeA-PAG pathway two days after predator stress (Figure 4), complementing those studies that have replicated this finding at 1, 9 and up to 12

days post predator stress [9, 23, 32]. A novel finding was the fading, but still present, potentiation in the left CeA-PAG of predator stressed rats. The presence of potentiation in the left hemisphere adds to previous studies showing left CeA-PAG one day after predator stress [27], but fading completely by 9 days [7]. Present findings suggest a left hemisphere potentiation lasting at least two days.

The presence of bilateral CeA-PAG pathway potentiation in predator stressed rats and the unilateral induced right CeA-PAG pathway potentiation in Handled CREB rats at the time of anxiety testing may account for some of the differences in open arm avoidance, risk assessment, and startle response between groups. This especially concerns the absence of reduced risk assessment in the Handled CREB group, since NMDA block in the left dorsolateral amygdala 30 minutes prior to predator stress prevents stress effects on risk assessment [26]. Moreover path analysis suggest that changes in open arm exploration and risk assessment may depend on bihemispheric changes in limbic transmission in the early stages after predator stress [27].

Long lasting potentiation in the right CeA-PAG pathway by predator stress has been suggested to reflect some, but not all, of the anxiogenic neuroplastic changes after predator stress [9, 23]. Taken together present findings lend strong support to this view.

4.3. Effects of Viral Vectoring CREB on pCREB lir. Given that predator stress increases pCREB lir selectively in the right lateral column of the PAG, and that CeA-PAG potentiation persists longer in the right hemisphere, it has been suggested that increased production of pCREB underlies right CeA-PAG potentiation. Furthermore, degree of pCREB expression and right CeA-PAG potentiation correlate highly with the same measures of the predator stress experience suggesting a strong relationship between these two phenomena [23].

In the present study, densitometry analysis revealed a right over left lateral PAG increase in pCREB lir in both Handled CREB and predator stressed groups. Thus increasing CREB expression directly and genetically in the right lateral PAG also increased pCREB in a pattern similar to predator stress in a group which had not been predator stressed. Moreover, in Handled CREB rats, the increase of pCREB in the right but not left lateral column of the PAG is consistent with potentiation in the right but not left CeA-PAG pathway in this group. The fact that pCREB expression in the right lateral column in the Handled CREB group was intermediate, neither differing from the predator stressed group nor the Handled GFP controls, is consistent with their milder than predator stressed rats increase in anxiety in the EPM and acoustic startle tests. Taken together, these results support the suggestion that elevated pCREB leads to neuroplastic changes that induce right CeA-PAG potentiation and increased anxiety [23, 32].

This conclusion must be tempered by the reduced power associated with lateral column significant findings. The reduced power here likely reflects a reduced effect evidenced in the small mean differences encountered in the analyses. As pointed out above (Section 3.6) the mean of pCREB lir in Handled CREB rats measured at 5 days post HSV injection is likely an underestimate of its value at peak expression of CREB, which occurs at three days after HSV injection, when treatments occurred (stress or handling), and which fades thereafter [43]. Moreover, effects of predator stress on pCREB expression are evident at 20 minutes post stress and fade thereafter (20 and unpublished observations). Since transient NMDA receptor block prevents predator stress effects on brain and behavior and suppresses pCREB expression [7, 25, 26, 40], it is likely that changes in brain and behavior depend on immediate effects of increased pCREB expression, which in this study would have likely begun before the time of pCREB measurement. Further studies examining CREB and pCREB expression in lateral PAG at 1–3 days post HSV injection are required to clarify present findings.

Present findings mirror those seen in previous work with respect to the lateral column of the PAG. However, the dorsal column results in comparison require greater interpretation. The pattern of dorsal column pCREB changes stand in contrast to findings that predator stress alone does not alter pCREB lir in this column when measured 20 minutes after predator stress [23, 40]. In the current study predator stressed rats had elevated pCREB expression in the right dorsal PAG, while the two Handled groups had lower and similar levels of expression two days after treatment (Figure 5). A right over left hemisphere expression effect was observed in both the predator stressed and Handled CREB groups, similar to the lateral column. The fact that the right exceeds the left in the Handled CREB rats suggests that right lateral column pCREB enhancement may have spread to the dorsal column, but not enough to differ from the Handled GFP control. Other explanations include a potential leak up the cannula tract or the possibility that this is a function of CREB induction, since the predator stressed group demonstrated similar effects though more pronounced. The increase of right over left pCREB expression in predator stressed rats suggests that the EPM is having an effect on the dorsal column up to 24 hours later. This extends previous findings which showed that dorsal column pCREB was elevated bilaterally in predator stressed rats 20–25 minutes after exposure to the EPM which took place 7 days after predator stress [55]. Previous and present findings differ, however, in that in the present study, there was no pCREB increase over control in the left hemisphere in predator stressed rats. This suggests that an increased time interval between the predator stress experience and EPM testing may allow for left hemisphere pCREB levels to increase. Conversely, in the present study 24 hours elapsed between EPM testing and pCREB testing. Perhaps left dorsal column pCREB expression faded over this time interval. Further research into time course of pCREB changes following predator stress and EPM exposure seems warranted.

Though lateral and dorsal column findings are somewhat in line with previous wok, the results of the ventral column are not. In the present study pCREB expression in predator stressed rats was decreased in comparison to both Handled groups in the right hemisphere, and right and left hemisphere expression did not differ in predator stressed rats. Moreover, Handled groups displayed increased

pCREB expression in right over left hemispheres (Figure 5). There are discrepancies and similarities with previous work examining pCREB expression 20 minutes after handling or predator stress. Previous work showed no differences in pCREB expression between predator stressed and handled controls in ventral PAG of both hemispheres, with right hemisphere expression elevated over the left [23]. Perhaps differences in time of sampling pCREB expression accounts for the discrepancies between past and present findings, since pCREB in the present study was measured two days after treatment.

If decreases of pCREB expression in ventral PAG are normally delayed after predator stress (for which we have preliminary evidence, unpublished data), then present findings suggest such decreases are independent of enhanced pCREB expression in lateral PAG induced by direct genetic induction at least. If increase in lateral column and decrease in ventral column pCREB expression parallel enhancement and suppression of normal functioning, then one might suspect a shifting of defensive response bias toward avoidance of threatening stimuli and away from a relaxed immobility, along the lines of functional columnar differences in the dorsolateral and ventral PAG described by Depaulis and Bandler [47]. Further time course studies of shifting defensive response bias following predator stress seem warranted.

4.4. Summary and Conclusions. In summary, the present study demonstrated that directly inducing CREB (and pCREB) expression in the right lateral PAG reproduced behavioral, brain, and molecular changes that closely resemble those seen in predator stressed rats. These findings suggest increased CREB (and perhaps pCREB) expression in the lateral PAG is at least sufficient to produce brain and behavioral changes normally induced by a brief predator stress. Moreover, similar effects of inducing CREB expression in basolateral amygdala on EPM anxiety at least, have been reported by Wallace et al. [43]. Together these data support the idea that the CREB-pCREB pathways in the right lateral PAG, and perhaps amygdala, are important entry level molecular paths to lasting anxiogenic effects of predator stress. To the extent that predator stress models some aspects of PTSD, present finding point to CREB and pCREB pathways as possible new therapeutic targets.

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Review Article

Antiaversive Effects of Cannabinoids: Is the Periaqueductal Gray Involved?

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Cannabinoids play an important role in activity-dependent changes in synaptic activity and can interfere in several brain functions, including responses to aversive stimuli. The regions responsible for their effects, however, are still unclear. Cannabinoid type 1 (CB1) receptors are widely distributed in the central nervous system and are present in the periaqueductal gray (PAG), a midbrain structure closely involved in responses related to aversive states. Accordingly, exposure to stressful stimuli increases endocannabinoid (eCB) levels in the PAG, and local administration of CB1 agonists or drugs that facilitate eCB-mediated neurotransmission produces antinociceptive and antiaversive effects. To investigate if these drugs would also interfere in animal models that are sensitive to anxiolytic drugs, we verified the responses to intra-PAG injection of CB1 agonists in rats submitted to the elevated plus-maze, the Vogel punished licking test, or contextual aversive conditioning model. The drugs induced anxiolytic-like effects in all tests. The same was observed with the transient receptor potential vanilloid type 1 (TRPV1) antagonist capsazepine and with cannabidiol, a nonpsychotomimetic phytocannabinoid that produces anxiolytic-like effects after systemic administration in humans and laboratory animals. These results, therefore, suggest that the PAG could be an important site for the antiaversive effects of cannabinoids.

1. Introduction

Cannabis sativa plant has been used for various purposes since the dawn of civilizations [1, 2], although only in the middle of twentieth century were its chemical constituents identified. Among its major components, there are the phytocannabinoids cannabinol, cannabidiol (CBD), and Δ^9-tetrahydrocannabinol (Δ^9-THC), the latter accounting for most of cannabis effects [3–5]. The mechanisms of Δ^9-THC effects started to be unveiled in the late 80s, with the discovery of CB1 receptors [6, 7]. Soon afterwards, the first endogenous agonist (arachidonoyl ethanolamide, AEA) was isolated and named anandamide, after the Sanskrit word "ananda" for "bliss" [8]. A second endocannabinoid, 2-arachidonoyl glycerol [9], and another cannabinoid receptor, called CB2 [10], soon followed. Selective antagonists were developed, such as rimonabant and AM251, supporting the notion that the CB1 receptor is the major responsible for the behavioral effects of cannabinoids [11, 12]. The expression of this receptor is considerably high in several brain regions such as the basal ganglia, cerebral cortex, hippocampus, amygdale, hypothalamus, and periaqueductal gray (PAG) [13, 14].

CB1 receptors are believed to be located in presynaptic terminals [15]. They activate Gi proteins that inhibit adenylate cyclase and calcium channels and enhance potassium currents, thereby reducing neural firing and neurotransmitter release [16]. This complements the fact that endocannabinoids are synthesized on a stimulus-dependent manner in postsynaptic neurons and immediately diffuse to the synaptic cleft [16]. Thus, contrary to classical neurotransmitters, endocannabinoids act "on demand" as retrograde messengers, inhibiting neural activity. Their effects cease by internalization followed by hydrolysis in neurons. It is still controversial whether endocannabinoids move through the cell membrane passively or are internalized by a putative

transporter. Although the latter remains to be identified [17, 18], pharmacological tools were developed, such as AM404, which are able to inhibit it and, thereby, increase CB1 receptor activation by AEA [18]. Inside neurons, AEA and 2-AG are catabolized by fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MGL), respectively [19]. Possibly, FAAH is located in postsynaptic neurons, whereas MGL is expressed in the presynapse [17]. Selective inhibitors of either FAAH (URB597) or MGL (URB602) have been developed, which provide the possibility of enhancing CB1 receptor activation by increasing the brain levels of endocannabinoids. Studies with these drugs as well as with genetically modified mice have related endocannabinoids to several functions of the central nervous system (for review, see [20]).

Other putative components of this system are the transient receptor potential vanilloid type 1 (TRPV1), the peroxisome-proliferator activated receptor, and the G protein-coupled receptor GPR55. Although anandamide binds to all these receptors, their functions remain uncertain [21]. In addition, an allosteric site in the CB1 receptor has been identified [22] and there is the possibility that, contrary to the initial thoughts, CB2 receptors may indeed be relevant for behavioral responses [23, 24]. Finally, more substances have been proposed as endocannabinoids, such as arachidonoyl dopamine, virodhamine, and noladin ether [20].

2. Cannabinoids and Anxiety

Natural or synthetic cannabinoids or CB1 receptor antagonists often yield complex responses in experimental models of anxiety. As summarized in Table 1, several authors have noticed bell-shaped dose-response curves in animal models predictive of anxiogenic- or anxiolytic-like activity, namely, the elevated plus maze (EPM), the elevated zero maze (EZM), the light dark test (DLT), and the Vogel conflict test (VCT). CB1 receptor agonists tend to be anxiolytic in lower doses, whereas higher doses may be anxiogenic [25]. However, compounds that enhance endocannabinoid effects, such as inhibitors of AEA uptake or hydrolysis, appear to produce only anxiolytic effects without bell-shaped dose-response curves (Table 1).

The reasons for these complex effects remain unknown. One possibility could be that these drugs would interfere with diverse brain regions which have different roles in the modulation of anxiety-like responses. However, the sites responsible for the effects of cannabinoids remain poorly investigated. CB1 receptors, as well as the putative protein responsible for internalization of AEA and the enzyme FAAH, are expressed in several regions of CNS related to anxiety, aversion, and defensive behaviors, including the prefrontal cortex, amygdala, hippocampus, hypothalamus, and PAG [13, 14]. These structures are proposed to be part of a system responsible for the elaboration of behavioral and autonomic responses to aversive stimuli. They are possible neural sites whose malfunction would lead to psychiatric pathologies such as generalized anxiety and panic disorders [26]. In this context, anxiolytic drugs would act by normalizing the functions of these structures [27, 28]. Moreover, this brain aversive system would be responsible for behavioral suppression in animal models predictive of anxiolytic-like activity. Generally, models of experimental anxiety rely on exposing animals to situation that generates conflicts between approach and avoidance, which can be generated by the drive of exploring a new, though, aversive environment, or by a source of reward that is associated with punishment. Anxiolytic-like drugs injected either systemically or into these structures shift the conflict toward approach responses [27, 28]. Thus, these models provide invaluable insights into the neurobiology of anxiety and the pharmacology of anxiolytic compounds. As discussed below, we have used direct drug administration in animals submitted to these models for studying the possible role of the PAG in the antiaversive actions of cannabinoids.

3. Anxiolytic Effects of Cannabinoids in the Periaqueductal Gray

The PAG is a mesencephalic structure that surrounds the cerebral aqueduct and can be divided along its rostro-caudal axis into dorsomedial, dorsolateral (dlPAG), lateral, and ventrolateral columns [29]. It is an important site in ascending pain transmission and a major component of a descending pain inhibitory system. Moreover, this structure receives glutamatergic projection from forebrain regions and sends descendent pathways to motor outputs and to autonomic centres that control blood pressure and heart rate [26]. The dorsal columns (dPAG) are possibly responsible for the elaboration of active defensive behaviors (see [26], for review). Lesions of the dPAG inhibit fear and anxiety produced by stimulation of the amygdala whereas stimulation of this region induces threat display associated with vocalization and strong flight responses [26]. In the caudal ventrolateral PAG, however, immobility has been described as the main outcome of local stimulation [30].

CB1 receptors are distributed along the various columns of this structure [13]. Moreover, administration of CB1 agonists increases Fos expression [31] and brain metabolic activity in the PAG of rats [32], suggesting that this structure could be involved in the effects of systemically administered cannabinoids. In agreement with this proposal, injection of CB1 receptor agonists into the dlPAG of rats has been shown to induce antinociceptive responses [33] and electric stimulation of the dorsal and lateral columns induces antinociception via activation of CB1 receptors accompanied by local AEA release [34]. Furthermore, subcutaneous formalin injection, a painful stimulus, substantially increased the release of AEA in the PAG [34, 35].

Concerning the possible involvement of PAG-endocannabinoid system on modulation of anxiety-like behaviors, an initial study showed that local administration of HU210, a potent CB1 agonist, attenuated the flight responses induced by dPAG injections of the excitatory amino acid D,L-homocysteic acid (see [36, Table 2]). In a subsequent study, where the injections were restricted to the dorsomedial PAG, HU210 decreased hyperlocomotion

Table 1: Effects of cannabinoids and drugs that interfere with the endocannabinoid system in animal models predictive of anxiolytic- or anxiogenic-like activity. (AEA: AEA; Δ^9-THC: Δ^9-tetrahydrocannabinol; CBD: cannabidiol; EPM: elevated plus-maze; EXM: elevated X-maze; EZM: elevated zero-maze; VCT: Vogel conflict test; FC: fear conditioning; DLT: dark-light test; SI: social; NSF: novelty-suppressed feeding interaction.)

Drug	Test	Dose (species)		Reference
		Anxiolytic-like effects	Anxiogenic-like effects	
Phytocannabinoids				
Δ^9-THC	EPM		10–20 mg/kg (mouse)	[37]
	EPM		1–10 mg/kg (rat)	[37]
	DLT	0.3 mg/kg (mouse)	0.5 mg/kg (mouse)	[38]
	DLT	0.3 mg/kg (mouse)		[39]
	EPM		1–10 mg/kg (mouse)	[40]
	EPM	0.075–0.75 mg/kg (rat)		[41]
	EPM		0.5–2.5 mg/kg (rat)	[42]
	DLT		1.25–2.5 mg/kg (rat)	[42]
	EPM	0.075–1.5; 3* mg/kg (rat)		[43]
CBD	EPM	1–10 mg/kg (mouse)		[37]
	EPM	2.5–10; 20* mg/kg (rat)		[44]
	EPM	5 mg/kg (rat)		[45]
	VCT	10 mg/kg (rat)		[46]
	FC	10 mg/kg (rat)		[47]
CB1 agonists				
HU210	EXM		25 μg/kg, 10 days (rat)	[48]
	NSF	100 μg/kg/day–10 days (rat)		[49]
	EPM	10 μg/kg (rat)	50 μg/kg (rat)	[50]
WIN-55212	EPM	1–3 mg/kg (mouse)		[51]
	EPM	1–3; 10* mg/kg (mouse)		[40]
	EPM	1–3 mg/kg (mouse)	1–3 mg/kg (rat)	[52]
CP55940	EPM		75–125 μg/kg (rat)	[53]
	EPM		75 μg/kg (rat)	[54]
	EPM	1 μg/kg (rat)	50 μg/kg (rat)	[55]
	EPM	2.5–5 μg/kg (rat)	40 μg/kg (rat)	[56]
	SI		40 μg/kg (rat)	[57]
	EPM	0.1–0.3 mg/kg (mouse)		[40]
AEA	EPM		10 mg/kg (mouse)	[58]
	DLT	0.3 mg/kg (rat)		[59]
AEA uptake inhibitor				
AM404	EPM	5 mg/kg (rat)		[60]
	EPM	1–3; 10* mg/kg (mouse)		[40]
	EPM	0.75–1.25 mg/kg (rat)		[37]
AEA metabolism inhibitors				
URB597	EZM	0.1 mg/kg (rat)		[61]
	EPM	0.1–0.3 mg/kg (mouse)		[40]
	EPM	0.1 mg/kg (mouse)		[62]
	DLT	0.1–0.3 mg/kg (rat)		[55]
	EPM	1 mg/kg (mouse)		[63]
$AACOCF_3$	DLT	4 mg/kg (mouse)		[64]

Table 1: Continued.

Drug	Test	Dose (species)		Reference
		Anxiolytic-like effects	Anxiogenic-like effects	
CB1 antagonists				
Rimonabant	EPM		3 mg/kg (rat)	[65]
	EPM		3 mg/kg (rat)	[53]
	EPM	3 mg/kg (mouse)		[66]
	VCT	0.3–3 mg/kg (rat)		[67]
	EPM	10 mg/kg (rat)		[67]
	EPM		3–10 mg/kg (mouse)	[40]
AM251	EPM		3 mg/kg (mouse)	[51]
	EPM		1.3–3 mg/kg (mouse)	[68]
	EPM		3–10 mg/kg (mouse)	[40]
	EPM		1–3 mg/kg (mouse)	[52]
TRPV1 agonists				
Olvanil	EPM	5 mg/kg (rat)		[69]
TRPV1 antagonists				
Capzasepine	EPM	1–10 μg/kg (rat)		[69]

* Bell-shaped dose-response curve.

induced by aversive ultrasound stimulation, but failed to change freezing responses. Moreover, HU210 effects were not entirely blocked by previous local injection of a CB1 receptor antagonist [70].

Considering these initial results, we decided to further investigate a possible influence of the PAG-endocannabinoid system on anxiety-like behaviors in rats submitted to different animal models of anxiety (Table 2). First, we showed that AEA injected into the dlPAG increased the exploration of the open arms of the elevated plus maze (EPM) [71], a model based on a natural conflict between exploratory behavior and innate fear of open spaces. The effects of AEA were similar to those observed with classical anxiolytic benzodiazepines [72] and were blocked by previous treatment with AM251, a CB1 receptor antagonist. These effects were also potentiated by previous treatment with AM404, an inhibitor of AEA uptake/metabolism. AM404 by itself, however, was without effect in this model. AEA produced an inverted U-shaped dose-response curve, with higher doses being ineffective [71].

To confirm a possible anticonflict effect of AEA in the dlPAG, we used the Vogel conflict test (VCT) [73], an animal model of anxiety not based on innate fear but instead on suppression of punished responses learned during the test. In this model, water-deprived rodents are exposed to a conflict between licking the spout of a bottle containing water and receiving a mild shock on the tong [74]. Anxiolytics that potentiate the action of γ-aminobutyric acid such as the benzodiazepines typically increase the number of punished licks [75]. AEA also induced anxiolytic-like effects in the VCT at the same dose range observed in the EPM (Table 2). Different from the results obtained in the latter model, AM404 was also able to increase the number of punished licks (Table 2). Although the causes of these contradictory results are not clear, they could involve the distinct animal models of anxiety employed. Brain endocannabinoids have been proposed to act as a "stress buffer system" [76], recruited by highly demanding situations. It was possible that the VCT, by involving pain and water deprivation, engages the endocannabinoid system in the dlPAG to a greater extent than the EPM. Actually, as discussed above, painful stimuli such as those used in the VCT have already been showed to increase AEA in this region [77].

We have further investigated this effect by intra-dlPAG administration of AEA and AM404 in rats submitted to a contextual fear conditioning paradigm, an animal model that also involves pain exposure [78]. Animals re-exposed to an environment where they had being previously submitted to an aversive stimulation, such as electrical footshocks, show behavioral and cardiovascular changes characterized by immobility (freezing) and mean arterial pressure (MAP) and heart rate (HR) increases [79, 80]. Although electrical or chemical stimulation of the dorsal portion of PAG is usually related with flight reactions, it can also produce freezing responses and increased cardiovascular activity [26]. Re-exposure to an aversively conditioned context increases neuronal activity in the PAG [81, 82], and PAG lesions block freezing to aversively conditioned stimulus [83, 84]. dlPAG microinjection of AEA or AM404 blocked the expression of the conditioned aversive responses [78]. This effect was inhibited by local pretreatment with AM251, reinforcing the involvement of CB1 receptors.

Altogether, these results suggest that the endocannabinoid system in the dlPAG can modulate responses to aversive stimuli. The mechanisms of these effects are still unclear. Using brain slices of the rat PAG, Vaughan et al. [85] showed that cannabinoids act via CB1 receptors to inhibit GABAergic and glutamatergic synaptic transmission. The efficacy of endogenous cannabinoids was limited by uptake and breakdown since AEA was only able to inhibit evoked

Table 2: Effects of Cannabinoid-related drugs injected into the PAG of rats submitted to animal models of anxiety-related behaviors. (AEA: anandamide; ACEA: arachidonyl-2-chloro-ethylamide; CBD: cannabidiol; EPM: elevated plus-maze; VCT: Vogel conflict test; CFC: contextual fear conditioning; dlPAG: dorsolateral PAG; dPAG: dorsal (dorsolateral + dorsomedial) PAG; dmPAG: dorsomedial PAG; unpub: unpublished data.)

	Drug	PAG column	Test	Doses tested	Effect (effective dose)	Ref.
Phytocannabinoids	CBD	dlPAG	EPM, VCT	15–60 nmol	Anxiolytic (30 nmol*)	[86]
Endocannabinoids	AEA	dlPAG	EPM	0.05–50 pmol	Anxiolytic (5 pmol*,1)	[71]
			VCT	5 pmol	Anxiolytic	[73]
			CFC	5 pmol	Anxiolytic	[78]
CB1 receptor agonists	ACEA	dlPAG	EPM	0.05–5 pmol	Anxiolytic (0.05 pmol*)	[71]
	HU210	dPAG	dPAG chemical stimulation	0.1–5 μg	Attenuated flight responses (0.1–5 μg)	[36]
	HU210	dmPAG	Ultrasound-induced hyperlocomotion and freezing	5 μg	Decreased hyperlocomotion, but increased freezing**	[70]
CB1 receptor antagonist	AM251	dlPAG	EPM, VCT, CFC	1–300 pmol	No effect by itself, but blocked AEA and AM404 anxiolytic effects	[71, 73, 78]
	Rimonabant	dPAG	Ultrasom-induced hyperlocomotion and freezing	30 μg	No effect by itself	[70]
AEA uptake inhibitor	AM404	dlPAG	EPM	0.5–50 pmol	No effect by itself; potentiated the anxiolytic effect of AEA	[71]
			VCT	50 pmol	Anxiolytic	[73]
			CFC	50 pmol	Anxiolytic	[78]
AEA metabolism inhibitor	URB597	dlPAG	VCT	0.01–0.1 nmol	Anxiolytic (0.01 pmol*)	[73]
TRPV1 antagonists	Capsazepine	dlPAG	EPM, VCT	10–60 nmol	Anxiolytic (60 nmol)+	[87]

* Bell-shaped dose-response curve. Anxiolytic effect blocked by AM251 (100 pmol) and potentiated by AM404 (50 pmol).
+Capsazepine 10 nmol turned the higher, ineffective dose of CBD (60 nmol) into an anxiolytic one in the EPM [85].
**Not blocked by rimonabant 30 μg.

inhibitory postsynaptic currents in the presence of the AT inhibitor, AM404. Several studies indicate that GABA- and glutamate-mediated neurotransmissions in the dPAG play opposite roles. While the former tonically inhibits defensive responses, the latter facilitates them [26]. Thus, CB1-mediated inhibitory effects on these two neurotransmitter systems could be one of the explanations for the observed bell-shaped dose-response curve induced by AEA in this region as well as the contradictory results regarding the effects of cannabinoids on anxiety (see Table 1 and text bellow for a discussion on the possible involvement of TRPV1 receptors).

These mechanisms may explain the effects in the PAG, yet they do not necessarily apply to other brain regions. In some areas, the levels of CB1 receptor expression can be higher in GABAergic (particularly in cholecystokinin-containing basket cells) as compared to glutamatergic neurons, with cannabinoid effects favoring impairment of inhibitory mechanisms mediated by the former neuronal population [16]. However, it remains to be further investigated how these neural subpopulations contribute to specific behavioral effects of cannabinoids. In addition, GABAergic and glutamatergic neurons may have different sensitivity to CB1 agonists or antagonists depending on the species under investigation. For instance, Haller et al. [52] observed opposite effects in mice and rats tested with the same doses of a cannabinoid in models of anxiety-like behavior (see Table 1). Inhibitory and excitatory currents were differentially affected in the hippocampi of these species, providing a possible basis for the discrepancies in the behavioral responses. Since we have employed rats as subjects in all our experiments, studies in other species could further consolidate our

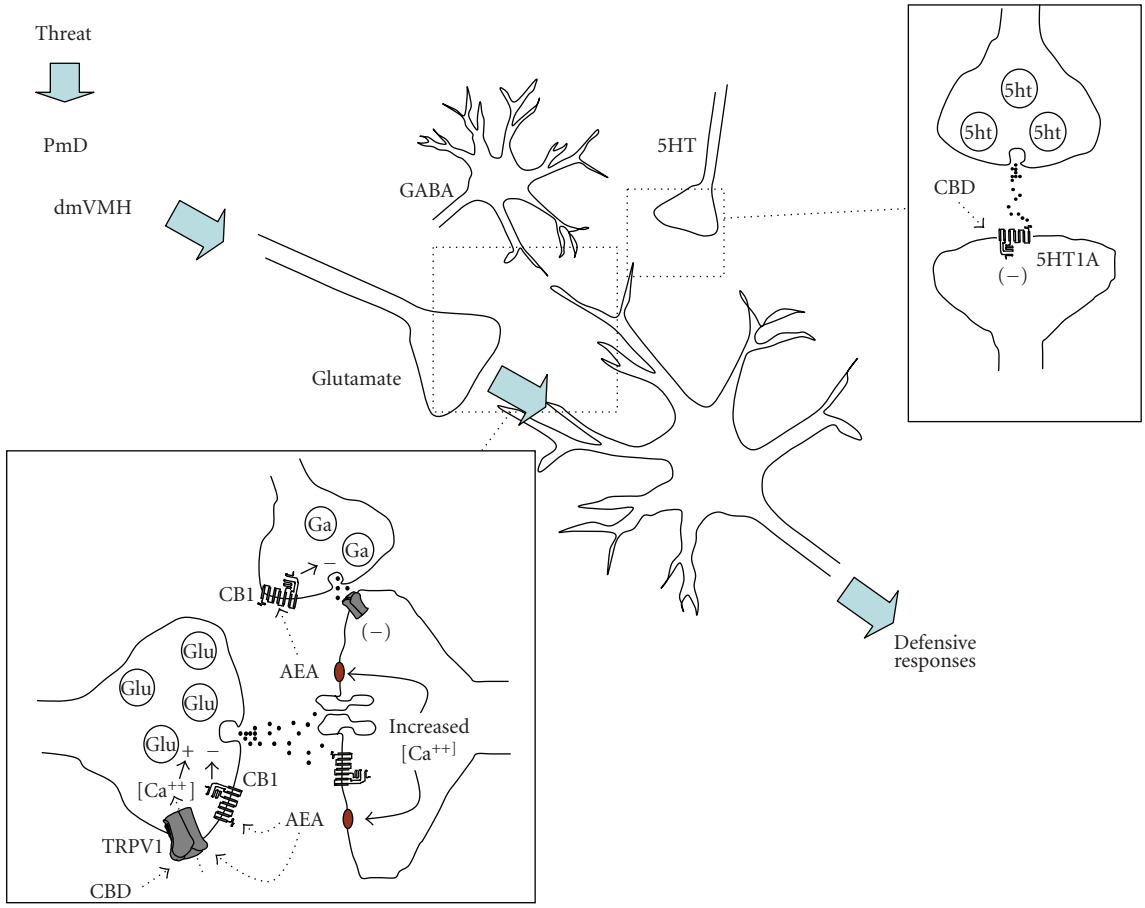

Figure 1: Possible effects of cannabinoids in the dlPAG. Glutamatergic inputs from forebrain structures such as the dorsomedial part of the ventromedial hypothalamic nucleus (dmVMH) and dorsal premammilary hypothalamic nucleus (PmD) activate a local neural substrate that mediates defensive responses [88]. This substrate is under GABAergic and serotonergic inhibitory influence [26]. Activation of CB1 receptors by cannabinoids such as AEA interferes with presynaptic glutamate (Glu) and GABA (Ga) neurotransmitter release. CB1-mediated decrease of glutamate release would promote anxiolytic-like effects. Activation of TRPV1 presynaptic receptors, on the other hand, would produce opposite effects. The anxiolytic effects of cannabidiol (CBD), a nonpsychotomimetic cannabinoid, in the dlPAG are not mediated by CB1 receptors, but probably involve activation of postsynaptic 5HT1A receptors. The bell-shaped dose-response curves observed with AEA and CBD may depend on activation of TRPV1 receptors. Regarding AEA, a presynaptic decrease of GABA release could also be related to this effect.

hypothesis that glutamatergic and GABAergic inhibitions would mediate anxiolytic- and anxiogenic-like effects of cannabinoids, respectively. For a more extensive discussion on the relevance of diverse neural subpopulations for the effects of cannabinoids, see [89].

4. Cannabidiol

Cannabidiol (CBD) is a major nonpsychotomimetic constituent of *Cannabis sativa* that is able to antagonize the anxiogenic and psychotomimetic effects of high doses of Δ^9-THC [90, 91]. It also promotes anxiolytic-like effects in several animal models (see [44–47], Table 1). In addition, CBD induces anxiolytic effects in healthy volunteers in the simulated public speaking test, a model of clinical anxiety, and in subjects submitted to a functional imaging analysis study [92, 93]. However, as commonly seem with other cannabinoids in animal models of anxiety, experiments with CBD yield bell-shaped dose-response curves, low doses being anxiolytic, and higher doses being ineffective [44]. The mechanisms for these actions remain poorly understood. CBD has low affinity for CB1 or CB2 receptors and could facilitate the endocannabinoid signalling by inhibition of AEA uptake or its enzymatic hydrolysis. It can also act as an agonist of TRPV1 or 5HT1A receptors [94, 95].

Considering that the PAG, in addition to CB1 [14], also expresses a significant number of TRPV1 and 5HT1A receptors [96, 97], we decided to verify if this region could be related to the effects of CBD. We found that CBD microinjections into the dlPAG produced anxiolytic-like effects in rats submitted to the EPM or the VCT [86] (Table 2). The effects in the EPM, however, also showed a bell-shaped dose-response curve, but were not blocked by previous local administration of AM251 [86], employed at the same dose that was able to antagonize the anxiolytic-like effects of AEA and AM404 (Table 2). The

anxiolytic-like effects of CBD, however, were prevented by WAY100635, an antagonist of 5HT1A receptors. Activation of these Gi-coupled-receptors enhances K+ currents and inhibits adenylyl cyclase activity [98]. They act as inhibitory autoreceptors in serotonergic neurons in the raphe nuclei but are also localized postsynaptically in several brain regions, including the PAG, amygdala, hippocampus, and frontal cortex. Actually, the PAG receives serotonergic projections from the dorsal raphe nuclei, and local activation of 5HT1A receptors promotes the control of anxiety states and the hypothalamus-pituitary-adrenal axis during stress responses [99]. Thus, 5HT1A receptors located in the PAG are possibly involved in the anxiolytic-like effects of CBD, a hypothesis corroborated by several studies showing that agonists of these receptors produce anxiolytic effects in the PAG [100, 101].

5. TRPV1 Receptors Methods

TRPV1 receptors belong to a large family of calcium-permeable cation channels [102]. They can be activated by elevation in temperature, pH decrease, or by exogenous ligands such as capsaicin, the pungent ingredient of red hot chilli peppers [103, 104]. They have been related to pain transmission and inflammatory responses in the peripheral nervous system. In addition to environmental stimuli, endocannabinoids such as AEA and N-arachidonyldopamine can also activate TRPV1 receptors. As a consequence, they can also be denominated endovanilloids [104, 105].

TRPV1 receptors are expressed in various brain regions related to anxiety, including the PAG [106, 107], where they can regulate glutamate release. Corroborating this proposal, local infusion of capsaicin produces antinociception by increasing glutamate release in this region [108]. In addition, activation of presynaptic TRPV1 receptors produced an excitatory effect in the firing activity of dlPAG neurons [109]. Glutamate is the main excitatory neurotransmitter in the central nervous system, and the injection of NMDA antagonist receptors into the dlPAG promotes anxiolytic effects in the EPM and VCT [110].

Few studies, however, have investigated the role of TRPV1 in anxiety. Systemic administration of capsazepine, a TRPV1 antagonist, induced anxiolytic-like effects in rats submitted to the EPM (Table 1) [69]. More recently, Marsch et al. [111] demonstrated that TRPV1-deficient mice show decreased anxiety in the EPM and light-dark test. Accordingly, the dual FAAH/TRPV1 blocker N-arachidonoyl-serotonin is able to induce CB1-mediated anxiolytic-like effects more potently than selective blockers of FAAH or TRPV1, further suggesting opposite roles for CB1 and TRPV1 receptors [112].

To further investigate the role of TRPV1 on anxiety modulation, we verified the effects of intra-dlPAG injection of capsazepine in rats submitted to the EPM and VCT. This drug decreased anxiety-like behaviors in both models (Table 2), suggesting that TRPV1 receptors facilitate anxiety responses in the PAG. The fact that AEA and CBD can also activate TRPV1 receptors [94, 104, 105] could help to explain the bell-shaped dose-response curves usually found with these compounds regarding their anxiolytic effects (Tables 1 and 2). In agreement with this proposal, it was recently showed that capsazepine blocks the anxiogenic effects of high doses of AEA in the prefrontal cortex [113]. It remained to be tested if similar effects could occur in the dlPAG. In an initial study, we confirmed this possibility, showing that intra-dlPAG pretreatment with an ineffective dose of capsazepine was able to turn the higher, ineffective dose of the CBD into an anxiolytic one (Table 2).

6. Conclusions

The pieces of evidence revised above suggest that the PAG, particularly its dorsolateral column, is involved in the modulatory effects of cannabinoids on defensive responses. This does not mean that the PAG is the only or the most relevant structure accounting for the antiaversive properties of cannabinoids. Other authors have also identified brain sites where CB1 receptor activation induces anxiolytic-like effects. Injection of low doses of Δ^9-THC either in the ventral hippocampus (5 μg) or in the prefrontal cortex (10 μg) resulted in anxiolytic-like effects; whereas in the amygdala (1 μg), opposite results were reported [114]. An early work has also shown anxiogenic-like effect of Δ^9-THC in this brain region [115] Moreover, intraprefrontal cortex injection of low or high doses of methanandamide induces CB1-mediated anxiolytic- or TRPV1-mediated anxiogenic-like effects, respectively [113]. Other authors have also investigated brain sites mediating nociceptive responses, antidepressive-like activity, and rewarding effects of cannabinoids [89].

In conclusion, local administration of CB1 agonists into the dlPAG produces anxiolytic-like effects in several animal models. These effects are prevented by AM251, indicating that they are being mediated by activation of CB1 receptors, possibly by presynaptic inhibition of glutamate release (see Figure 1). Results with AM404, an AEA metabolism/uptake inhibitor, also suggest that local synthesis of endocannabinoids in the dlPAG can modulate defensive responses, at least under high-aversive conditions. The results also showed that the dlPAG could be involved in the reported anxiolytic effects of CBD, a nonpsychotomimetic phytocannabinoid. This compound, however, appears to act by activating 5HT1A receptors (Figure 1). Finally, activation of vanilloid TRPV1 receptors in the dlPAG seems to facilitate defensive responses (Figure 1) and may be, in part, responsible for the bell-shaped dose-response curves of the anxiolytic effects of AEA and CBD. A balance between CB1- and TRPV1-activations is a possible mechanism through which endogenous AEA could control aversive responses.

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Research Article

Behavioral Consequences of Delta-Opioid Receptor Activation in the Periaqueductal Gray of Morphine Tolerant Rats

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Chronic morphine administration shifts delta-opioid receptors (DORs) from the cytoplasm to the plasma membrane. Given that microinjection of morphine into the PAG produces antinociception, it is hypothesized that the movement of DORs to the membrane will allow antinociception to the DOR agonist deltorphin II as a way to compensate for morphine tolerance. Tolerance was induced by twice daily injections of morphine (5, 10, or 20 mg/kg, subcutaneous) for 3.5 days. Microinjection of deltorphin into the vPAG 6 hours after the last morphine injection produced a mild antinociception that did not vary in a consistent manner across morphine pretreatment doses or nociceptive tests. In contrast, deltorphin caused a decrease in activity in morphine tolerant rats that was associated with lying in the cage. The decrease in activity and change in behavior indicate that chronic morphine administration alters DORs in the vPAG. However, activation of these receptors does not appear to compensate for the decrease in antinociception caused by morphine tolerance.

1. Introduction

Opioid receptors in the periaqueductal gray (PAG) contribute to a wide range of behaviors. These include nociceptive modulation, cardiovascular regulation, thermoregulation, and locomotor activity [1–5]. Although mu-opioid receptors (MOR) are known to contribute to PAG mediated antinociception [6, 7], less is known about the contribution of delta-opioid receptors (DORs). Although antinociception has been produced by the administration of DOR agonists into the PAG, these effects are mild compared to the antinociception produced by MOR agonists [8–10].

DORs are particularly interesting because the expression of these receptors is surprisingly dynamic. Chronic treatment with morphine causes the spinal density of DORs to shift from the cytoplasm to the plasma membrane [11, 12]. A similar shift in DORs from the cytoplasm [13] to the plasma membrane appears to occur in the PAG. Swim stress causes an increase in DOR density in the plasma membrane of PAG neurons [14]. *In vitro* recordings show that DOR agonists do not alter GABAergic synaptic transmission in PAG neurons from drug-naive animals [15–17], but inhibit GABAergic IPSCs in mice treated chronically with morphine [18].

The behavioral significance of enhanced DOR expression in the PAG has not been characterized. The increased expression of DOR in the PAG of morphine tolerant rats could be a compensatory mechanism for the loss of antinociception at the mu-opioid receptor. Increased expression of DORs in the spinal cord has been shown to enhance the antinociceptive effect of intrathecal administration of the DOR agonist deltorphin II [19].

The objective of the present study was to determine the behavioral consequences of activating DORs in the PAG following induction of morphine tolerance. Given the widespread effects mediated by the PAG, mobilization of DORs to the plasma membrane could contribute to a wide range of behaviors. The enhanced antinociceptive effects of DOR agonists at the spinal level [19] suggest that the administration of DOR agonists into the vPAG of morphine tolerant rats will produce antinociception. This hypothesis will be tested by examining the antinociceptive and locomotor effects of microinjecting the DOR agonist deltorphin into the vPAG of rats made tolerant to morphine.

2. Methods

Male Sprague-Dawley rats (240–360 g) were anesthetized with pentobarbital and implanted with a guide cannula aimed at the ventrolateral PAG using stereotaxic techniques (from lambda: AP = +1.2 mm, ML = 0.6 mm, and DV = −4.6 mm). The guide cannula was 9 mm long and affixed to two screws in the skull with dental cement. Rats were handled daily for one week following surgery. All injections and testing were conducted during the dark phase of a 12-hour light/dark cycle in a dimly illuminated room. Experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory animals. Efforts were made to minimize the number and potential suffering of the experimental subjects.

2.1. Materials. Nociception was assessed using the hot plate, tail withdrawal, and formalin tests. The hot-plate test (IITC, Woodland Hills, Calif, USA) consisted of measuring the latency for a rat to lick a hind paw when placed on a 52°C plate. Tail withdrawal measured the latency to move the tail when placed in 52°C water. The formalin test consisted of rating pain behavior on a 0–3 scale following injection of formalin (2% in 50 ul) into the plantar surface of the hind paw [20]. The values on this scale are 0 = normal behavior; 1 = paw touches the ground without bearing weight; 2 = paw does not touch the ground; 3 = paw is above the ground and licked.

2.2. Microinjection Procedure. Four days after surgery, an injection cannula was inserted through the guide cannula, but no drug was injected. This process habituates rats to the injection procedure and diminishes behavioral effects produced by cell damage on the test day. Testing began one week following surgery, deltorphin II (1 μg/0.5 μl) or saline was microinjected into the vPAG. An 11 mm injection cannula was inserted into the guide cannula while the rat was gently restrained by hand. The injection cannula extended 2 mm beyond the end of the guide cannula. Drugs were injected at a rate of 0.1 ul/10 s. The injection cannula remained in place an additional 20 seconds to minimize drug flow up the cannula track. The stylet was reinserted into the guide cannula and the rat was returned to its home cage.

Experiment 1. Repeated Morphine Injections. The objective of this experiment was to determine the behavioral effects of microinjecting deltorphin into the vPAG in rats made tolerant to repeated subcutaneous injections of morphine. Morphine (5, 10, or 20 mg/kg) or saline (1 ml/kg) was administered twice a day (at 9:30 and 15:00) for 3.5 days. Nociception was assessed with the hot plate and tail flick tests 30 minutes after the injection on trials 1 and 7, but not after injections on trials 2–6. This procedure limits changes in nociception from repeated testing [21, 22]. Six hours after the last subcutaneous injection, all rats were injected with deltorphin (1 μg/0.5 μl) into the vPAG. Nociception was assessed using the hot-plate test 20 minutes later. A subset of these rats was tested again on the hot plate 50 minutes after deltorphin administration (N = 9, 5, 10, and 8 for groups tested with saline, 5, 10, and 20 mg/kg of morphine, resp.). Nociception was assessed using the formalin test in the other rats (N = 8, 6, and 9 for groups tested with saline, 10, and 20 mg/kg of morphine, resp.).

Locomotor activity was assessed for 30 minutes beginning immediately after the 20 minutes hot-plate test. Activity was assessed by placing the rat into a chamber (25.1 × 47 cm) with 7 photobeams spaced 5.1 cm apart (San Diego Instruments, San Diego, Calif, USA). The average number of photobeams disrupted each minute was measured and averaged over 10 minute intervals for 30 minutes. The behavior of the rat was examined every 5 minutes during the locomotor test in an attempt to determine the reason for the changes in locomotion (e.g., grooming, sleeping, and freezing). Normal behavior was defined as walking, sniffing, and grooming.

Experiment 2. Continuous Morphine Administration. Rats were surgically implanted with a guide cannula aimed at the vPAG as described in Experiment 1. One week later, tolerance was induced by implanting two 75 mg morphine pellets under the skin of the upper back while rats were briefly anesthetized with halothane. Control rats were implanted with two placebo pellets. Nociception was assessed using the hot-plate test 2 hours following pellet implantation.

Rats were returned to the test room 3 days after pellet implantation and allowed to habituate for 30 minutes. Nociception was assessed at the end of this period using the hot-plate test to determine whether tolerance had developed. Following this baseline test, both morphine and placebo-treated rats were injected with deltorphin (1 ug/0.5 ul) into the vPAG. Rats were returned to their cage immediately following the injection. Nociception was assessed using the hot-plate test 30 and 60 minutes after the deltorphin microinjection.

2.3. Histology. Following testing, rats were given an overdose of halothane (Sigma, St. Louis, Mo, USA). The microinjection site was marked by injecting cresyl violet (0.2 μl) into the PAG. The brain was removed, placed in formalin (10%), sectioned coronally (50 μm), and viewed under a microscope to localize the injection site [23]. Only rats with injection sites in or immediately adjacent to the vPAG were included in data analysis.

2.4. Data Analysis. The effects of morphine pretreatment were compared to saline or placebo-treated controls using a t-test or analysis of variance. The Bonferroni and Tukey tests were used for post hoc comparisons. Statistical significance was defined as a probability of less than .05.

3. Results

Experiment 1. Repeated Morphine Injections. Systemic administration of high doses of morphine (5, 10, and 20 mg/kg) produced maximal antinociception on trial 1 (see Figure 1). A significant decrease in antinociception was evident with

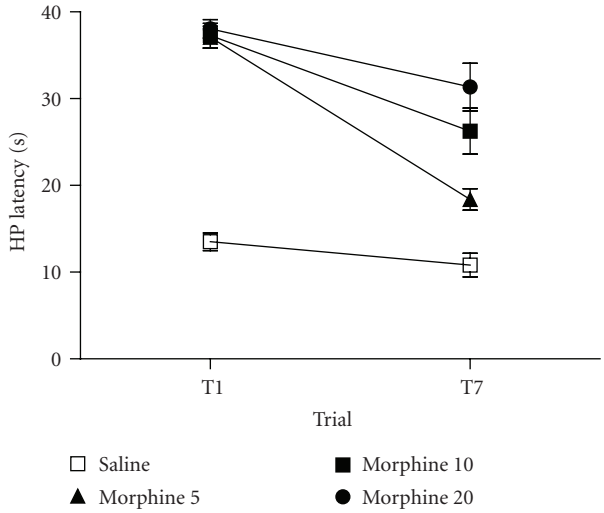

Figure 1: Tolerance to antinociception develops with repeated injections of morphine. The hot-plate latency following morphine administration on trial 7 was significantly less than on trial 1 for the morphine-treated groups ($F(1, 39) = 31.38$, $P < .01$). Antinociception was still evident with the administration of large doses of morphine (10 and 20 mg/kg), but not as pronounced as on trial 1. Large sample sizes were used for most groups ($N = 19$, 5, 19, and 17 for rats pretreated with saline and morphine at 5, 10, and 20 mg/kg, resp.) because subsets of these rats were subsequently tested using either the formalin test or repeated hot-plate tests.

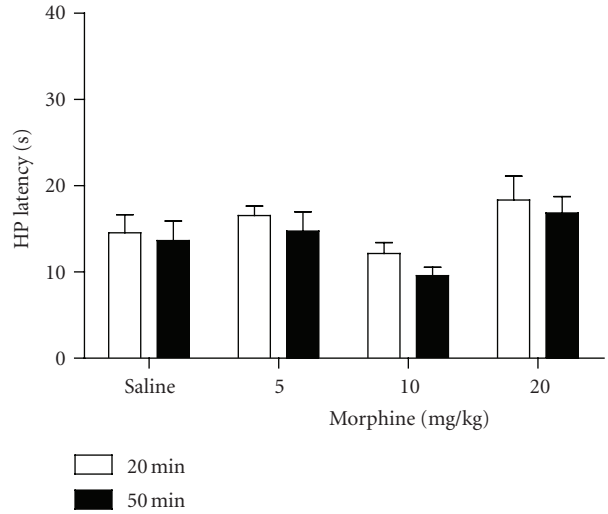

Figure 2: Changes in nociception following microinjection of deltorphin into the vPAG. Nociception was assessed 20 and 50 minutes after deltorphin microinjection into the vPAG of rats made tolerant to morphine. Pretreatment with morphine produced a slight decrease in hot-plate latency in rats made tolerant to 10 mg/kg of morphine compared to the slight increase in rats made tolerant to 20 mg/kg of morphine, although neither of these changes differed significantly from the effects of deltorphin administration in saline-pretreated controls. All rats were tested on the hot plate 20 minutes after deltorphin microinjection (see sample sizes in the caption of Figure 1), but only a subset was tested at 50 minutes for the saline ($N = 9$), 5 ($N = 5$), 10 ($N = 10$), and 20 ($N = 8$) mg/kg morphine groups.

repeated administration from trial 1 to 7 ($F(1,56) = 53.446$, $P < .05$). The magnitude of the decrease in antinociception was dose dependent ($F(3,56) = 5.536$, $P < .05$). That is, the lowest dose (5 mg/kg) produced the least antinociception and the highest dose (20 mg/kg) produced the greatest antinociception in morphine tolerant rats.

Microinjection of deltorphin into the vPAG of morphine naive rats produced a slight increase in hot-plate latency. Rats pretreated with saline showed a significant increase in hot-plate latency following microinjection of deltorphin from 10.8 (see trial 7 in Figure 1) to 14.5 seconds (20-minute test in Figure 2) (one-tailed $t(18) = 1.925$, $P < .05$).

The effect of microinjecting deltorphin into the vPAG of morphine tolerant rats varied with the pretreatment dose (see Figure 2). A 2×2 ANOVA revealed a significant difference in hot-plate latency between the pretreatment groups ($F(3,84) = 3.203$, $P < .05$), but no difference in hot-plate latency between the 20- and 50-minute tests ($F(1,84) = 0.866$; $P > .05$). The difference between groups was caused by a slight increase in the hot-plate latency of rats pretreated with 20 mg/kg of morphine and a slight decrease in latency in rats pretreated with 10 mg/kg (see Figure 2). Although the difference between these groups was statistically significant (Bonferroni, $t = 2.420$, $P < .05$), neither the 10 mg/kg ($t = 0.96$ and 1.15 for 20 and 50 minutes, resp.) nor the 20 mg/kg ($t = 1.49$ and 0.87 for 20 and 50 minutes, resp.) groups differed from the saline-pretreated group. Moreover, these changes in nociception were quite small compared to the antinociception produced by systemic administration of morphine on trial 7 (26.2 ± 2.7 and 31.3 ± 2.7 seconds following 10 and 20 mg/kg doses of morphine).

A similar difference between pretreatment groups was evident when nociception was assessed with the tail flick test 50 minutes after deltorphin administration ($F(2,52) = 8.699$, $P < .05$). Microinjection of deltorphin into the PAG of rats pretreated with 20 mg/kg of morphine caused a small, but significant, increase in tail flick latency (4.4 ± 0.2 seconds) compared to rats pretreated with saline (3.5 ± 0.2 seconds) or 10 mg/kg of morphine (3.4 ± 0.2 seconds) (Tukey test, $P < .05$ for both comparisons).

Nociception was assessed using the formalin test in the rats not tested on the hot plate at 50 minutes. Formalin was injected 5 minutes following the 20 minute hot-plate test so the first and second phases [20] could be assessed 25–29 and 40–44 minutes after microinjection of deltorphin. In contrast to the hot-plate test, pain ratings on the formalin test did not differ between the pretreatment groups on either the first (mean ratings = 1.65, 1.60, and 1.73) or the second (mean ratings = 0.40, 0.63, and 0.67) phases ($F(2,20) = 0.272$, $P = .76$).

Locomotor activity was assessed during the 30 minutes following the 20 minute hot-plate test in the subset of rats not injected with formalin. Microinjection of deltorphin into the vPAG caused a significant decrease in activity in the morphine compared to the saline-pretreated rats ($F(2,29) = 6.84$, $P < .05$). This decrease was similar in rats

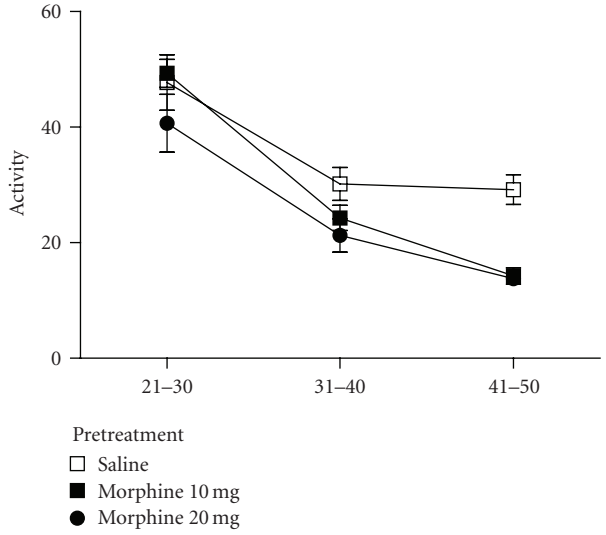

Figure 3: Decrease in locomotion following microinjection of deltorphin into the vPAG. Microinjection of deltorphin (1 μg/0.5 μl) into the vPAG caused a decrease in locomotion in rats pretreated with morphine (N = 13 and 9 for rats pretreated with 10 and 20 mg/kg) compared to rats pretreated with saline (N = 11). This decrease in activity is caused by a change in normal behavior to lying and crouching in the cage following deltorphin administration (see Table 1).

Table 1: Percentage of rats showing normal and abnormal behavior following deltorphin microinjection into the vPAG in morphine-pretreated rats.

	Crouching/lying	
Pretreatment	25–39 minutes	40–54 minutes
Saline	0% (0/6)	0% (0/6)
Morphine 10 mg	20% (1/5)	67% (6/9)
Morphine 20 mg	36% (4/11)	40% (6/15)

Note: sample sizes are shown in parentheses.

pretreated with 10 and 20 mg/kg of morphine (see Figure 3). The greatest decrease in activity was evident at the latest time period (41–50 minutes) for both pretreatment groups.

The decrease in activity was accompanied by a change in behavior in which rats tended to crouch or lie down in the activity chamber. An increase in the number of rats crouching or lying increased in rats pretreated with 10 and 20 mg/kg of morphine beginning approximately 25 minutes after the deltorphin microinjection (see Table 1). This shift in the number of rats displaying normal exploratory behavior to crouching and lying increased further during the last 15 minutes of the test (40–54 minutes after the deltorphin microinjection).

Experiment 2. Continuous Morphine Administration. Implantation of morphine pellets produced an increase in hot-plate latency compared to placebo-treated rats when assessed at 1 hour (36.1 ± 3.9 versus 20.5 ± 2.4 seconds; $t(8) = 3.865, P < .05$). Tolerance to the antinociceptive effect

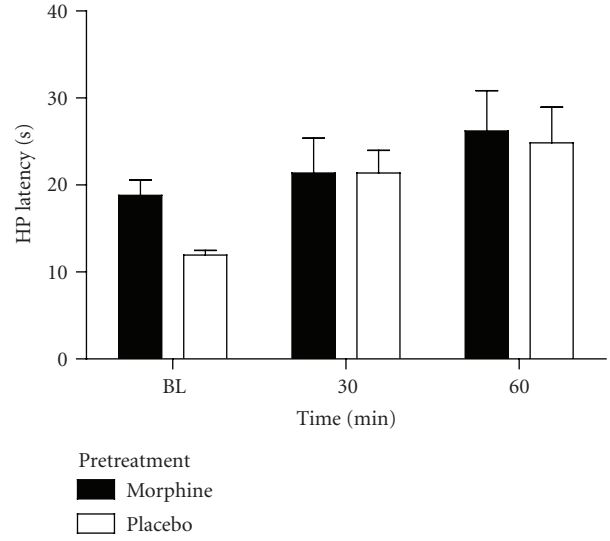

Figure 4: No change in nociception following deltorphin microinjection into the vPAG in rats treated with continuous morphine. Rats implanted with morphine pellets 3 days prior to this test had higher baseline hot-plate latency than rats implanted with placebo pellets indicating that tolerance to the antinociceptive effects was not complete. Microinjection of deltorphin into the vPAG caused an increase in hot-plate latency in rats with placebo pellets (N = 5), but did not increase the hot-plate latency for rats with morphine pellets (N = 5) above the baseline latency. That is, there was no additional antinociception by injecting deltorphin into the vPAG of rats receiving morphine.

of morphine was evident by day 4 as indicated by a decrease in hot-plate latency to 18.8 ± 1.8 seconds. Microinjection of deltorphin into the PAG caused a modest, but significant, increase in hot-plate latency compared to baseline latency ($F(2,16) = 5.806, P < .05$). Although the baseline hot-plate latency for rats treated with morphine pellets was greater than in placebo-treated rats (see Figure 4), this difference did not reach statistical significance using the Bonferroni test (t = 1.482 nanoseconds). However, microinjection of deltorphin into the vPAG of placebo-treated rats caused a significant increase in hot-plate latency at the 60-minute time point compared to the baseline (Bonferroni, t = 3.044. $P < .05$). There was no difference in mean hot-plate latency following deltorphin administration between morphine and placebo-treated rats ($F(1,8) = 0.791, P > .05$), suggesting that deltorphin administration did not increase antinociception beyond what was already present in rats with morphine pellets.

4. Discussion

The present data demonstrate that repeated morphine administration alters the response of vPAG neurons to the DOR agonist deltorphin. Although PAG neurons contribute to a wide range of behaviors [24], the change in response to deltorphin microinjection was specific to locomotor activity. Microinjection of deltorphin into the vPAG produced a consistent decrease in activity in rats pretreated with morphine.

This decrease in activity was caused by a drastic change in behavior from exploring and grooming to crouching and lying along the edge of the cage. In contrast, microinjection of deltorphin produced a mild antinociception that was not altered in a consistent manner by prior morphine administration.

MOR agonists such as morphine are the most effective treatment for pain. The descending modulatory system that runs from the PAG to rostral ventromedial medulla (RVM) to spinal dorsal horn plays an important role in the antinociceptive effects of both MOR and DOR agonists [9, 25–28]. The antinociception produced by microinjection of DOR agonists into the PAG is weak compared to morphine administration [9, 10]. However, DORs are located in the PAG [13] and these receptors appear to move from the cytoplasm to the plasma membrane following stress [14]. The density of DORs on the membrane has also been shown to increase in spinal neurons following chronic exposure to morphine [11, 12].

The present data show that microinjection of deltorphin into the vPAG had modest effects on nociception. Rats pretreated with 10 mg/kg of morphine showed a slight hyperalgesia compared to saline-pretreated rats injected with deltorphin into the vPAG. This effect seems to be dose and test dependent. Rats pretreated with 20 mg/kg of morphine showed a slight increase in hot-plate latency following deltorphin microinjection. No changes in nociception were evident on the formalin test. The lack of effect of deltorphin in modulating nociception is surprising given that spinal administration of deltorphin following chronic morphine administration produces antinociception [11].

The lack of a consistent change in nociception following deltorphin administration could be caused by an inability of the morphine administration procedure to mobilize DORs to the plasma membrane. Although possible, this explanation seems unlikely given that a decrease in activity was produced by microinjection of deltorphin into the PAG. Moreover, we used two different procedures to induce tolerance (repeated injections and continuous administration) that closely match previous studies reporting changes in DORs [11, 12, 18]. Finally, mobilization of DORs to the plasma membrane is associated with morphine tolerance and the rats in the present study showed clear signs of tolerance to the antinociceptive effects of morphine.

In vitro electrophysiological recordings reveal DOR-mediated inhibition of GABAergic IPSCs in tissue from mice pretreated with morphine [18], but no effect of DOR agonists in PAG slices from animals that have not been exposed to morphine [15–17]. This inhibition of GABA input is similar to the effect produced by administration of MOR agonists into the PAG [29]. These data suggest that microinjection of DOR agonists into the PAG of morphine tolerant rats should produce antinociception. However, the present data show no consistent antinociceptive effect following deltorphin microinjection into the PAG of morphine tolerant rats.

In contrast, microinjection of deltorphin into the vPAG of morphine pretreated-rats caused a clear and consistent decrease in locomotor activity. Acute administration of morphine into the vPAG also produces a decrease in activity [4]. Thus, it appears that DORs compensate for the locomotor, but not the antinociceptive effects associated with morphine tolerance in the vPAG. The immobility produced by morphine microinjection into the vPAG appears to be part of a defensive freezing response [30–33]. However, the decrease in activity produced by microinjection of deltorphin into the vPAG reported here does not appear to be caused by fear-induced freezing. Microinjection of deltorphin caused rats to crouch and lay along the edge of the cage as if the rats were ill or dysphoric. This effect appears to be consistent with previous research showing that deep tissue pain sufficient to induce recuperative behavior activates vPAG neurons [34]. Given that stress increases the density of DORs on the plasma membrane [14], activation of these receptors may contribute to recuperative behavior.

One hypothesis is that the recuperative behavior mediated by the vPAG is part of a coordinated response triggered by severe hemorrhage that includes hypotension. Severe blood loss has been shown to activate neurons in the vPAG [35], and inactivation of the vPAG [2, 36] or microinjection of the DOR antagonist naltrindole into the PAG [37] blocks the hypotension produced by hemorrhage. Future studies are needed to determine whether activation of DORs in the PAG alters blood pressure.

5. Conclusion

The decrease in locomotor activity caused by microinjection of deltorphin into the vPAG of morphine tolerant rats is consistent with previous data showing that DOR density on the plasma membrane increases following chronic morphine administration. PAG DORs could contribute to morphine tolerance [38–40], behavioral changes related to stress [14], or hypovolemic shock [35, 37], but do not appear to contribute to antinociception. That is microinjection of deltorphin into the PAG did not produce antinociception regardless of how tolerance was induced (repeated injections or continuous administration), test used to assess nociception (hot plate, tail flick, and formalin tests), or test times (20 and 50 minutes).

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Review Article

The Role of the Periaqueductal Gray in the Modulation of Pain in Males and Females: Are the Anatomy and Physiology Really that Different?

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Anatomical and physiological studies conducted in the 1960s identified the periaqueductal gray (PAG) and its descending projections to the rostral ventromedial medulla (RVM) and spinal cord dorsal horn, as a primary anatomical pathway mediating opioid-based analgesia. Since these initial studies, the PAG-RVM-spinal cord pathway has been characterized anatomically and physiologically in a wide range of vertebrate species. Remarkably, the majority of these studies were conducted exclusively in males with the implicit assumption that the anatomy and physiology of this circuit were the same in females; however, this is not the case. It is well established that morphine administration produces greater antinociception in males compared to females. Recent studies indicate that the PAG-RVM pathway contributes to the sexually dimorphic actions of morphine. This manuscript will review our anatomical, physiological, and behavioral data identifying sex differences in the PAG-RVM pathway, focusing on its role in pain modulation and morphine analgesia.

1. Introduction

It was first reported that electrical stimulation of the midbrain periaqueductal gray (PAG) produced profound analgesia in the male rat in 1969 [1]. Since then, the anatomical and physiological organization of the PAG and its descending projections to the rostral ventromedial medulla (RVM) and dorsal horn of the spinal cord have been well characterized in a variety of species, including the rat [2–9], cat [10–18], primate [19, 20], and rabbit [21] (see Figure 1). The PAG-RVM-spinal cord pathway comprises an essential neural circuit for opioid-based antinociception [6, 18, 22]. Intra-PAG administration of the mu opioid receptor (MOR) agonist morphine, the most commonly prescribed opiate for persistent pain relief, produces naloxone-reversible analgesia [23] as well as naloxone-reversible excitation of RVM neurons [7, 24, 25]. Similarly, lesions of the PAG or intra-PAG administration of MOR antagonists [26–29] attenuate the antinociceptive effects of systemic morphine across a wide range of analgesiometric tests [30]. Studies utilizing autoradiography, immunohistochemistry, and in situ hybridization have shown that the PAG contains a high density of MOR [31–38], with approximately 27–50% of PAG neurons retrogradely labeled from the RVM expressing MOR [35, 37].

While it is well established that the PAG-RVM-spinal cord pathway is essential for the analgesic actions of both systemic and intra-PAG morphine, these early studies were conducted exclusively in male subjects. Only recently have studies begun including "sex" as an independent variable, and it is becoming increasingly clear that morphine does not produce the same degree of antinociception in males and females, especially following the induction of persistent pain. Sex differences in morphine potency were first reported in rodents in the late 1980s, when it was shown that systemic morphine administration produced a significantly greater degree of antinociception in males using acute pain assays [39–42]. This phenomenon has been repeated in multiple studies employing animal models of pain, including orofacial [43] and visceral [44, 45]

pain models, as well as persistent somatic pain models [38, 46–52]. Although results on the contrary are also reported, generally these studies have shown that morphine produces a significantly greater degree of analgesia in males in comparison to females. Indeed, we have recently reported that male rats have a significantly higher MOR expression in the PAG, which is positively correlated with morphine analgesia in male but not female rats [38].

Recently, clinical studies in humans have also reported sex differences in morphine analgesia. Of the limited number of studies that examined "gender" or "sex" as an independent variable, it has been reported that males experience greater morphine analgesia compared to females [53–55]. In fact, one study reported that females required 30% more morphine to reach the same level of analgesia as males [55]. Similar to the rodent literature, the results in human studies are not unequivocal. Sarton et al. [56] reported greater morphine analgesia in females, while two studies reported no sex difference [57, 58]. Sex differences in morphine consumption also have been reported [59]; however, given that the majority of negative side effects associated with morphine consumption, including nausea, headache, and dysphoria [57, 60], are exacerbated in females compared to males, morphine consumption is not a reliable indicator of morphine analgesia.

Sex differences in opioid analgesia are not limited to mu opioid agonists. In both human and animal studies, sex differences in the analgesic effects of kappa or delta opioid agonists have also been reported, although again, not without controversy [61–65]. Several factors are likely to contribute to the disparate results between studies reporting the presence or absence of a sex difference in opioid analgesia, including differences in the type of pain being examined (e.g., experimental acute pain versus postoperative pain versus a chronic pain state), the route of drug administration (e.g., oral versus intravenous versus intrathecal), the strain differences in the rodents studies, and the efficacy of the opiate being administered. Sex differences in basal pain sensitivity, as well as estrous cycle effects, may also contribute [54, 55, 57, 66–79].

While it is clear that sex differences in opioid analgesia are not a simple and straightforward phenomenon, when sex differences are reported, they are not trivial in magnitude. In our persistent inflammatory [38, 46] and visceral pain [44, 45] studies, the ED_{50} for females is twice the ED_{50} of males. Similarly, morphine is approximately 5-fold more potent in producing antihyperalgesia in arthritic males compared to arthritic females [52]. Sex differences in morphine analgesia are not due to sex differences in the pharmacokinetics of morphine in humans [56] or rodents [50]. Rather, sex differences in morphine analgesia are likely related to the inherent differences in how the central nervous system of males and females respond to opiates. To date, the mechanism(s) underlying the sexually dimorphic actions of morphine remain unknown.

Given that the PAG and its descending projections to the RVM and dorsal horn of the spinal cord provide a primary pathway for the actions of opiates in pain modulation, inherent differences in this pathway could contribute to the sexually dimorphic actions of morphine. Thus, we tested three hypotheses: (1) are there sex differences in the anatomical organization of the PAG-RVM pathway? (2) is there a sexually dimorphic response of the PAG-RVM output neurons to persistent pain? (3) does the administration of morphine differentially engage the PAG-RVM pathway in male and female rats?

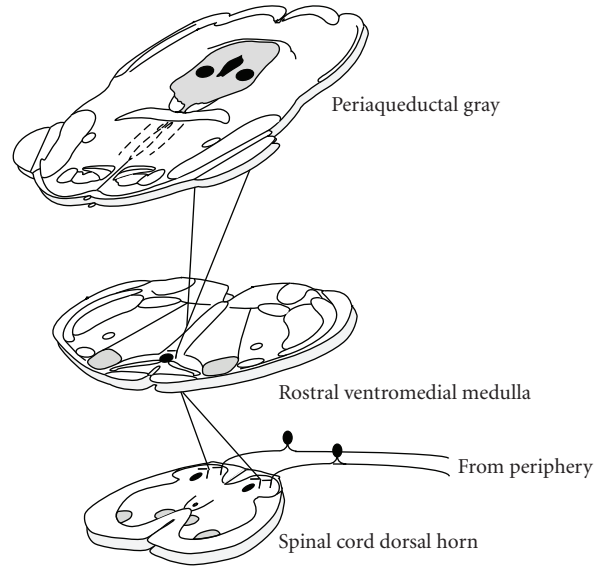


Figure 1: A schematic of the descending inhibitory pathway for pain modulation illustrating the projections from the midbrain periaqueductal gray to the brainstem RVM and the spinal cord dorsal horn.

2. Sexually Dimorphic Organization of a Descending Pain Inhibitory Pathway

We used neuroanatomical tract-tracing techniques to examine whether there were qualitative and/or quantitative differences in the neural projection from the PAG to the RVM in male and female rats. Consistent with previous anatomical studies [2, 80, 81], we reported that the dorsomedial, lateral and ventrolateral PAG heavily project to the RVM in both male and female rats [82]. While no qualitative sex differences were noted in the overall distribution of PAG-RVM projection neurons, females had significantly more PAG-RVM output neurons across the rostrocaudal axis of the PAG compared to males [83, 84] (Figures 2(a)–2(c)). The average number of retrogradely labeled cells across the rostrocaudal extent of the PAG was greater by a third in female compared to male rats (Figure 3(a)). The most prominent sex difference in retrograde labeling was observed within the lateral and ventrolateral regions of the PAG, an area known to contain a dense distribution of MOR [34, 37].

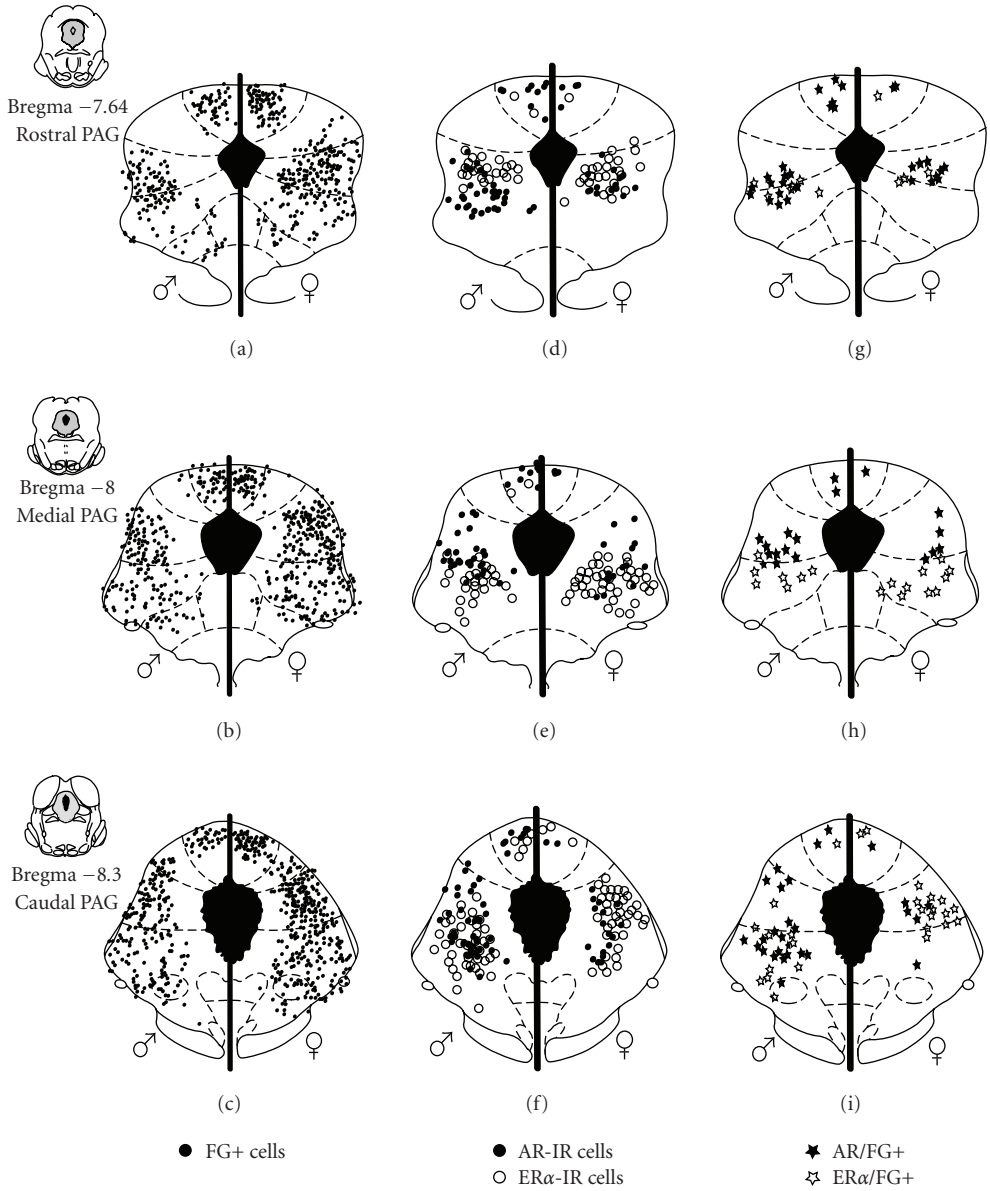
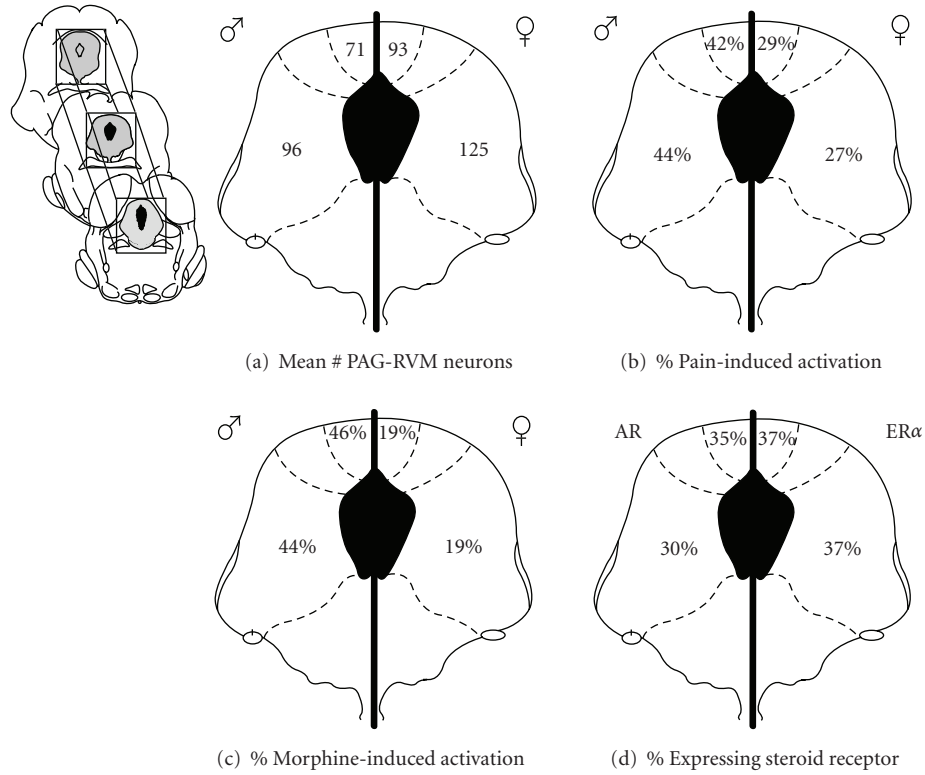


Figure 2: (a)–(c) Distribution of cells retrogradely labeled (FG+) from the RVM in males (left) and females (right) at three representative rostrocaudal levels of the periaqueductal gray. Each black circle represents one FG+ cell. (d)–(f) Distribution of PAG cells that were immunoreactive for AR (closed circles) or ERα (open circles). (g)–(i) Distribution of PAG cells retrogradely labeled from the RVM that were also immunoreactive for AR (closed stars) or ERα (open stars).

3. Sexually Dimorphic Response of the PAG-RVM Pathway to Persistent Inflammatory Pain

Inflammatory pain results in the activation of descending modulatory circuits [8, 85] and contributes to both hyperalgesia and antinociception [86–89]. We found that the persistent inflammatory pain induced by injection of complete Freund's adjuvant (CFA) into the rat hindpaw caused extensive activation of PAG neurons as measured by Fos labeling. Interestingly, this activation was comparable (both quantitatively and qualitatively) in male and female rats [82]. However, when the analysis was restricted to PAG neurons retrogradely labeled from the RVM, while females have almost twice the number of PAG-RVM output neurons in comparison to males, very few of these cells in female rats expressed inflammation-induced Fos, suggesting that this circuit is preferentially activated in males (Figure 3(b)). Indeed we found that, overall, persistent inflammatory pain activated approximately 43% of PAG-RVM neurons in the dorsomedial, lateral and ventrolateral PAG of males, but only half as many PAG-RVM output neurons were activated by inflammatory pain in females. Activation of the PAG and its descending outputs to the RVM results primarily in



(a) Mean # PAG-RVM neurons (b) % Pain-induced activation

(c) % Morphine-induced activation (d) % Expressing steroid receptor

Figure 3: (a) Mean number of PAG cells retrogradely labeled from the RVM across the rostrocaudal axis in males (left) and females (right). (b) Percentage of Fos-positive neurons that were retrogradely labeled from the RVM in males (left) and females (right) following twenty-four hours of inflammation. (c) Average of the percentage of AR (left) and ERα (right) receptor-expressing PAG cells retrogradely labeled from the RVM. (d) Percentage of Fos-positive neurons that were retrogradely labeled from the RVM in males (left) and females (right) following twenty-four hours of inflammation and one hour of morphine (5 mg/kg).

the inhibition of dorsal horn neuronal responses to acute noxious stimuli [90–95]; therefore, one would predict that given the greater activation of the circuit in males than females, males should have displayed reduced hyperalgesia following induction of plantar inflammation. However, in our behavioral studies, we found no sex differences in either baseline withdrawal latencies or in CFA-induced hyperalgesia. Therefore, our finding that the PAG-RVM descending circuit is not being engaged to the same degree by persistent inflammatory pain in males and females suggests that there is an alternative mechanism for endogenous pain modulation in female rats [96–99].

We have recently begun exploring this possibility using combinatorial anterograde and retrograde tract-tracing in combination with persistent pain-induced Fos labeling. The results of these studies suggest that there are indeed sex differences in both the efferent and afferent projections of the PAG. Specifically, the amygdala, ventromedial hypothalamus, and periventricular nucleus project more heavily to the PAG in females than males. In contrast, the medial preoptic area, parabrachial nucleus, and locus coeruleus project more heavily to the PAG in males than females [100]. In addition, our data indicate that the projections to the parabrachial nucleus, locus coeruleus, and the A5/A7 noradrenergic cell group appear to be greater in males (Loyd and Murphy, unpublished observations). Obviously, further research on the anatomy and physiology of pain modulatory circuits in females is warranted.

4. Sex Differences in the Activation of the Descending Inhibitory Pathway by Morphine

Although sex differences in PAG-RVM output neuron activation do not appear to contribute to sex differences in pain, they do appear to contribute to sex differences in morphine analgesia. Until recently, all studies examining the mechanisms of morphine action in the PAG were conducted exclusively in males; therefore it was unknown whether morphine administration has the same physiological effect on PAG neurons in females. Electrophysiological studies of PAG neurons are limited because they examine the response of a single neuron [7, 17, 101–109]. We have addressed this problem by using tract-tracing techniques and Fos labeling to measure the activity of *populations* of PAG-RVM neurons in the PAG of males and females.

Systemic morphine administration attenuates the persistent pain-induced Fos expression within the PAG of male but not female rats [82] and is consistent with our data showing that the ED_{50} for systemic morphine is approximately twofold higher in females compared to males whether administered systemically [46] or directly into the

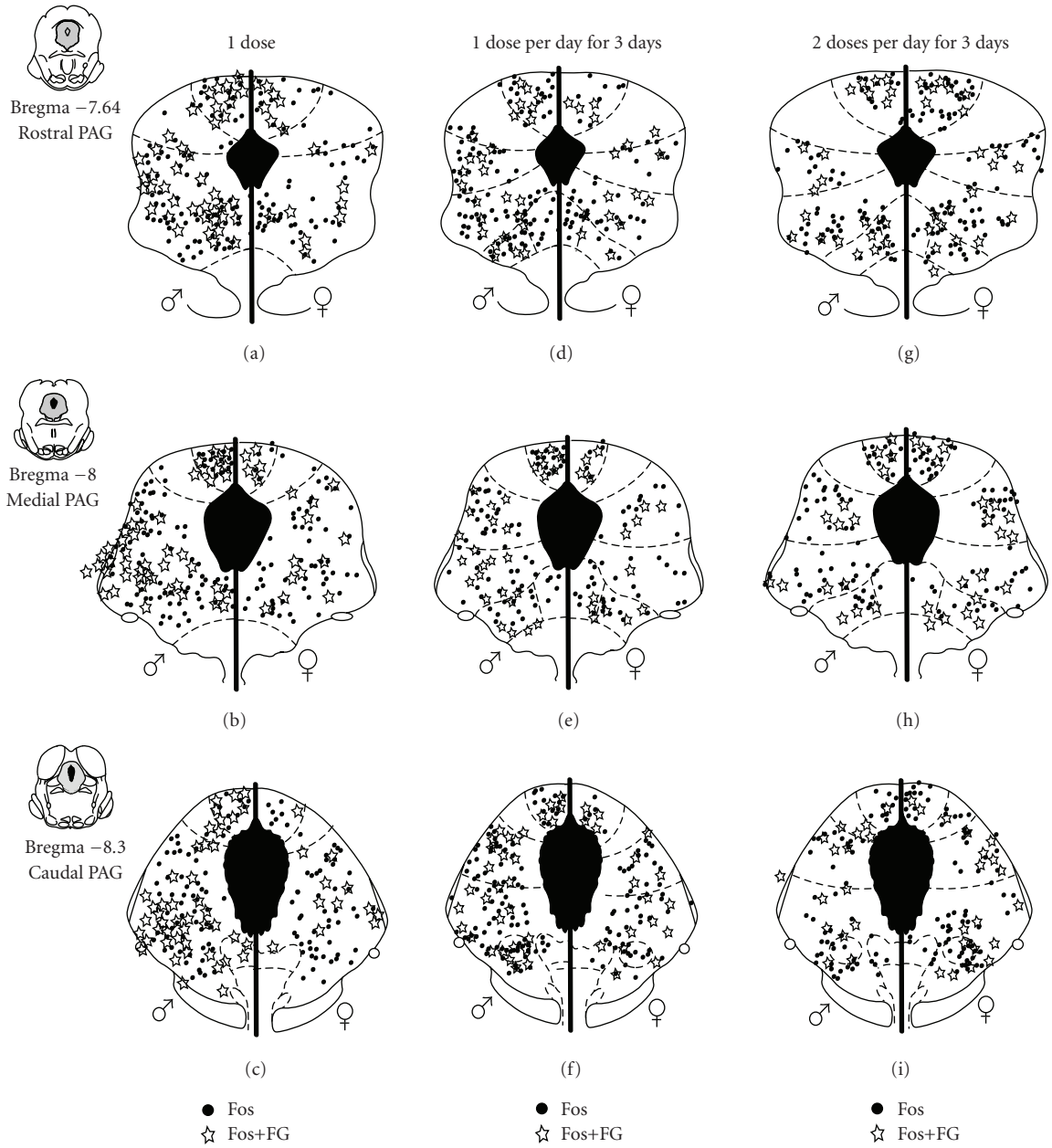


Figure 4: Distribution of PAG cells expressing Fos (black circles) and cells retrogradely labeled from the RVM expressing Fos (stars) following: (a)–(c) one 5 mg/kg dose of morphine; (d)–(f) one 5 mg/kg dose of morphine per day for three consecutive days; (g)–(i) or two 5 mg/kg doses of morphine per day for three consecutive days in males (left) and females (right) at three representative rostrocaudal levels of the PAG.

PAG [38]. Interestingly, morphine administration, in the absence of pain, resulted in a twofold greater activation of PAG neurons compared to saline administration [84]. No sex difference was observed in the activation of PAG neurons by morphine (see the black circles in Figures 4(a)–4(c)), suggesting that in the absence of pain, morphine is equipotent in its ability to depolarize PAG neurons. However, when the analysis was limited to PAG neurons projecting to the RVM, the number of neurons activated by morphine was consistently and significantly higher in males compared to females (see the stars in Figures 4(a)–4(c)) [84]. Indeed, approximately half of PAG-RVM neurons in males were activated by morphine, whereas only 20% were activated in females (see Figure 3(c)). These results corroborate previous studies demonstrating that morphine results primarily in the net excitation of PAG-RVM neurons, most likely through the removal of tonic GABA inhibition [35, 104, 110, 111]. The finding that very few PAG-RVM neurons were activated by morphine in females suggests that morphine may be limited in effectiveness as a pain modulator.

Given that more PAG neurons project to the RVM in female compared to male rats, it is possible that pain

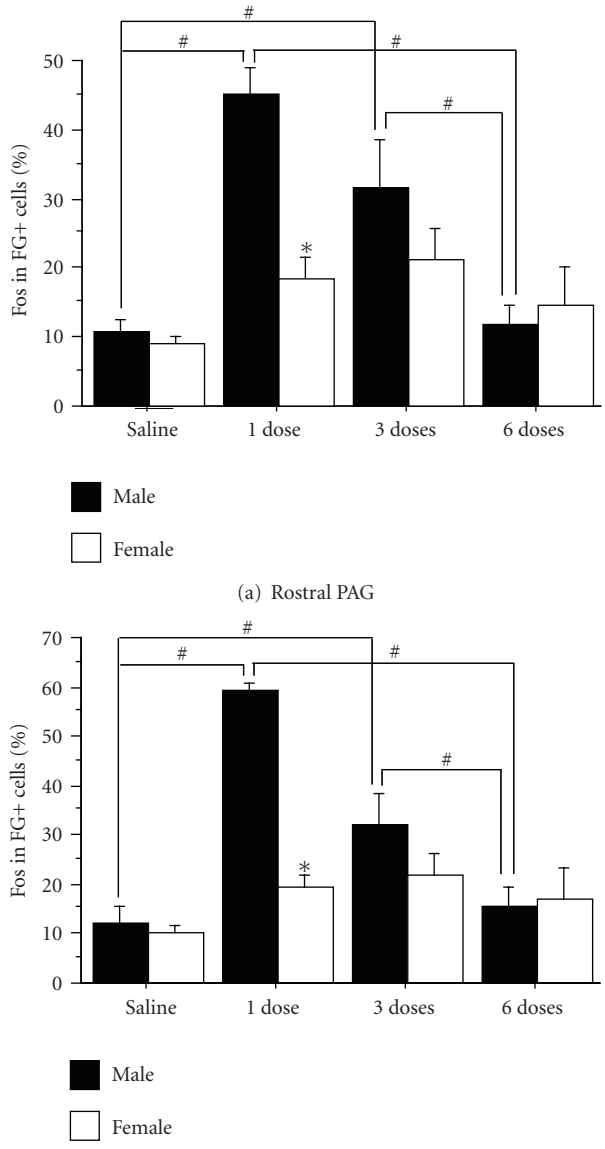


Figure 5: Percentage of Fos-positive neurons that were retrogradely labeled from the RVM (%Fos in FG+ cells) in male (solid bars) and female (open bars) rats injected with either morphine or saline once or twice daily for three days for the rostral ((a); Bregma −6.72, −7.04, −7.74) and caudal ((b); Bregma −8.00, −8.30, −8.80) PAG. A decrease in labeling is evident with an increase in the number of morphine injections for male rats. The # indicates a significant effect of treatment and the ∗ indicates a significant effect of sex. *Saline: morphine naïve; 1 dose: saline pretreatment followed by one dose of morphine; 3 doses: one dose of morphine per day; 6 doses: two doses of morphine per day.*

modulation in females is less dependent on opioids. If this is the case, then direct activation of PAG output neurons should produce greater antinociception in females, not males. Microinjection of the GABA antagonist bicuculline into the PAG produces antinociception [110, 112] by disinhibiting output neurons. Surprisingly, even though females have more output neurons, the antinociceptive effect of microinjecting bicuculline into the PAG is greater in males [113].

5. Sex Differences in the Development of Tolerance to Morphine

Repeated or continuous administration of morphine into the ventrolateral PAG of male rats has been shown to result in the development of tolerance [26, 114–118]. In addition, blocking opioid binding sites in the ventrolateral PAG attenuates the development of tolerance to systemically administered morphine [26]. Tolerance appears to be mediated by a reduction in MOR signaling efficacy in PAG neurons [119], an effect that is reversed when MOR coupling is enhanced via upregulated adenylate cyclase activity [120]. If the PAG-RVM pathway is essential for the development of tolerance, then activation of the PAG-RVM pathway by morphine should decline as tolerance develops, and changes in the activation of this pathway would correlate with sex differences in the development of tolerance to morphine. These hypotheses were tested in male and female rats using behavioral testing (hot plate) and immunohistochemistry to map the activation of the PAG-RVM pathway following repeated morphine administration.

Morphine was administered once or twice a day for three days in rats that had previously received retrograde tracer injections into the RVM. To examine the activation of PAG-RVM neurons during the development of tolerance, males and females were both administered 5 mg/kg of morphine, the ED_{50} for males. Repeated administration of systemic morphine induced tolerance in males to a significantly greater extent than in females [83], consistent with previous research administering equipotent doses of morphine to examine sex differences in tolerance [47]. The half maximal antinociceptive effect of a single injection of morphine following the development of morphine tolerance was two times greater for female compared to male rats. In parallel, the activation of PAG-RVM neurons was significantly attenuated following repeated morphine administration in males [83]. While there was no sex difference in the activation of the PAG following three doses or six doses of morphine over three days (see the black circles in Figures 4(d)–4(i)), the activation of the PAG-RVM projection neurons steadily declined in males only (see the stars in Figures 4(d)–4(i)). Activation of the PAG-RVM pathway by morphine in female rats was minimal, and therefore did not decline significantly following repeated administration of morphine (Figure 5; previously published [83]).

While together, these data provide compelling support for a central role of the PAG in the development of morphine tolerance; these studies administered the *male* ED_{50} dose of morphine. While a single administration of this dose of morphine resulted in comparable activation of the PAG in males and females, it was suboptimal in producing behaviorally defined antinociception in females and may account for why females did not develop tolerance to the same degree as males. Future studies employing sex-specific ED_{50} doses are clearly warranted.

6. Role of Gonadal Hormones in Sex Differences in Morphine Analgesia

Studies in rodents indicate that sex differences in the organizational and activational effects of the gonadal hormones estradiol and testosterone influence morphine analgesia. For example, male rats castrated at birth demonstrate decreased morphine potency in adulthood, while female rats masculinized at birth demonstrate greater morphine potency in adulthood [121, 122]. Similarly, morphine is less effective in gonadectomized adult males and is more effective in ovariectomized adult females [40, 123–128]; these effects can be reversed with hormone replacement [44, 123, 129]. Moreover, the antinociceptive potency of morphine has been reported to be greater during diestrus, when circulating estradiol levels are lowest [43, 124, 125, 127, 130], which is corroborated by our recent findings that MOR expression in female rats is the highest during diestrus compared to proestrus and estrus [38]. Recently, it was reported that microinjection of morphine directly into the PAG produces less antinociception during estrus (after estradiol peaks), while there was no sex difference in morphine potency between diestrus females and males [131]. We have recently reported similar findings in which the antihyperalgesic effects of intra-PAG morphine were significantly greater in females in diestrus in comparison to proestrus and estrus [38].

The anatomical substrate(s) whereby gonadal steroids influence pain and analgesia is unknown. Both androgen (AR) and estrogen receptorsα (ERα) have been localized in the PAG in the male rat [132]. Although it is not known if these receptors are present in the female rat, they have been localized in other species including the female cat [12], golden hamster [133], guinea pig [134], and the rhesus monkey [135, 136]. To date, however, the anatomical distribution of both types of steroid receptor within the PAG in reference to cells projecting to the RVM is not known.

We have combined neuroanatomical tract-tracing techniques and steroid receptor immunohistochemistry to characterize the expression of AR and ERα in the PAG-RVM pathway of male and female rats [137]. In these studies, we found that males had a significantly greater number of AR immunoreactive neurons localized within the dorsomedial, lateral and ventrolateral PAG compared to females. Interestingly, both the qualitative and quantitative expression of ERα in the PAG was comparable between the sexes (see Figures 2(d)–2(f)). Both receptor types were preferentially localized within the dorsomedial, lateral and ventrolateral subdivisions of the PAG and increased in density along the rostrocaudal axis of the PAG with the highest expression localized within the caudal PAG. In addition, 30–37% of PAG-RVM output neurons expressed AR or ERα (Figure 3(d)) with the highest density of colabeling in the lateral/ventrolateral region of PAG. ERa and AR colocalization in PAG neurons projecting to the RVM was comparable between the sexes [137] (Figures 2(g)–2(i)). The high density of steroid receptors localized on PAG-RVM output neurons may contribute to our observed sex differences in morphine analgesia. Although there was no sex difference in the anatomical localization of gonadal steroid receptors in the PAG despite the higher density of AR in males, 27–50% of PAG-RVM neurons contain MOR [37]. Given that morphine activates more of these neurons in male compared to female rats, the interaction between morphine and sex hormones is likely greater in the PAG of male compared to female rats.

There are several mechanisms whereby gonadal steroids may modulate opioid-sensitive PAG-RVM output neurons, thereby potentially resulting in a dimorphic response to morphine. First, estradiol has been shown to uncouple the MOR from G protein-gated inwardly rectifying potassium channels [138] resulting in an attenuation of morphine-induced hyperpolarization. Second, estradiol has also been shown to induce MOR internalization [139], thereby reducing available opioid binding sites on the cell membrane. Interestingly, ERα is required for estradiol-induced MOR internalization [140] supporting the hypothesis that colocalization of MOR and ERα in the PAG-RVM output neurons may provide a pain modulatory mechanism. Interestingly, administration of estradiol to gonadectomized males reinstates morphine analgesia while dihydrotestosterone does not [141], suggesting that estrogens affect morphine potency in both male and female rats [130].

7. Conclusions

Research spanning for four decades has shown that the PAG and its descending projections to the RVM and spinal cord dorsal horn constitute an essential neural circuit for opioid-based analgesia. During the last half of that period, numerous rodent and human studies have established sex differences in the antinociceptive and analgesic effects of morphine; however, the neural mechanisms underlying the sexually dimorphic actions of morphine remain poorly understood. It is now clear that the anatomical and physiological characteristics of the PAG and its descending projections to the RVM are sexually dimorphic, with clear biological consequences in terms of morphine potency. Our studies, as well as those of others, have shown that morphine is less potent in females compared to males in the alleviation of persistent pain. Future research efforts utilizing female subjects in both the investigation of persistent pain mechanisms and identification of both effective and potent pain therapeutics are clearly warranted.

Acknowledgments

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Review Article

Progesterone Withdrawal-Evoked Plasticity of Neural Function in the Female Periaqueductal Grey Matter

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Cyclical changes in production of neuroactive steroids during the oestrous cycle induce significant changes in $GABA_A$ receptor expression in female rats. In the periaqueductal grey (PAG) matter, upregulation of $\alpha 4\beta 1\delta$ $GABA_A$ receptors occurs as progesterone levels fall during late dioestrus (LD) or during withdrawal from an exogenous progesterone dosing regime. The new receptors are likely to be extrasynaptically located on the GABAergic interneurone population and to mediate tonic currents. Electrophysiological studies showed that when $\alpha 4\beta 1\delta$ $GABA_A$ receptor expression was increased, the excitability of the output neurones in the PAG increased, due to a decrease in the level of ongoing inhibitory tone from the GABAergic interneurones. The functional consequences in terms of nociceptive processing were investigated in conscious rats. Baseline tail flick latencies were similar in all rats. However, acute exposure to mild vibration stress evoked hyperalgesia in rats in LD and after progesterone withdrawal, in line with the upregulation of $\alpha 4\beta 1\delta$ $GABA_A$ receptor expression.

1. Introduction

The periaqueductal (PAG) grey matter is involved in regulating a remarkable number of bodily functions. Circuits controlling nociception, temperature regulation, micturition, vocalisation, cardiorespiratory function, and sexual behaviours are all dependent on the functional integrity of this midbrain region [1–5]. The PAG is also involved in producing emotional changes, particularly those associated with fear and defensive behaviour [6, 7], and has the ability to modulate activity in its various control circuits to orchestrate changes in the behavioural response pattern that are appropriate to an ever-changing environment [8]. In females, the PAG operates within a constantly changing hormonal milieu that results from the cyclical changes in production of neuroactive gonadal steroids during the menstrual cycle (oestrous cycle in animals). The lipophilic nature of these molecules means that they readily gain access to the brain from the circulation [9]. Here we review the results of our recent investigations into the functional consequences of changes in circulating levels of progesterone during the oestrous cycle in female rats. These experimental studies have revealed remarkable hormone-linked changes in the intrinsic excitability of the PAG circuitry that are reflected by significant changes in the behaviour of the animal.

2. Cyclical Changes in Progesterone Secretion in Females

In women, production of progesterone undergoes substantial changes during the menstrual cycle. Plasma levels of the steroid remain at a constant low level during the first half of the cycle. Following ovulation, secretion of progesterone by the corpus luteum increases, resulting in elevated blood plasma levels [10]. In the absence of a fertilised ovum the corpus luteum then degenerates, with an associated rapid fall in plasma progesterone production prior to menstruation.

It has long been recognised that the cyclical production of gonadal hormones during the menstrual cycle can trigger significant changes in psychological status. In up to 75% of women, the late luteal phase of the cycle, when progesterone levels decline rapidly, is associated with the development of adverse psychological symptoms; these may include irritability, mood swings, aggression, and anxiety

[11]. Additionally, bodily changes such as breast tenderness and bloating may occur and responsiveness to painful stimuli becomes enhanced [12, 13]. Importantly, symptoms fail to develop in women during anovulatory cycles [14] indicating a causal relationship between changes in gonadal steroid levels and brain function. The oestrous cycle of rodents acts as a suitable model of the human menstrual cycle, and offers the opportunity to study hormone-induced plasticity of brain function within intact circuits and to relate this to a behavioural outcome. The late dioestrus (LD) phase in rats, when progesterone levels are falling naturally, correlates with the premenstrual phase in women and increased anxiety and aggression have been reported to develop during dioestrus in rats [15–17]. In rats progesterone levels also fall rapidly during proestrus following the preovulatory surge [18]. However, the dynamics of this short-lasting surge in progesterone production are not sufficient to trigger the long-lasting changes in GABAergic function that accompany LD (see below, also [19] for discussion of this point).

3. Neural Actions of the Progesterone Metabolite Allopregnanolone

Within the brain, progesterone produces genomic effects via neuronal nuclear-bound receptors. In addition, it can also act at the cell membrane level. These nongenomic effects are mediated not by progesterone itself but via the actions of its neuroactive metabolite 3 alpha-hydroxy-5 alpha-pregnan-20-one (allopregnanolone, ALLO). ALLO is a steroidal compound that is synthesized *de novo* within the brain from progesterone via the actions of 5α-reductase and 3α-hydroxysteroid dehydrogenase (for review, see [20]). ALLO acts at two sites on the $GABA_A$ receptor. The first is an activation site that produces direct receptor activation and the second is a potentiation site at which ALLO acts as a powerful positive allosteric modulator to enhance the effects of GABA [21]. Potentiation occurs in response to low nanomolar concentrations of ALLO [22] whereas higher, micromolar concentrations, as seen for example at parturition [23], are required for direct activation of $GABA_A$ receptors [24]. The levels of ALLO present in the brain are influenced by the circulating levels of progesterone and we have shown recently that ALLO from the plasma gains ready access to the PAG where it produced a decrease in neuronal excitability via a $GABA_A$-mediated mechanism [25]. In other brain structures, changes in the concentration of ALLO following fluctuations in the level of circulating progesterone have been shown to trigger upregulation of $GABA_A$ receptor subunit expression that leads to changes in neural excitability and behaviour [26].

4. Progesterone Withdrawal-Induced Plasticity of GABA Receptor Function in the PAG

$GABA_A$ receptors are pentameric structures that surround a single chloride channel. Although most receptors are comprised of only 3 subunit types, the large pool of available subunits means that receptors can be comprised of many different subunit combinations [27]. The functional characteristics of individual receptor subtypes are determined by their subunit composition. There are several indications that fluctuations in the concentrations of progesterone play a major role in determining the temporal pattern of expression of certain subunits of the $GABA_A$ receptor. For example, withdrawal of cultured cerebellar granule cells or cortical neurones from long-term treatment with progesterone was accompanied by upregulation of α4 subunit mRNA [28, 29]. Similarly, hippocampal tissue from progesterone-withdrawn rats showed upregulation of both α4 and δ subunit mRNA [26, 30]. These effects were mediated not by progesterone itself but by its neuroactive metabolite ALLO and presumably represent a response of the neurone in an effort to maintain homeostasis. Thus progesterone influences neural function directly via a genomic action at the nuclear progesterone receptor and indirectly via a nongenomic action of ALLO at the membrane-bound $GABA_A$ receptor.

The PAG is another brain region that shows a susceptibility to phasic changes in the ambient level of progesterone and hence ALLO. Using immunohistochemistry, we found that 24-hour withdrawal of female rats from long-term dosing with exogenous progesterone ($5\,mg\,Kg^{-1}$ IP twice daily for 6 days) leads to upregulation of α4, β1, and δ $GABA_A$ receptor subunit protein in neurones in the PAG (Figure 1) [31]. Within the PAG, subunit-immunoreactive neurones were distributed throughout all subdivisions (Figure 2). However, upregulation after progesterone withdrawal was most marked in the dorsolateral column [32] where the density of GABAergic neurones, in which most $\alpha4\beta1\delta$ receptors are expressed [31], was the greatest [33].

We have been able to translate the findings obtained using an exogenous dosing regime to the natural fluctuations in the hormone that occur during the oestrous cycle. During LD, when plasma levels of progesterone are falling, a parallel upregulation of α4, β1, and δ $GABA_A$ receptor subunit protein occurred within the PAG [34]. This suggests that new $GABA_A$ receptors with the $\alpha4\beta1\delta$ subunit configuration had been formed in response to the falling steroid levels in the brain. $GABA_A$ receptors containing δ subunits are likely to be extrasynaptically located [35] and those with the $\alpha4\beta1\delta$ configuration are characterised by an extremely low EC_{50} for GABA [36]. The $\alpha4\beta1\delta$ $GABA_A$ receptors in the PAG should therefore be activated by the level of GABA present in the extracellular fluid and be responsible for mediating tonic currents [35, 36]. Expression of α4, β1, and δ subunit protein was confined predominantly to the GABAergic neuronal population in the PAG [32]. These two factors, that is, sensitivity to GABA and cellular location, may provide an important key to the functional consequences of progesterone withdrawal-induced receptor plasticity. In functional terms, increased expression of $\alpha4\beta1\delta$ receptors on GABAergic neurones would be expected to lead to a reduction in the level of their activity, thus disinhibiting the output neurones within the PAG by reducing the level of ongoing GABAergic tone (Figure 3). Hence, one would expect to see an increase in the excitability of the various neural control systems located within the PAG. The PAG is organised functionally in terms of a number of longitudinal

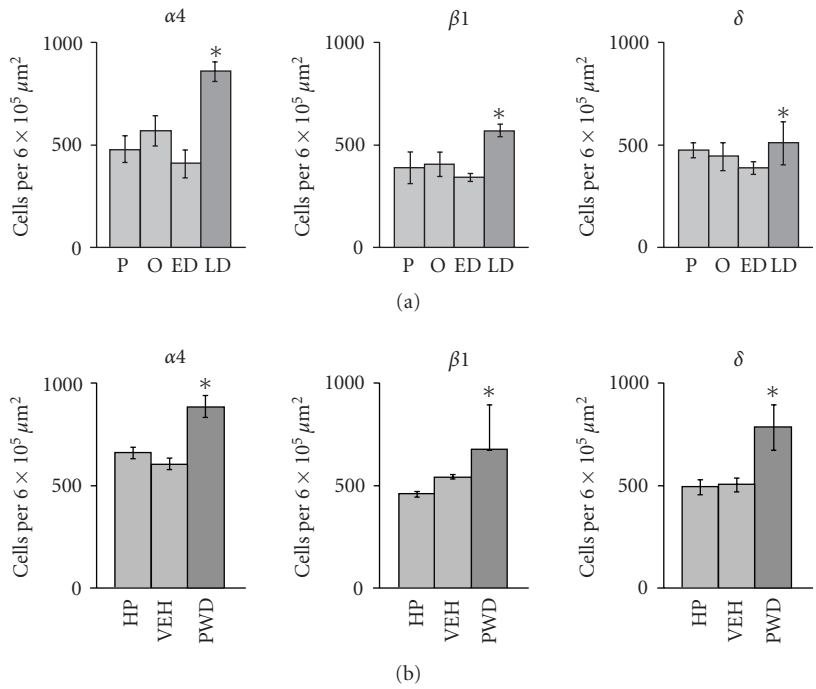

Figure 1: Density of $\alpha 4$, $\beta 1$, and δ $GABA_A$ receptor subunit-immunoreactive neurones in the PAG in (a) spontaneously cycling female rats and (b) rats that had undergone a progesterone withdrawal regime. Abbreviations: P: proestrus; O: oestrus; ED: early dioestrus; LD: late dioestrus; HP: high progesterone; VEH: vehicle treated; PWD: progesterone-withdrawn. $n = 5$ for each group, all values mean ± SEM. $^*P < .05$, post hoc Fischer test after significant ($P < .05$) one-way ANOVA. Data redrawn from [31, 32].

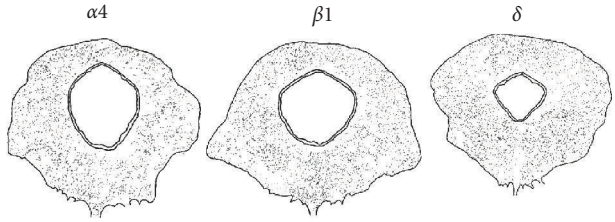

Figure 2: Camera lucida reconstruction showing distribution of $\alpha 4$, $\beta 1$, and δ $GABA_A$ receptor subunit positive neurons in representative sections taken from the mid PAG level from a rat in early dioestrus. The figure is adapted from [31].

columns that integrate and control diverse aspects of its function [37]. The oestrous cycle-linked upregulation of $\alpha 4$, $\beta 1$, and δ $GABA_A$ receptor subunit protein was seen in all regions of the PAG with little evidence of somatotopic localisation. Hence, the functional consequences of receptor plasticity are likely to be widespread and not confined to any one aspect of PAG function.

5. Oestrous Cycle-Linked Changes in Neural Excitability of the Female PAG

The excitability of the neural circuits of the PAG is normally regulated by tonic activity of GABAergic neurones. At the neuronal level, the presence of ongoing GABAergic inhibition within the PAG was revealed by the increase in firing rate of output neurones in the presence of a $GABA_A$ antagonist [19]. In the conscious rat, the functional importance of the tonically active GABAergic control system is manifested by the dramatic behavioural changes elicited by microinjection of $GABA_A$ antagonists into this region [39–42]. To date, most of these studies have been restricted to male animals. However, given the plasticity of the GABAergic control system in the PAG in females, changes in the functional excitability of the neural circuitry might be expected to occur at different stages of the oestrous cycle. Indeed, our electrophysiological studies have shown that GABA tone in the PAG in females is reduced during LD [19] and also in oestrus, although the latter effect is unlikely to be related to plasticity of $\alpha 4\beta 1\delta$ receptor expression (for a discussion on this point see [19]). Changes in the intrinsic level of GABAergic tone in the PAG have the potential to impact significantly on the wide range of the behaviours that are controlled by this region. Indeed, even in the anaesthetised preparation, we were able to show changes in responsiveness of single neurones in the PAG to the panicogenic and pronociceptive CCK_2 receptor agonist pentagastrin at different stages of the cycle [19].

6. Behavioural Consequences of Steroid Hormone Withdrawal-Evoked Changes in PAG Function

The PAG is a source of multiple descending control pathways to the spinal cord that exert both inhibitory and facilitatory influences on the spinal processing of nociceptive

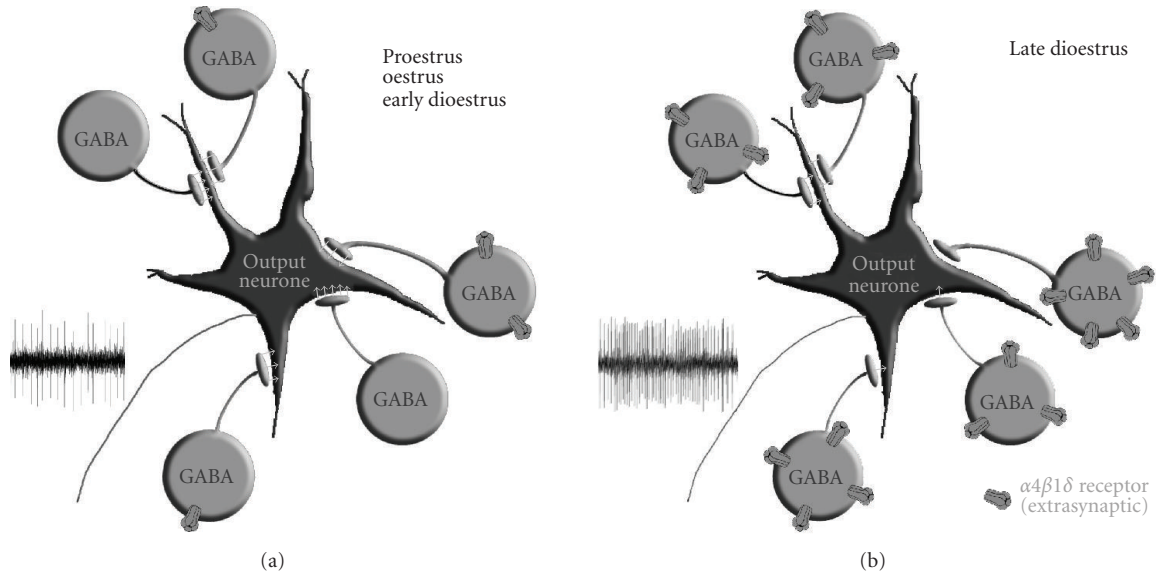

Figure 3: Cartoon to depict changes in $GABA_A$ receptor expression in the PAG at different stages of the oestrous cycle. $\alpha4\beta1\delta$ $GABA_A$ receptors are expressed mainly by GABAergic interneurones where they are extrasynaptically located and mediate tonic currents. The excitability of output neurons from the PAG is limited by spontaneous activity in GABAergic interneurones. (a) When expression of $\alpha4\beta1\delta$ $GABA_A$ receptors is low during proestrus (Pro), oestrus (O), and early dioestrus (ED), high levels of activity in the interneurone population limit the excitability of the output neurons. (b) When progesterone levels fall during late dioestrus (LD), increased expression of $\alpha4\beta1\delta$ receptors leads to an increase in tonic current carried by GABAergic cells, thus limiting their on-going activity. The output neurones therefore become intrinsically more excitable, and their threshold for activation is lowered. The figure is adapted from [38].

information [43]. Both pro- and antinociceptive control systems are thought to be tonically active under normal conditions but the balance between them is in a state of constant dynamic flux [44, 45]. We have recently begun to investigate the behavioural consequences of steroid hormone-evoked changes in the excitability of the circuitry in the conscious animal, focussing on changes in responsiveness to painful stimuli as well as on indices of anxiety.

The tail flick latency (TFL) in response to radiant heat applied to the underside of the tail elicits a withdrawal reflex that is a commonly used index of sensitivity to acute cutaneous pain in conscious rats [46]. We compared TFLs in rats that had undergone a progesterone withdrawal regime ($5\,mg\,Kg^{-1}$ IP twice daily for 6 days followed by 24-hour withdrawal) with those obtained from rats in proestrus (Pro) and LD. These two stages of the oestrous cycle were chosen as being representative of low and high expressions of $\alpha4\beta1\delta$ $GABA_A$ receptors in the PAG (see Figure 1). Since changing hormone levels might also induce changes in stress or anxiety levels in rats used for nociception testing, which could potentially influence their perception of pain [46], we also observed the behaviour of animals in a $1\,m \times 1\,m$ open field arena to assess intrinsic anxiety levels [47].

Experiments involving nociceptive testing were carried out under the UK Animals (Scientific Procedures) Act 1986 and conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1985). Female Wistar rats were habituated to a restraining tube. TFLs were measured at 5-minute intervals over a 15-minute period, that is 3 tail flick tests, to obtain a basal value for TFL. There was no difference in basal TFLs between any of the different groups of spontaneously cycling rats or the progesterone-treated animals (Figure 4(a)). Similarly, there was no difference in any of the behavioural indices measured in the open field (Table 1). We next investigated interactions between anxiety and nociception. The rats were subjected to mild nonnoxious stress by vibrating the restraining tube with the rat inside it for 5 minutes at 4 Hz [48]. During the vibration stress, the rats showed signs of anxiety that included micturition, defaecation, and vocalisation. Immediately following the vibration stress, tail flick testing was resumed. Rats that were undergoing progesterone-withdrawal had developed a poststress hyperalgesia that was manifested as a significant decrease in tail flick latency that persisted for 10 minutes (Figure 4(b)). Rats maintained on the progesterone dosing schedule failed to develop hyperalgesia (Figure 4(b)). Spontaneously cycling rats in Pro (low $\alpha4\beta1\delta$ $GABA_A$ receptor expression in the PAG) were compared with rats in LD (high $\alpha4\beta1\delta$ $GABA_A$ receptor expression in the PAG) using the same protocol. Rats in LD displayed a significant hyperalgesia following the vibration stress, whereas rats in Pro showed no change in TFL (Figure 4(b)).

These experiments failed to show any change in baseline thermal nociception in female Wistar rats at different stages of the oestrous cycle. In contrast, other investigators have been able to detect oestrous cycle-linked differences in sensitivity to pain [49–53]. However, the reports of differences in nociception with respect to cycle stage were equivocal.

Table 1: Behavioural indices in open field test for rats in proestrus, late dioestrus, and after progesterone withdrawal. All values mean ± SEM. Late dioestrus (LD, $n = 15$), proestrus (Pro, $n = 22$), high progesterone (HP, $n = 7$).

Measure	Group		
	LD	Pro	HP
Total distance travelled (cm)	3827 ± 228	3737 ± 205	4328 ± 208
Average speed (cm s^{-1})	12.85 ± 0.76	12.91 ± 0.70	14.45 ± 0.70
Time in central zone (%)	2.56 ± 0.51	1.96 ± 0.25	2.82 ± 0.56
Time in middle zone (%)	10.00 ± 1.98	7.74 ± 1.42	6.75 ± 0.94
Time in outer zone (%)	86.60 ± 2.24	89.09 ± 1.73	90.85 ± 1.43
Number of central zone re-entries	4.25 ± 0.64	4.10 ± 0.62	3.57 ± 0.57
Time rearing (%)	18.53 ± 2.35	19.13 ± 2.01	14.13 ± 1.26
Time freezing (%)	0.30 ± 0.30	0.54 ± 0.44	n/a
Time grooming (%)	7.68 ± 1.51	6.48 ± 1.48	4.93 ± 1.38

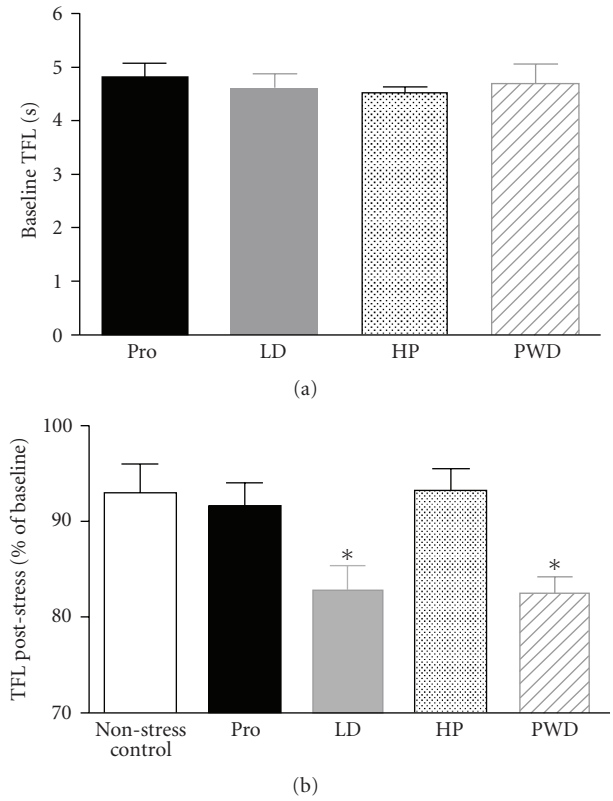

Figure 4: (a) Baseline tail flick latency in spontaneously cycling female Wistar rats in proestrus (Pro) and late dioestrus (LD) and in rats undergoing progesterone treatment (HP) or withdrawal from an exogenous progesterone dosing regime (PWD). Data show mean values of three readings taken at 5-minute intervals. All values mean ± SEM. Pro: $n = 13$, LD: $n = 12$, HP: $n = 8$, PWD: $n = 7$. (b) Change in tail flick latency (TFL) following 5-minute exposure to nonnoxious vibration stress in normally cycling and progesterone-withdrawn female Wistar rats. Data represent mean values obtained during 10 minutes immediately poststress as a percentage of the mean baseline level measured during 10 minutes prior to the stress. A control group comprising rats in proestrus and late dieostrus received no vibration stress. All values mean ± SEM; no stress (control): $n = 12$, Pro: $n = 13$, LD: $n = 12$, HP: $n = 8$, PWD: $n = 7$. $^*P < .05$, post hoc Dunnett's test in comparison to control group after significant ($P < .05$) one-way ANOVA.

At best, it seems that cycle stage may influence responsiveness to pain in rats but only in some strains and under specific experimental conditions. In terms of PAG function, this suggests either that there is very little oestrous cycle-linked change in tonic descending control of spinal nociceptive processing or, alternatively, that the activity in the control systems is altered during the cycle but in such a way that there is little change in the net balance of control at the spinal cord level.

In line with the lack of oestrous cycle effect on basal pain sensitivity, we were also unable to detect any differences in basal anxiety levels using the open field test. This finding is supported by previous studies [54, 55] but not by the work of Frye et al. [56] who reported an increase in anxiety-related behaviour in a brightly lit open field during the dark phase of the day in rats in dioestrus. However, this study may not be directly comparable to the present work, since testing in bright light, as opposed to the relatively subdued lighting (60 lux) used in the present study, would be inherently more stressful to the rats [57] by compounding the anxiogenic effects of bright light and exposure to a novel environment.

Interestingly, in the present study oestrous cycle-related differences in responsiveness to pain became apparent in the setting of a mild stress that increased anxiety levels. Moreover, the oestrous cycle-linked hyperalgesia appeared only during LD, when $\alpha 4\beta 1\delta$ receptor expression in the PAG would be upregulated. Rats undergoing withdrawal from progesterone, in which $\alpha 4\beta 1\delta$ receptor expression in the PAG would also be upregulated, showed a hyperalgesia, indicating that the effect seen in the spontaneously cycling animals was likely to be hormone-linked. During the oestrous cycle, levels of a number of gonadal hormones change [18]. Oestradiol in particular has been shown to affect GABAergic function [58]. However, at the time when $\alpha 4\beta 1\delta$ $GABA_A$ receptor expression increase during LD, plasma oestradiol levels are low and stable, suggesting that changes in progesterone rather than oestrogen are responsible for the changes in PAG function during LD. The results of our most recent work indicate that the PAG may be involved in mediating the oestrous cycle-linked hyperalgesia in the setting of mild anxiety. We have found that exposure to the vibration stress regime elicits differential expression of the immediate early gene c-*fos* in the ventrolateral PAG in rats in Pro and LD (Lovick and Devall, unpublished work). The ventrolateral PAG contains neurons that are a source of descending facilitation of spinal nociceptive inputs [59, 60]. Thus steroid hormone-linked changes in the excitability of descending control systems from the PAG may alter the level of descending control over spinal nociceptive processing and contribute to the hyperalgesia that develops in LD or during progesterone withdrawal.

Recent imaging studies in humans have also implicated the PAG in anxiety-induced hyperalgesia. Anticipatory anxiety in expectation of receiving a painful cutaneous thermal stimulus led not only to a heightened pain experience when the stimulus was delivered, that is hyperalgesia, but was also associated with activation of the PAG [61]. In women, increased responsiveness to noxious stimulation has been reported consistently during the luteal phase of menstrual cycle [12, 13]. In many women, anxiety levels are raised during the late luteal phase [11]. In any experimental scenario involving pain testing in human subjects, a degree of anxiety or apprehension is almost inevitable. Thus it is possible that the reported menstrual cycle-related differences in pain sensitivity in women may to a large extent be secondary to changes in anxiety levels rather than a primary response to changing hormone levels.

In female rats, falling progesterone levels can produce remarkable changes in the functional characteristics of neurones in the PAG, which may underlie certain oestrous cycle-linked changes in behaviour. These findings have implications for the design and interpretation of behavioural studies in female rodents in which the stage of the oestrous cycle may be a significant confounding influence. It is also possible that such hormone-linked changes may underlie the development of menstrual cycle-related disorders in susceptible women.

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Research Article

Afferent Connections to the Rostrolateral Part of the Periaqueductal Gray: A Critical Region Influencing the Motivation Drive to Hunt and Forage

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Previous studies have shown that a particular site in the periaqueductal gray (PAG), the rostrolateral PAG, influences the motivation drive to forage or hunt. To have a deeper understanding on the putative paths involved in the decision-making process between foraging, hunting, and other behavioral responses, in the present investigation, we carried out a systematic analysis of the neural inputs to the rostrolateral PAG (rlPAG), using Fluorogold as a retrograde tracer. According to the present findings, the rlPAG appears to be importantly driven by medial prefrontal cortical areas involved in controlling attention-related and decision-making processes. Moreover, the rlPAG also receives a wealth of information from different amygdalar, hypothalamic, and brainstem sites related to feeding, drinking, or hunting behavioral responses. Therefore, this unique combination of afferent connections puts the rlPAG in a privileged position to influence the motivation drive to choose whether hunting and foraging would be the most appropriate adaptive responses.

1. Introduction

Previous studies from our laboratory, examining the neural basis of morphine-induced inhibition of maternal behavior, brought up the suggestion of a rather unsuspected and integrative role of the periaqueductal gray (PAG) in influencing the selection of adaptive behavioral responses [1, 2].

Examining the neural basis underlying maternal behavior inhibition by low doses of morphine in morphine-experienced dams, we found that morphine treatment induces a behavioral switch from maternal to predatory behavior. Hence, morphine-challenged dams, tested in an environment containing both pups and roaches (which served as prey), clearly preferred hunting instead of nursing [2]. We have further shown that, under physiological conditions, there is a natural endogenous opioid tone that may be able to stimulate hunting in lactating dams [3].

The results of behavioral, neuronal immediate early gene activation, and lesion experiments indicate that a particular site in the PAG, at the level of the oculomotor nucleus, located in the outer half of the lateral column, and referred to as the rostrolateral PAG (rlPAG), should be responsible for this switching from maternal behavior to prey hunting in morphine-treated dams [2]. First, we showed that the rlPAG upregulates Fos expression in lactating rats acutely challenged with morphine [1], similar to what had been found for animals performing insect hunting [4]. Next, by testing morphine-treated dams in an environment containing pups and roaches, we were able to show that

lesions of the rlPAG, but not other parts of the PAG, impaired predatory hunting and restored the maternal response [2]. These findings support the idea that this opioid sensitive PAG site is critical for influencing the motivation drive to hunt and forage; and should be a nodal part of a neural circuit involved in the decision-making process between hunting, foraging, and other behavioral responses.

To start unraveling this circuit, in the present study, we performed a comprehensive investigation on the rlPAG afferent connections, using the Fluorogold as retrograde tracer. A number of retrograde tract-tracing studies have investigated the afferent sources of inputs to the PAG, but they used a much less sensitive retrograde tracer (i.e., the retrograde transport of the horseradish peroxidase) and were based on large injection sites encompassing different PAG functional domains [5]. The retrograde tract-tracing method using Fluorogold as a tracer, and revealed by immunohistochemical procedures, is one of the most sensitive retrograde tract-tracing tools available [6], and yields relatively small injection sites, a feature particularly suitable for investigating the afferent connections of relatively small sites, such as the rlPAG, in the present case.

Overall, the present results support the idea that the rlPAG combines a unique set of inputs rendering this region particularly suitable for influencing the decision-making process between hunting, foraging, and other behavioral responses.

2. Materials and Methods

2.1. Animals. Subjects were adult female Wistar rats (n = 18) weighing 190–220 g and approximately 90 days of age at the beginning of the experiments. Food and water were available ad libitum to the animals in light-controlled (06:00 AM to 06:00 PM) and temperature-controlled (23–25°C) rooms. Conditions of animal housing and all experimental procedures were conducted under institutional guidelines of the Committee on Animals of the (Colégio Brasileiro de Experimentação Animal, Brazil) and the Committee on the Care and Use of Laboratory Animal Resources, National Research Council.

2.2. Retrograde Tracing Experiments. Animals were anesthetized with a mixture of ketamine (Vetaset; Fort Dodge Laboratory, Campinas, Brazil) and xylazine (Rompum, 1:2 v/v; 1 mL/kg body weight; Bayer; Sao Paulo, Brazil), and unilateral iontophoretic deposits of a 2% solution of Fluorogold (Fluorochrome Inc., Colo, USA) were placed stereotaxically into the rlPAG (2.9 mm rostral to the interaural line, 0.65 mm from the midline, and 4.5 mm ventral to the surface of the brain). Deposits were made over 5 minutes through a glass micropipette (tip diameter, 25 μm) by applying a +3 μA current, pulsed at 7-second intervals, with a constant-current source (Midgard Electronics, Wood Dale, Ill, USA, model CS3). After a survival time of 7–12 days, the animals were deeply anesthetized with sodium pentobarbital (65 mg/kg, IP) and perfused transcardially with a solution of 4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4; the brains were removed and left overnight in a solution of 20% sucrose in 0.1 M phosphate buffer at 4°C. The brains were then frozen, and five series of 30-μm-thick sections were cut on a sliding microtome in the transverse (frontal) plane and collected from the caudal medulla through the rostral tip of the prefrontal cortex. One complete series was processed for immunohistochemistry with an antiserum directed against Fluorogold (Chemicon International, Calif, USA) at a dilution of 1:5000. The antigen-antibody complex was localized with a variation of the avidin-biotin complex system (ABC) [7], with a commercially available kit (ABC Elite Kit, Vector laboratories, Calif, USA). The sections were mounted on gelatin-coated slides and then treated with osmium tetroxide to enhance visibility of the reaction product. Slides were then dehydrated and cover slipped with DPX. An adjacent series was always stained with thionin to serve as reference for cytoarchitecture.

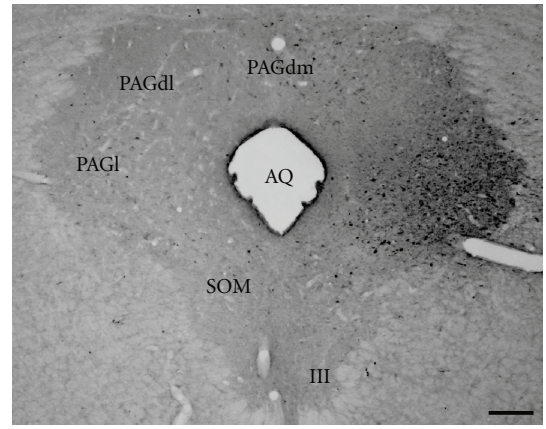


Figure 1: Brightfield photomicrograph illustrating the appearance of Fluorogold (FG) injection site in experiment PAGlFG15. Scale bar = 200 μm

Sections were examined under a microscope with bright- and dark-field illumination. Fluorogold deposits in the injection sites, and the distribution of retrogradely labeled neurons, were plotted with the aid of a camera lucida onto maps prepared from adjacent thionin-stained sections. The distribution of retrograde labeling was transferred onto a reference atlas of the rat brain [8]. The figures were prepared using Adobe PhotoShop (v.5.5; Adobe Systems, San Jose, Calif, USA) for photomicrographs and Adobe Illustrator (v.10, Adobe Systems) for drawings.

3. Results

The distribution of neurons projecting to the rlPAG region was examined by using Fluorogold. In five experiments, the deposit of the tracer appeared to be confined almost entirely to the rlPAG (located in the outer half of the lateral column at the levels of the oculomotor nucleus). The appearance of a representative Fluorogold injection site in the rlPAG is illustrated in Figure 1, and the distribution of retrogradely labeled neurons from this experiment is illustrated schematically in Figure 2. The results of this experiment are described in detail, because the injection site was virtually confined

to the rlPAG. Furthermore, in this experiment, the pattern of retrograde labeling was representative of that one seen in each of the other experiments with deposits centered in the rlPAG. The following is a summary of regions that appear to send fibers to the rlPAG.

3.1. Telencephalon. Retrogradely labeled neurons were found in the isocortex, amygdala and in the septal region. No retrogradely labeled cells were found in the hippoccampal formation.

In the isocortex, a large number of retrogradely labeled neurons was observed in the prelimbic, infralimbic, anterior cingulate, and secondary motor areas (Figures 2(a), 2(b), 2(c) and 3(a), 3(b)), in addition to substantial labeling in the gustatory and visceral areas (Figures 2(b), 2(g), 2(h), and 2(i)). A few retrogradely labeled neurons were found in the posterior part of agranular insular area (Figure 3(c)) and primary motor, perirhinal, and ectorhinal areas. All of the cortical labeled cells were pyramidal neurons of the layer V.

In the lateral septal nucleus, a relatively sparse number of marked cells were observed in the rostral part of the nucleus, distributed mainly through the dorsal region of the ventrolateral zone (Figures 2(b), 2(c)). In the septal region, we have also observed a substantial number of retrogradely marked neurons in the posterior division of the bed nuclei of the stria terminalis, particularly in the interfascicular nucleus, and also, to a lesser degree, in the transverse nucleus (Figures 2(e), 2(f)). In the amygdala, a large number of retrogradely labeled cells were found to be restricted to the medial part of the central amygdalar nucleus (Figures 2(g), 2(h), 2(i), and 4(a)).

3.2. Diencephalon

Thalamus. At the thalamus, a substantial retrograde labeling was found in the ventral part of the zona incerta (Figures 2(h), 2(i), 2(j)). No marked cells were observed in the dorsal thalamus.

Hypothalamus. In thepreoptic region, a substantial retrograde labeling was found in the median preoptic nucleus (Figures 2(c), 2(d), and 4(b)), in addition to a sparse number of marked cells in the anteroventral preoptic nucleus (Figures 2(c) and 2(d)).

At the anterior hypothalamic levels, the anterior part of the anterior hypothalamic nucleus presented a dense cluster of retrogradely labeled cells, which were distributed in the region that seems to overlap, at least partially, with a territory known to contain a large number of neurons expressing enkephalin [9] (Figures 2(e), 2(f), 2(g), and 4(c)). Moreover, at these levels, a substantial retrograde labeling was also found in the lateral hypothalamic area immediately dorsal to the optic tract and the supraoptic nucleus, which seems to correspond to a region densely targeted by the lateral component of retinohypothalamic tract [10] (Figures 2(f), 2(g), and 4(c)).

At tuberal levels, a dense number of retrogradely labeled cells was found in the anterior part of the ventromedial nucleus, in addition to a moderate number of marked cells in the retrochiasmatic area, the ventrolateral and central parts of the ventromedial nucleus, the tuberal nucleus, and rostral parts of the posterior hypothalamic nucleus (Figures 2(g), 2(h), 2(i), 2(j), and 4(d)). Furthermore, at these levels, we have found a large number of retrogradely labeled cells in the lateral hypothalamic area, distributed in the dorsal, suprafornical, justadorsomedial, and justaventromedial areas (Figures 2(g), 2(h), 2(i), and 2(j)).

At the mammilary levels, a large number of labeled neurons were found in the dorsal premammilary nucleus, mostly distributed in the dorsal part of the nucleus (Figure 2(k)). Finally, at these levels, we have found moderate retrograde labeling in the parasubthalamic nucleus, in addition to sparse labeling in the subfornical region of the lateral hypothalamic area (Figures 2(j) and 2(k)).

Brainstem. At mesodiencephalic levels, a large number of marked cells were found in the precommissural nucleus (Figure 2(l)). In the midbrain, at the injection site level, substantial retrograde labeling was found in the lateral part of the intermediate layer of the superior colliculus (Figure 2(m)). Additionally, at this level, a moderate number of retrogradely labeled neurons were also found in the PAG, which appeared to be distributed within the dorsomedial part (Figure 2(m)). Proceeding caudally, at the intermediate rostrocaudal levels of the dorsal raphe nucleus, a moderate number of marked cells was found in the ventrolateral part of the PAG (Figure 2(n)).

At rostral pontine levels, a few labeled cells were found in the laterodorsal tegmental nucleus, as well as in the central lateral, dorsal lateral, and ventral lateral parts of the parabrachial nucleus (Figure 2(o)).

It should be noted that, in the experiments with Fluorogold deposits centered in the rlPAG, retrograde labeling was mostly ipsilateral. However, some of the main sources of projections to this area, including the prefrontal cortex, retrochiasmatic area, anterior hypothalamic nucleus, ventromedial hypothalamic nucleus, lateral hypothalamic area, zona incerta, precommissural nucleus, and the PAG, also displayed conspicuous retrograde labeling contralateral to the injection site.

4. Discussion

The results of the present retrograde axonal tract-tracing study suggest that the rlPAG receive inputs from several widely distributed areas in the forebrain and, to a lesser extent, from the brainstem, as well. Prefrontal cortical areas represent the major telencephalic source of inputs to the rlPAG. In addition, clear telencephalic inputs appear to arise from the gustatory, visceral, and perirhinal cortical areas, as well as from the medial part of the central amygdalar nucleus and the interfascicular nucleus of the bed nuclei of the stria terminalis. In the diencephalon, massive inputs to the rlPAG arise from several hypothalamic sites, including the median preoptic nucleus, anterior hypothalamic nucleus, retrochiasmatic area, anterior, and ventrolateral parts of the ventromedial nucleus, dorsal premammillary nucleus (PMd), and several districts of the lateral hypothalamic area.

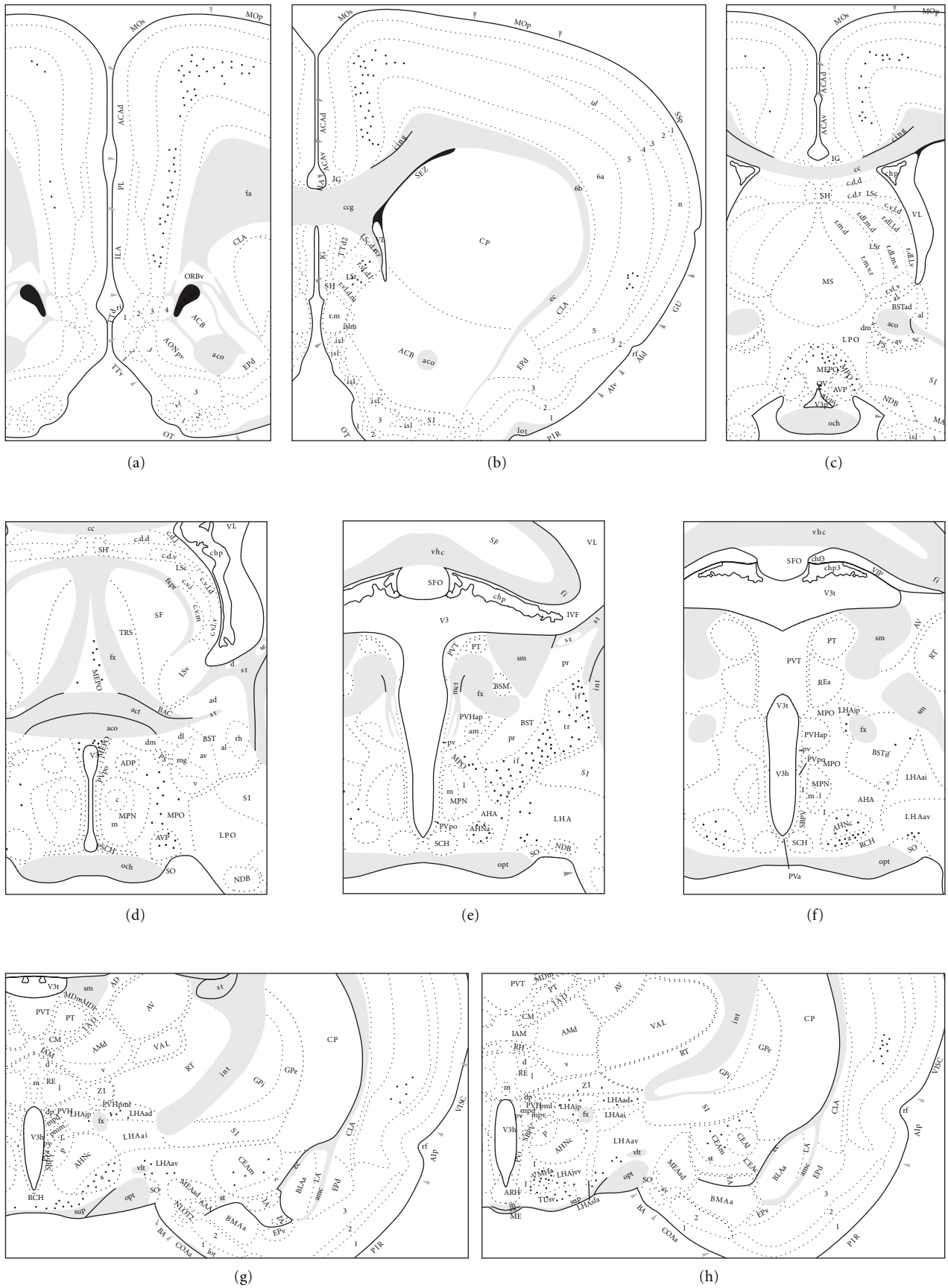


Figure 2: Continued.

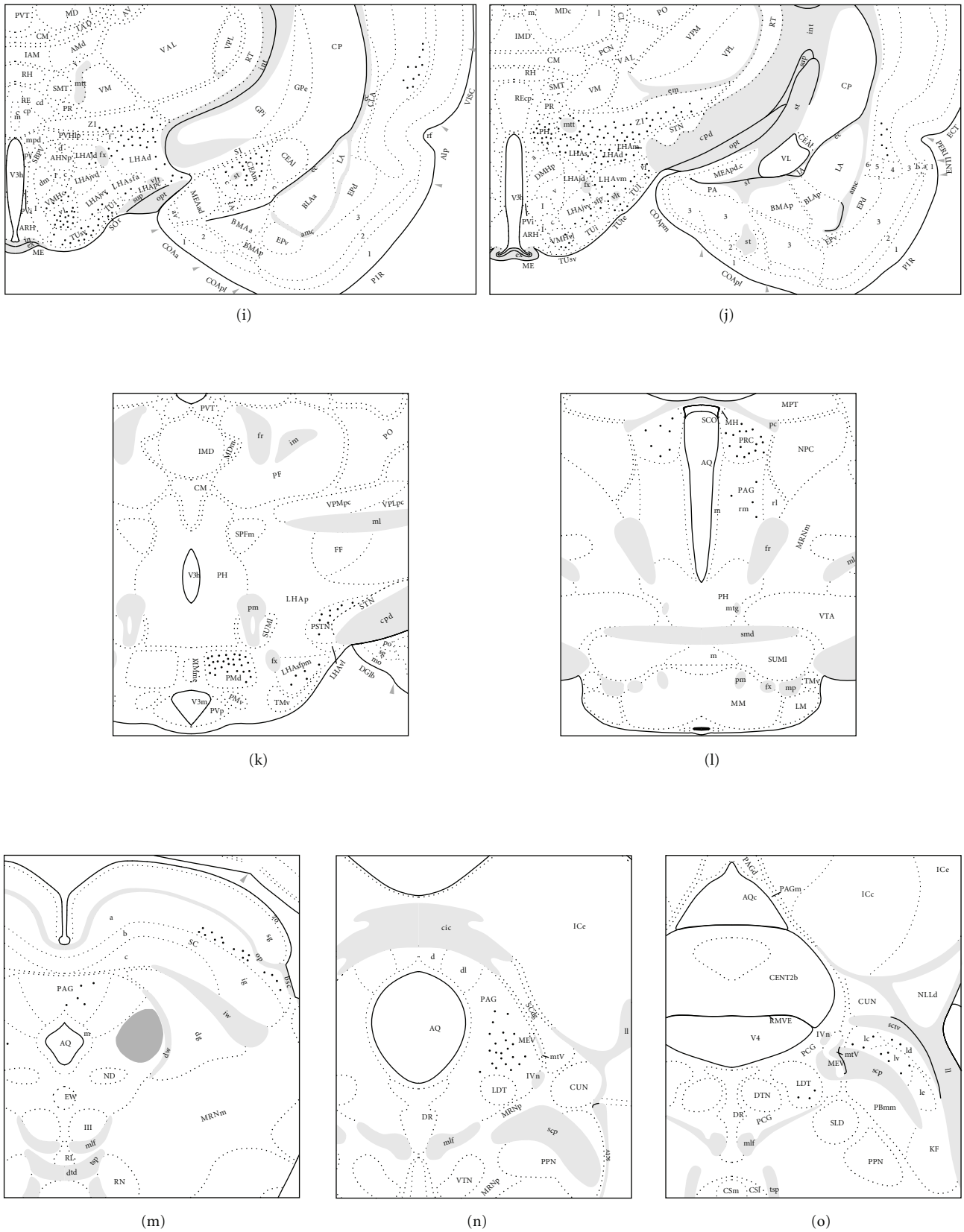
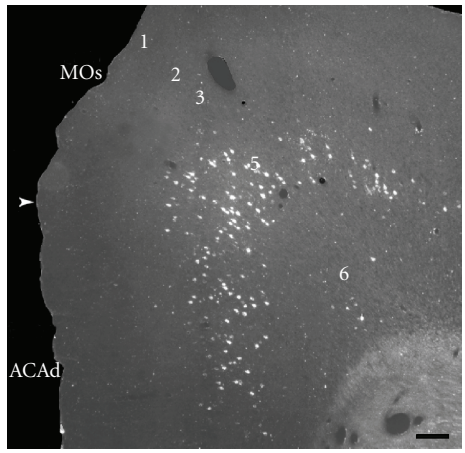
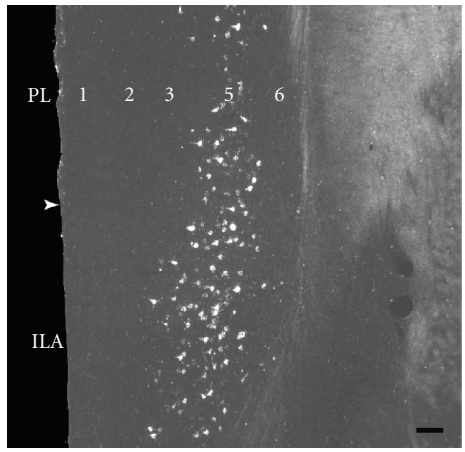


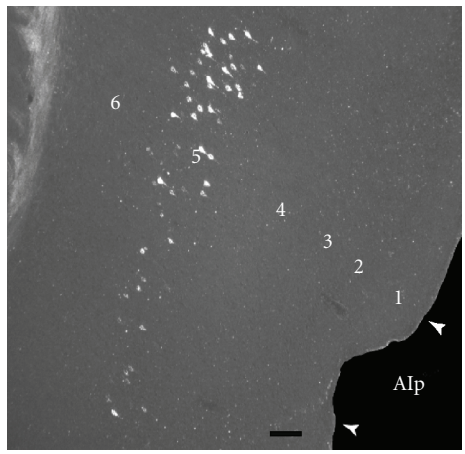
Figure 2: Inputs to the rlPAG. The distribution of retrogradely labeled neurons (black dots) in experiment PAGlFG15 plotted onto a series of standard drawings of the rat brain arranged from the rostral (a) to caudal (o) levels. The dark gray area indicates the FG injection site in this experiment. For abbreviations, see Supplementary Material available online at doi:10.1155/2009/612698.



(a)



(b)



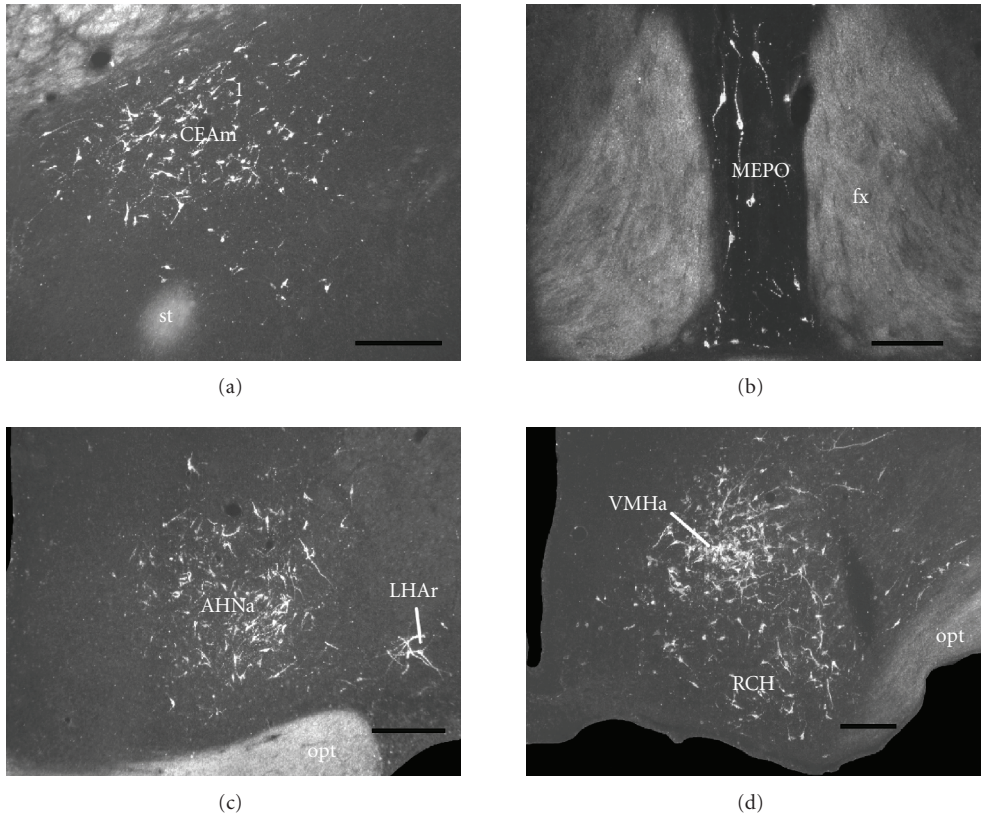
(c)

Figure 3: Dark field photomicrographs showing (a) the distribution of retrogradely labeled cells within the ipsilateral secondary motor area and the dorsal part of the anterior cingulated area; (b) the prelimbic and infralimbic areas; (c) and the visceral area. Scale bars = 200 μm.

In contrast, the rlPAG appears to receive inputs from considerably fewer brainstem sites, where significant retrograde labeling was found in other parts of the PAG (i.e., the dorsomedial and ventrolateral parts) and in the intermediate layers of the lateral part of the superior colliculus.

In agreement with previous anterograde tract-tracing studies, medial prefrontal cortical areas, including the infralimbic, prelimbic, anterior cingulated, and secondary motor areas, appear to represent one of the most important afferent sources of projections to the rlPAG [11–20]. In line with previous anterograde findings, we have also found that the rlPAG receives projections from visceral, gustatory, and perirhinal cortical areas [12–14, 17]. In the septal region, a substantial number of retrograde labeled cells were found in the interfascicular nucleus of the BST, a finding likewise supported by previous PHAL studies [21]. The present results also revealed that the medial part of the central nucleus of the amygdala is an important source of telencephalic inputs to the rlPAG. This finding has also been confirmed by means of PHAL anterograde tract-tracing method, which showed that the medial part of the central nucleus of the amygdala provides a substantial terminal field to the lateral PAG [22].

The present findings also revealed that the rlPAG is targeted by several hypothalamic districts. At preopotic levels, the rlPAG seems to receive substantial inputs from the median preoptic nucleus, in addition to somewhat sparse inputs from the anteroventral preoptic nucleus. Both of these projections have been confirmed by previous PHAL studies [23]. At anterior hypothalamic levels, a prominent group of retrogradely labeled cells was found in the anterior part of the anterior hypothalamic nucleus, in a region containing a characteristic cluster of enkephalinergic cells [9]. We were able to confirm this projection by means of PHAL deposits placed in the anterior part of the anterior hypothalamic nucleus, which yielded a distinct terminal field in the rlPAG [S. R. Mota-Ortiz and N. S. Canteras, personal observation]. In addition, at these levels, we have found that the rlPAG receives inputs from the retrochiasmatic area and a lateral hypothalamic region immediately adjacent to the optic tract and the supraoptic nucleus, which receives direct projections from the lateral part of the retinohypothalamic tract [10] and corresponds to the so-called retinoceptive region of the lateral hypothalamic area. Both the projections from the retrochiasmatic area and from the retinoceptive region of the lateral hypothalamic area to the rlPAG have been previously confirmed through PHAL experiments [24, N. S. Canteras, personal observation]. At tuberal levels, in agreement with the results of our retrograde transport experiments, previous evidence based on PHAL anterograde tract-tracing indicates that the rlPAG receives dense projections from the anterior part of the ventromedial nucleus and the posterior hypothalamic nucleus, in addition to somewhat weaker inputs from the ventrolateral part of the ventromedial nucleus and the adjacent tuberal nucleus [25, 26]. In addition, at these levels, we found that the rlPAG receives substantial inputs from the dorsal, suprafornical, justadorsomedial, and justaventromedial areas of the lateral hypothalamus, as well as immediately adjacent parts of the zona incerta. These projections have been confirmed in PHAL studies [27, M. Goto, personal observation]. Notably,



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

Figure 4: Dark field photomicrographs showing (a) the distribution of retrogradely labeled cells within the ipsilateral medial part of the central nucleus of the amygdala; (b) the median preoptic nucleus; (c) the anterior part of the anterior hypothalamic nucleus and the retinoceptive region of the lateral hypothalamic area; (d) and the anterior part of the ventromedial hypothalamic nucleus and the retrochiasmatic area. Scale bars = 200 μm.

this region of the lateral hypothalamus overlaps with the region of cells expressing melanin-concentrating hormone (MCH) and hypocretin/orexin [28]. Moreover, in agreement with previous PHAL studies [29], we have also found, in the lateral hypothalamic area, that the parasubthalamic nucleus represents another source of inputs to the rlPAG. Finally, at mammilary levels, we found a distinct projection mostly from the dorsal part of the PMd. The PMd represents one of the main hypothalamic sources of inputs to the PAG [5], and previous PHAL studies have shown that the dorsal and ventral parts of the nucleus provide a differential pattern of projection to the PAG, and confirmed that the dorsal part of the PMd provides a strikingly dense projection to the rlPAG [30].

The present results also revealed that a small number of brainstem sites appear to innervate the rlPAG. Evident retrograde labeling was found in the precommissural nucleus, the dorsomedial and ventrolateral parts of the PAG, and the lateral part of the intermediate layer of the superior colliculus. All of these projections have been confirmed through PHAL anterograde tract-tracing method [31, S. R. Mota-Ortiz and N. S. Canteras, personal observation] In addition, we have also found that the rlPAG receives relatively sparse projections from the laterodorsal tegmental nucleus and the lateral part of the parabrachial nucleus. Unfortunately, to our knowledge, these projections remain yet to be demonstrated with anterograde tracer studies.

4.1. Differential Inputs to Other PAG Parts: Comparison with the Afferent Connections to the Dorsolateral PAG (dlPAG). As mentioned above, previous retrograde tract-tracing studies have investigated the afferent sources of inputs to the PAG, but they were based on large injection sites, providing just an overall picture of the afferent inputs to the PAG without differentiating among the particular PAG domains [5]. By determining the specific set of afferent inputs to the rlPAG, we have provided important information to distinguish this PAG site from adjacent different functional domains, such as the immediately adjacent dorsolateral PAG (dlPAG). In contrast to the rlPAG, the dlPAG upregulates Fos expression in response to both actual and contextual predatory threats [32–34], and seems to be responsible for organizing the expression of unconditioned and conditioned antipredatory responses [33]. As for the rlPAG, the dlPAG also receives substantial inputs from the anterior cingulate, prelimbic, and infralimbic areas [11, 12, 19, 20], but in sharp contrast to what has been found for the rlPAG, the visceral area appears to project only sparsely to the dlPAG [13]. Additionally, in contrast to the rlPAG, the dlPAG does not seem to receive substantial inputs from the amygdala,

and seems to be densely innervated by medial hypothalamic sites involved in processing predatory cues, including the posterior part of the anterior hypothalamic nucleus, the dorsomedial part of the ventromedial nucleus and the dorsal premammillary nucleus [25, 35, 36]. Notably, the projection from the dorsal premammillary nucleus to the dlPAG arises chiefly from the ventrolateral part of the nucleus, which is the hypothalamic site most responsive to the predator or its cues [30]. In the zona incerta, differently from what we have just found for the rlPAG, the dlPAG seems to receive dense projections from the rostral zona incerta, also referred to as the incertohypothalamic area [37]. The superior colliculus also has a differential pattern of projection to the PAG, where the lateral part of the intermediate layer projects to the rlPAG, as presently shown, and the medial part of the intermediate and deep layers target the dorsal PAG [38]. Notably, the medial part of the intermediate and deep layers of the superior colliculus respond to visual-threatening stimuli, such as suddenly expanding shadows in the upper visual field (which look like an approaching predator), and, via a projection to the dlPAG, may exert a marked influence on the control of defensive responses [38].

The evidence just discussed supports the idea that different functional PAG domains are likely to have particular sets of afferent inputs, and next, we will provide a discussion on how the diverse inputs to the rlPAG may be related to its function on hunting and foraging behavior.

4.2. Functional Considerations. As commented in the Introduction, the rlPAG is an opioid sensitive site, which is critical for influencing the motivation drive to hunt and forage, and is likely to be part of a neural circuit involved in the decision-making process between hunting, foraging, and other behavioral responses. The present findings help to reveal this complex network, and reinforce the rlPAG's nodal role in integrating a wealth of different kinds of information likely to influence foraging or hunting activity.

One of the main findings of the present investigation is that the rlPAG appears to be particularly driven by inputs from medial prefrontal cortical areas. In the present context, it is noteworthy that, in the prefrontal cortex, the anterior cingulate, the prelimbic, and the infralimbic cortices have been associated with diverse emotional, cognitive, and mnemonic functions that underlie attentional and decision-making processes [39, 40]. In addition to the prefrontal cortex, the rlPAG also seems to be influenced by the septohippocampal system via a projection from the enkephalinergic region of the anterior hypothalamic nucleus [36]. Of particular relevance in the present account, the septohippocampal system has been shown to have a role in prioritizing the temporal order of motivated responses, which, in the present case, seems to utilize an enkephalinergic pathway. Here, it is noteworthy to recall that the opioidergic influence on the rlPAG has been shown to control the selection of adaptive behavioral responses, switching from maternal to hunting behavior [1].

As commented in the Introduction, the rlPAG upregulated Fos expression in animals performing insect hunting, and in the present study, we have shown that the rlPAG integrates inputs from a number of neural sites related to the circuitry underlying predatory hunting. Thus, we have presently found that the medial part of the central amygdalar nucleus provides a significant projection to the rlPAG. The medial part of the central amygdalar nucleus is part of a distinct amygdalar circuit comprising; the anterior part of the cortical nucleus, the anterior part of the basomedial nucleus and the posterior part of the basolateral nucleus, which has been shown to be mobilized during insect predation and seems to be particularly involved in processing visceral, gustatory and olfactory information [41–44]. The central nucleus—the main output way station of this amygdalar circuit—has been shown to be involved in controlling feeding behavior [45, 46]. In particular, the nucleus appears to integrate food hedonic values [47, 48] and to influence searching and consumption of palatable food through a pathway involving opioidergic neurotransmission [48, 49]. In the context of feeding behavior, it is also particularly relevant to point out that, according to the present findings, the rlPAG is also in a position to integrate visceral and gustatory information from the visceral and gustatory cortical areas, and to a lesser degree, from the parabrachial area [50].

The rlPAG also seems to be innervated by lateral hypothalamic regions activated during predatory hunting, namely, the parasubthalamic nucleus and the region containing cells expressing MCH and hypocretin/orexin [44]. The parasubthalamic nucleus is also targeted by the medial part of the central amygdalar nucleus, and provides inputs to hindbrain control regions involved in modulating digestive and metabolic responses occurring in both cephalic and consummatory phases of feeding behavior [29]. The lateral hypothalamic region containing cells expressing MCH and hypocretin/orexin appears to be involved in controlling arousal and exploratory activity related to feeding behavior [27, 51, 52]. In fact, this lateral hypothalamic region may also represent an important interface for the hypothalamic periventricular sites involved in the control of homeostatic feeding [53]. Moreover, according to the present findings, the rlPAG also receives direct inputs from the retrochiasmatic region, which has also been included in the hypothalamic circuit controlling feeding [54].

We have presently shown that the rlPAG is also targeted by the lateral part of the intermediate layer of the superior colliculus, which we have also previously shown to be mobilized during insect hunting [55]. In fact, cells in this collicular region seem to respond to moving objects in the temporal visual field, and we have found that animals bearing lesions in this collicular region, besides failing to orient themselves toward the moving prey, were also clearly less motivated to pursue the roaches [I. C. Furigo and S. R. Mota-Ortiz, personal observation] perhaps reflecting the functional role of the superior colliculus—rlPAG pathway.

The rlPAG is also significantly targeted by hypothalamic districts that do not seem to be directly involved in controlling feeding or hunting behavior, namely, the median preoptic nucleus, the retinoceptive region of the lateral hypothalamic, area and the dorsal premammillary nucleus. The median preoptic nucleus is classically known to respond

to plasma osmolarity and influence drinking behavior [23], and this pathway to the rlPAG may be thought of as having some role in the search of water supplies. Conversely, the projection from the retinoceptive region of the lateral hypothalamic area may bring the information regarding the environmental luminescence, which may be thought of as having a direct impact on foraging activity [N. S. Canteras, personal observation] . Finally, the dorsal part of the dorsal premammillary nucleus provides an important projection to the rlPAG. Unfortunately, to the best of our knowledge, the potential roles of this path remain yet to be determined.

Here, it is important to consider that functional studies using electrical or chemical stimulation have suggested a role for the lateral PAG in defensive responses. In the rat, chemical stimulation with kainic acid (KA) in the region of the rlPAG produced strong backward defense [56], a kind of response that would be expected when the animal is exposed to a predator. However, this finding is hard to conciliate with the fact that rlPAG does not seem to be activated in response to predator exposure [32], as would be expected. In reality, the effects of KA injection in this region should be taken very cautiously since the immediately adjacent dlPAG presents a much heavier binding to KA [57] when compared to the rlPAG. Actually, the dlPAG appears as the PAG site presenting the largest activation to predator exposure [32], and, as previously discussed, its hodological pattern is fully compatible with its role in antipredatory defense.

In addition, the concept of a distinct functional column for the entire lateral PAG should also be revised. In fact, the role here assigned to the rlPAG does not seem to apply to the caudal half of the lateral PAG, since lesions encroaching upon this latter PAG region do not seem to affect the motivational drive to hunt [2, M. H. Sukikara and S. R. Mota-Ortiz, personal observation]. Conversely, there is a prominent Fos upregulation in the caudal, but not in the rostral, lateral PAG in response to predator exposure [32, 33].

Overall, the present findings support the idea that the rlPAG has a central role in a complex network controlling the decision-making process between hunting, foraging, and other behavioral responses. On one hand, the rlPAG appears to be importantly driven by medial prefrontal cortical areas involved in controlling attentional and decision-making processes. On the other hand, the rlPAG receives a wealth of information from different neural sites related to feeding, drinking, or hunting behavioral responses. Therefore, this unique combination of afferent connections puts the rlPAG in a privileged position to influence the motivation drive to choose whether hunting and foraging are the most appropriate adaptive responses.

Acknowledgments

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