Advances in Pharmacological Sciences

$GABA_A$ Receptor Subtypes: Novel Targets for Novel Medicines

Guest Editors: Naheed R. Mirza, John Atack, and Keith Wafford

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2012, Article ID 529861, 2 pages
doi:10.1155/2012/529861

Editorial

$GABA_A$ Receptor Subtypes: Novel Targets for Novel Medicines

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Received 14 December 2011; Accepted 14 December 2011

$GABA_A$ receptors are ligand gated ion channels that are targeted by drugs with multiple therapeutic applications. Most notably, such drugs include the benzodiazepines, the hypnotic Z-drugs (zolpidem, zopiclone, and zaleplon), and the barbiturates. The benzodiazepines rapidly achieved preeminence as minor tranquilizers when they were introduced in the 1960s but thereafter lost favour, in a story that exemplifies how a "good drug can go bad" [1]. Nevertheless, benzodiazepines remain a mainstay of psychopharmacology but the decline in their public image is encapsulated by the colourful comment of Gorman [2] who stated that "Prescribing benzodiazepines is like watching pornography. If you ask a person at random if he watches pornography, he will vehemently deny it, but someone must be because it is a billion dollar a year business. Similarly, if you ask a physician if he prescribes Valium or Xanax or Ativan, he or she will say of course not.... Yet, like pornography, benzodiazepine prescriptions generate billions of dollars of revenue around the world, so somebody must be prescribing them". Recently, there has been a resurgence of interest in $GABA_A$ receptor pharmacology which has formed the basis for the development of novel modulators of discrete populations of this receptor family [3].

Over the last 25 years or so considerable advances in the molecular biology of $GABA_A$ receptors have identified multiple subtypes of $GABA_A$ receptor. Hence, $GABA_A$ receptors are heteropentameric assemblies of proteins derived from a family comprising 16 members ($a_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, θ, π). Despite the enormous number of theoretical pentameric combinations, thankfully only around 20 configurations have been described in native receptors [4]. A variety of approaches have been used to develop a greater understanding of the roles of these different subtypes in the physiology and pathophysiology of the CNS. These strategies include the generation of transgenic mouse models that have been used to delineate the functions of different subtypes, and the identification of subtype-selective pharmacological compounds that have formed the basis of hypotheses that subtype-selectivity can lead to therapeutic selectivity (e.g., anxioselective drugs without sedation). A combination of these strategies has resulted in emerging scientific evidence for novel therapeutic applications for subtype-selective drugs (e.g., in neuropathic pain, autism, schizophrenia, Alzheimer's disease, and stroke), and consistent with these various hypotheses clinical data on subtype-selective drugs in human trials indicate a novel pharmacology relative to known drugs targeting $GABA_A$ receptors. In this special issue, the articles focus on various aspects of the molecular genetics as well as preclinical and clinical pharmacology of $GABA_A$ receptors.

S. Nickoll's et al. in his paper focus on the novel therapeutic opportunity afforded by drugs that positively modulate $GABA_A$-α_2/α_3 receptors selectively as novel analgesics without sedation, cognition impairment, or abuse. Testing such compounds in animal models of inflammatory and neuropathic pain, complemented by in vivo electrophysiology, they highlight the need for sufficient efficacy at relevant subtypes of $GABA_A$ receptor in addition to selectivity. C. Vinkers and B. Olivier explore mechanisms underlying benzodiazepine tolerance and conclude that no unifying underlying mechanisms can be discerned but emphasize that since this is an important therapeutic issue

with respects to currently marketed GABAergic drugs, it is an important area to investigate with respects to emerging subtype selective compounds.

$GABA_A$-α_5 and $GABA_A$-δ receptors are located extrasynaptically, mediate tonic inhibitory neurotransmission in the CNS, and have different biophysical, physiological, and pharmacological properties relative to $GABA_A$-$\alpha_1/\alpha_2/\alpha_3$ receptors which are predominantly synaptically located. J. Braudeau et al. focus on selective negative modulators of $GABA_A$-α_5 receptors as a unique cognition enhancing strategy in Down's syndrome (DS), by demonstrating that the selective compound α5IA induces gene expression (c-fos and Arc) and rescues impaired gene expression in the Ts65Dn mouse model of DS. The compound α5IA has already shown evidence of cognition enhancement in man. Currently, Roche pharmaceuticals is assessing a compound (RG1662) in Down's syndrome, and by extension the relevance of this line of research in potentially reducing cognitive burden in Alzheimer's disease is clear. A. Clarkson reviews the complex role of $GABA_A$ receptors in cerebral ischaemia/stroke, suggesting that in an animal model of stroke, treatment with the α_5-selective negative allosteric modulator L-655708 can enhance functional recovery when given after a delay but not, interestingly, when given at the time of stroke. The assumption is that the attenuation of extrasynaptic GABAergic function can be beneficial by counteracting an increase in tonic inhibition that results from increased GABA concentrations in the peri-infarct region. Consequently, therapeutic strategies based upon a disinhibition of $GABA_A$-α_5 or $GABA_A$-δ receptors may aid functional recovery in vivo.

A. C. Errington et al. suggest negative modulators of $GABA_A$-δ receptors as a novel treatment option for absence epilepsy given that enhanced GABAergic function has been demonstrated in animal models of absence epilepsy, and that compounds enhancing $GABA_A$ function can engender absence seizures. Certainly this suggestion is contrary to the classical approach of treating seizures by increasing inhibitory neurotransmission, but the authors elegantly demonstrate the relevance of key thalamocortical circuits in generating EEG signatures common to animals and man and the high expression and relevance of a $\alpha_4\beta_2\delta$ receptor population in this circuit. M. W. Hulin et al. focus on literature implicating $GABA_A$ receptors and neurosteroids in mediating actions of alcohol. They describe the neurosteroid dehydroepiandrosterone (DHEA) which decreases alcohol intake in rats and broadly behaves as a negative modulator of $GABA_A$ receptors, although genomic effects of this molecule complicate matters. Nonetheless, the authors emphasize the potential role of $GABA_A$-δ receptors in mediating the effects of DHEA in reducing alcohol intake; albeit this is a controversial area.

X. Chen et al. use a comparative pharmacology approach in human volunteers to demonstrate that subtype-selective $GABA_A$ drugs engender pharmacodynamic effects that only partly overlap with those of benzodiazepines. For example, the $GABA_A$-α_2/α_3 selective drug TPA023 induces impairment of saccadic peak velocity but does not induce body sway or impair attention in volunteers. The novel pharmacological profile of subtype selective compounds in man mirrors preclinical data using transgenic mouse models and subtype-selective tools, indicating that molecular neuroscience can be translated to the clinic and guide drug development.

The paper by B. H. Bentzen and M. Grunnet gives some insight into the drug development of new chemical entities, specifically focusing on cardiovascular (CV) safety. Clearly many benzodiazepines were registered for clinical use in a regulatory environment considerably different from today's. Therefore, in developing novel subtype-selective modulators, assumptions cannot be made in terms of safety and toxicology. These authors use anaesthetized animals and isolated hearts to emphasize that the CV effects of benzodiazepines are highly dependent on the conscious state of the animal, with implications for CV assessment of future novel GABAergic molecules.

Given the wide-spread therapeutic success of GABAergic drugs across a variety of indications and an emerging understanding of the pharmacology of subtype-selective drugs preclinically and clinically, $GABA_A$ receptors are clearly a highly druggable target class for which preclinical to clinical transition/translation strategies exist—desirable qualities in today's highly complex drug development and regulatory environment. The articles in this special issue give a snapshot of the diverse range of topics being explored regarding the therapeutic utility of selectively targeting $GABA_A$ receptors, providing a ground base of understanding for potentially important drugs of the future.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2012, Article ID 416864, 19 pages
doi:10.1155/2012/416864

Review Article

Mechanisms Underlying Tolerance after Long-Term Benzodiazepine Use: A Future for Subtype-Selective $GABA_A$ Receptor Modulators?

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Received 7 July 2011; Revised 10 October 2011; Accepted 2 November 2011

Academic Editor: John Atack

Despite decades of basic and clinical research, our understanding of how benzodiazepines tend to lose their efficacy over time (tolerance) is at least incomplete. In appears that tolerance develops relatively quickly for the sedative and anticonvulsant actions of benzodiazepines, whereas tolerance to anxiolytic and amnesic effects probably does not develop at all. In light of this evidence, we review the current evidence for the neuroadaptive mechanisms underlying benzodiazepine tolerance, including changes of (i) the $GABA_A$ receptor (subunit expression and receptor coupling), (ii) intracellular changes stemming from transcriptional and neurotrophic factors, (iii) ionotropic glutamate receptors, (iv) other neurotransmitters (serotonin, dopamine, and acetylcholine systems), and (v) the neurosteroid system. From the large variance in the studies, it appears that either different (simultaneous) tolerance mechanisms occur depending on the benzodiazepine effect, or that the tolerance-inducing mechanism depends on the activated $GABA_A$ receptor subtypes. Importantly, there is no convincing evidence that tolerance occurs with α subunit subtype-selective compounds acting at the benzodiazepine site.

1. Introduction

Shortly after their development in the 1960s, benzodiazepines became very popular as they exerted many desirable effects such as reduction of anxiety, anticonvulsant properties, and myorelaxation combined with a rather low toxicity [1]. However, their use is associated with many side effects precluding their long-term use, including sedation, amnesia, cognitive impairment, and ataxia. Even though guidelines generally recommend limiting benzodiazepines to short-term use, long-term use still often occurs. Chronic benzodiazepine treatment can result in the development of benzodiazepine dependence [2]. DSM-IV criteria for benzodiazepine dependence consist of various psychological (behavioral) and physical symptoms, including tolerance, withdrawal symptoms when drug intake is stopped and dose escalation [3]. Indeed, chronically treated patients become less sensitive to some effects of benzodiazepines (tolerance) which may include anticonvulsant, sedative, hypnotic, and myorelaxant effects of benzodiazepines. Also, benzodiazepine discontinuation may result in the appearance of a characteristic withdrawal syndrome with heightened anxiety, insomnia, and sensory disturbances [4]. In fact, tolerance and withdrawal could be two manifestations of the same compensatory mechanism, with withdrawal occurring when the counterbalancing benzodiazepine effect is absent [5]. This is supported by the fact that acutely induced benzodiazepine effects are opposite to the withdrawal symptoms, and that changes in glucose use in the Papez circuit (including the cingulate cortex and mammillary body) were also observed on withdrawal, implying a common circuitry in the withdrawal process [6]. However, physical dependence

(usually defined by withdrawal symptoms) does not require the presence of tolerance, and tolerance may develop without any signs of physical dependence [7].

Presently, despite decades of basic and clinical research, our understanding how benzodiazepines tend to lose their efficacy over time (i.e., tolerance) is at least incomplete. Here we review the current knowledge on the neuroadaptive mechanisms underlying benzodiazepine tolerance. This paper does not specifically address the addictive properties of benzodiazepines and their effects on the dopamine system or their abuse liability potential (including their nonmedical use in popular culture), which are described in detail elsewhere [8–10].

Benzodiazepine tolerance is considered to constitute an adaptive mechanism following chronic treatment, and it may thus be regarded as an example of neuronal plasticity. Efforts have been made to explain tolerance at the molecular or functional level of the $GABA_A$ receptor because classical (nonselective) benzodiazepines modulate inhibitory $GABA_A$ receptors possessing α_1, α_2, α_3, or α_5 subunits. On the other hand, the excitatory glutamate system has also been implicated to play a role in the development of benzodiazepine tolerance [5]. Enhanced understanding of the dynamic process leading to reduced benzodiazepine efficacy following chronic treatment could accelerate the development of compounds that would maintain efficacy during chronic treatment [11]. Indeed, increasing knowledge on the specific functions of different $GABA_A$ receptor subunits has led to a breakthrough of novel and more selective drugs acting at the benzodiazepine site of the $GABA_A$ receptor. It is interesting but beyond the scope of the review to draw a comparison between benzodiazepine tolerance and alcohol tolerance as alcohol (albeit with low potency) acts at the $GABA_A$ receptor [12].

Firstly, we will discuss the molecular basis of the $GABA_A$ receptor system before taking a closer look at the clinical aspects of the development of benzodiazepine tolerance. Then, the putative molecular mechanisms underlying benzodiazepine tolerance will be extensively discussed, followed by a section specifically addressing the issue of tolerance development with novel and more selective benzodiazepines in the light of the putative tolerance mechanisms associated with classical benzodiazepines. From a clinical perspective, the understanding of tolerance is important because long-term benzodiazepine treatment with continuing efficacy—using either existing or novel and more selective drugs—could offer potential benefits to several groups of patients.

2. Benzodiazepines and the $GABA_A$ System

2.1. $GABA_A$ Receptors. $GABA_A$ receptors constitute the major fast inhibitory neurotransmitter system in the brain. They are composed of five transmembrane-spanning subunits that assemble to form a ligand-gated chloride channel with various possible subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, and π) resulting in $GABA_A$ receptor heterogeneity [13]. Binding of GABA to the $GABA_A$ receptor increases the influx of negatively charged chloride ions, resulting in an inhibitory postsynaptic signal (IPSP). Although in theory a vast number of subunits combinations could be expected,

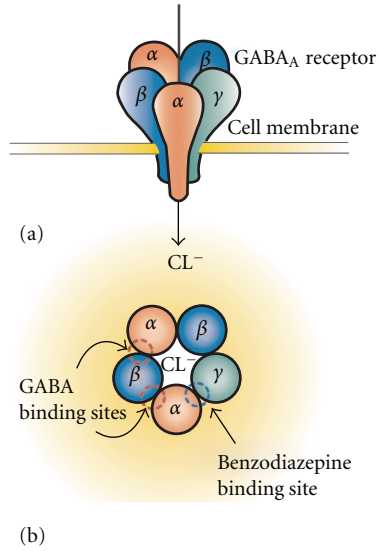

Figure 1: Representation of the $GABA_A$ receptor structure. The inhibitory $GABA_A$ receptor consists of five subunits that together form a ligand-gated chloride (Cl) channel (a). When GABA binds (between the α and the β subunit of the $GABA_A$ receptor), chloride ions flow into the neuron, resulting in a hyperpolarization of the cell membrane (a). Classical nonselective benzodiazepines allosterically enhance the inhibitory actions of GABA by binding between the α_1, α_2, α_3, or α_5 subunit and the γ subunit (b). Although the $GABA_A$ receptor displays a large molecular heterogeneity depending on the subunit composition, the most common subtype is a pentamer with 2α, 2β, and 1γ subunit.

$GABA_A$ receptors are found in typical subunit compositions with the most common receptor subtype being composed of two α, two β, and one γ subunit [14] (Figure 1). In situ hybridization and immunohistochemical studies have shown that $GABA_A$ receptor subunits display a distinct CNS distribution with a differential cellular localization pattern, suggesting that $GABA_A$ receptor subunits have a specialized function (Table 1) [14]. Overall, a high expression of GABAergic subunits is present in the cortex, hippocampus, and basal ganglia [15]. Of the GABAergic subunits, α_1, β_1, β_2, β_3, and γ_2 subunits are found throughout the brain. In contrast, the α_2, α_3, α_4, α_5, α_6, γ_1, and δ subunits have a specific regional expression pattern. The α_1 subunit is highly coassembled with β_2 and γ_2 subunits and is synaptically located on neuronal cell bodies. $GABA_A$ receptors that contain an α_2 or α_3 subunit are less abundant and are codistributed with the β_3 and γ_2 subunits. The α_2 subunit is present in the cortex, hippocampus, amygdale, and hypothalamus, and often its expression is negatively correlated with the expression of α_1 subunits. The expression of the α_3 subunit is highest in the cortex, hippocampus,

Table 1: Localization of common $GABA_A$ receptor subtypes in the brain (adapted from [19]).

Subtype	Frequency	Localization
$\alpha_1\beta_2\gamma_2$	Major (60%) synaptic	Cerebral cortex (layer I–VI), hippocampus, striatum, cerebellum, amygdala, brainstem.
$\alpha_2\beta_n\gamma_2$	Minor (15–20%) synaptic	Cerebral cortex (layers I–IV), hippocampus, striatum, hypothalamus, amygdala.
$\alpha_3\beta_n\gamma_2$	Minor (10–15%) synaptic	Cerebral cortex (layers V-VI), hippocampus, cerebellum, amygdala, brainstem (including raphe nuclei and locus coeruleus), spinal cord.
$\alpha_4\beta_n\delta/\gamma$	Minor (<10%) extrasynaptic	Hippocampus (dentate gyrus), thalamus, cortex.
$\alpha_5\beta_n\gamma_2$	Minor (<5%) extrasynaptic	Cerebral cortex, hippocampus, amygdala, hypothalamus, spinal cord.
$\alpha_6\beta_n\gamma 2/\delta$	Minor (<5%)(extra) synaptic	Cerebellum.

amygdala, thalamus, and brainstem, although it is also expressed in monoaminergic neurons (e.g., the raphe nuclei and the locus coeruleus in the brainstem) and cholinergic neurons in the forebrain. α_5 subunits are predominantly expressed in the hippocampus where they comprise 15–20% of the diazepam-sensitive $GABA_A$ receptors [16]. Regarding cellular localization, cortical and hippocampal pyramidal cells receive input from morphologically distinct GABAergic interneurons that innervate different pyramidal cell parts depending on the type of interneuron (e.g., chandelier and basket cells) with a specialized postsynaptic expression of α subunits [17, 18].

Thus, $GABA_A$ receptor subtypes probably possess diverging functional properties dependent on the subunit composition, contributing to the GABA signaling complexity [13]. Additionally, $GABA_A$ receptors are found synaptically as well as extrasynaptically. Synaptic receptors usually contain γ subunits and mediate fast phasic inhibition accompanied by transient high GABA concentrations [16]. By contrast, GABA has higher potency (at μM concentrations) at extrasynaptic $GABA_A$ receptors that usually contain a δ subunit, preferentially assemble with α_6 or $\alpha_4$subunits and have slow desensitization kinetics [20]. Also, α_5 subunits may be localized extrasynaptically [21]. Extrasynaptic tonic inhibition—which is not modified by benzodiazepines—is suggested to modulate excitability of neuronal networks throughout the brain.

2.2. Benzodiazepines from a Nonspecific towards a Subunit-Specific Pharmacology. Classical benzodiazepines allosterically modulate GABA-induced IPSPs by binding to the benzodiazepine site of $GABA_A$ receptors that contain an α_1, α_2, α_3, or α_5 subunit in combination with a β and a γ_2 subunit (Figure 1). The exact binding site of benzodiazepines at the $GABA_A$ receptor is located between the α and γ subunit. In contrast, benzodiazepines do not interact with $GABA_A$ receptors that contain an α_4- or α_6-subunit. In addition to benzodiazepines, other drug classes can bind to the $GABA_A$ receptor complex, including several anticonvulsants, ethanol, barbiturates, neurosteroids, and some anesthetics [15]. The fact that classical benzodiazepines nonselectively bind to different α subunits led to the hypothesis that the pharmacological profile with anxiolytic, sedative, anticonvulsive and myorelaxant properties may be further dissected. Both genetic and pharmacological approaches explored the hypothesis that α subunits differentially contribute to the different effects of classical benzodiazepines The genetic approach consisted of point mutations into specific α subunits (α_1(H101R), α_2(H101R), α_3(H126R), and α_5(H105R)), turning them functionally insensitive to benzodiazepines without altering their GABA sensitivity [22]. Pharmacological research on the $GABA_A$ receptor has focused on the development of compounds that show differential efficacy across the various α subunits [13]. Such drugs generally bind with equal affinity to all α subunits (i.e., α_1, α_2, α_3, and α_5 subunits), but selectively alter the capacity to increase GABA binding to one or more of them. Using this strategy, various efficacy-selective (and some affinity-selective) compounds have been developed with preferential agonistic activity at the α_1 (zolpidem and zaleplon), $\alpha_{2/3}$ (TPA023, L838, 417, and SL651498), or inverse agonistic activity the α_5 subunit (α5IA, L-655,708, and MRK-016) (see also Table 2).

In line with a specific central localization and distribution of GABAergic subunits, these genetic and pharmacological approaches have demonstrated that different α subunits of the $GABA_A$ receptor mediate the distinct effects of benzodiazepines. Specifically, α_1-containing $GABA_A$ receptors probably mediate the sedative, amnesic, and anticonvulsant actions of classical benzodiazepines [13, 23]. In contrast, muscle relaxation and anxiety reduction after benzodiazepine administration was primarily ascribed to α_2 (and possibly α_3) subunit activation [24], whereas α_5 subunit-containing $GABA_A$ receptors appear to be involved in learning and memory [25, 26].

In light of the topic of this review, studies investigating the contribution of GABAergic subunits in benzodiazepine abuse liability, drug reinforcement, and tolerance development are of particular interest. Unfortunately, studies applying genetic and subtype-selective methodologies to examine the development of tolerance are scarce. One study using α subunit point mutation mice implicated a critical role for α_5 subunits together with α_1 subunits in the decreasing sedative efficacy of the classical benzodiazepine diazepam after chronic treatment [27]. We will discuss this finding in detail later in this paper. Studies on the background of physical dependence and abuse liability using subtype-selective $GABA_AR$ modulators are more abundant. Using self-administration studies, it was shown that efficacy at α_1-containing $GABA_A$ subtypes significantly contributed to the reinforcing effects and withdrawal symptoms of benzodiazepines [8, 28, 29]. Specifically, TPA123, which still possesses 23% intrinsic activity at α_1 subunits still led

Table 2: Summary of novel $GABA_A$ receptor subtype selective compounds.

Target	Name	Efficacy (compared to a classical benzodiazepine)	Affinity/Remarks	Ref
α_1	Zolpidem	Comparable at $\alpha_1/\alpha_2/\alpha_3/\alpha_5$	5-10-fold higher affinity for α_1 versus $\alpha_{2/3}$ > 1000 fold higher affinity for α_1 versus α_5	[15]
$\alpha_{2/3}$	TPA023	α_1 (0%), α_2 (11%), α_3 (21%), α_5 (5%)	Equivalent affinity	[8]
$\alpha_{2/3}$	TPA123	α_1 (23%), α_2 (35%), α_3 (43%), α_5 (19%)	Equivalent affinity. Reinforcing efficacy and physiological dependence remained present	[8]
$\alpha_{2/3}$	L838,417	α_1 (1.5%), α_2 (43%), α_3 (43%), α_5 (39%)	Equivalent affinity	[23]
$\alpha_{2/3}$	SL651498	α_1 (45%), α_2 (115%), α_3 (83%), α_5 (50%) Compared to zolpidem for α_1 efficacy	5–10-fold increased affinity for $\alpha_{2/3}$, 10–20 fold lower affinity for α_5	[31]

to benzodiazepine-like drug reinforcement and withdrawal symptoms, whereas TPA023 with 0% α_1 intrinsic activity did not, even at full $GABA_A$ receptor-binding capacity [8]. However, there is still the possibility that the lower α_2 and α_3 efficacy of TPA023 may have contributed to the absence of drug reinforcement and withdrawal. In support, L-838,417 also led to continued self-administration, even though it lacks efficacy for the α_1 subtype [30]. In any case, the α_5 subunit may not be directly involved in the abuse potential of classical benzodiazepines as the α_1-preferring hypnotic zolpidem with no affinity for the α_5 subunit still led to self-administration in primates [30]. This finding is surprising as it suggests that the α_5 subunit may be involved in tolerance development but not in drug reinforcement. Consequently, these processes could be independently mediated, even though they are both incorporated in the definition of benzodiazepine dependence.

2.3. GABA Metabolism. As benzodiazepines enhance the inhibitory effects of GABA and shift the GABA concentration-response curve to the left, the synaptic GABA concentration affects benzodiazepine efficacy. GABA is converted from glutamate by the enzyme glutamic acid decarboxylase (GAD) that maintains intracellular levels of GABA and exists in two independent isoforms (GAD_{65} and GAD_{67}). In contrast to the localization of GAD_{67} in the neuronal body, GAD_{65} is primarily expressed in axon terminals, suggesting a role for GAD_{65} in synaptic neurotransmission and a more general role for GAD_{67} in regulating GABA synthesis [32]. Synaptic GABA is removed from the cleft into the presynaptic axon terminals by GABA transporters (GATs). So far, four GAT subtypes have been identified, with the highly expressed GAT_1 and GAT_4 being the most widely distributed [33].

3. The Development of Benzodiazepine Tolerance

Before examining the possible mechanisms underlying the development of benzodiazepine tolerance after long-term exposure, it is important to review its evidence and determine whether it is clinically relevant. Overall, there is little doubt that benzodiazepines are acutely effective in reducing anxiety, sleep latency and preventing convulsions. The tolerance that is eventually thought to develop appears to occur at different rates and to a different degree for each of the benzodiazepine effects [34]. Preclinical studies have shown that tolerance to the sedative and hypnotic effects occurs rather rapidly, followed by tolerance to the anticonvulsant effects, whereas tolerance to the anxiolytic effects of benzodiazepines are absent or partially develop after long-term treatment (for reviews, see [34–36]). As these preclinical studies have already been extensively reviewed, and novel preclinical studies on benzodiazepine tolerance have been limited in the last years to our knowledge, it is beyond this paper to reproduce all preclinical data on tolerance development. In general, preclinical studies are in agreement with the clinical divergent picture, even though in most preclinical studies, tolerance is not directly related to the applied dose, dosing interval, or the drug's plasma levels or half-life. Here, we will focus on the clinical evidence for (the rate of) tolerance development for each benzodiazepine action, even though we will also include preclinical studies when clinical studies are lacking or inconclusive.

3.1. Clinical Studies on Sedative and Hypnotic Tolerance. A study in low-dose benzodiazepine-dependent subjects showed a complete loss of hypnotic activity independent of the half-life of the prescribed benzodiazepine, even though a substantial suppression of REM sleep still occurred [37]. Also, other studies have shown that chronic users displayed no increase in sedation or motor impairment after the acute application of a benzodiazepine [38–40]. Moreover, tolerance to benzodiazepine-induced decreased reaction speed was shown after 10 days of alprazolam treatment [41]. Oral administration of triazolam, a short acting benzodiazepine, initially improved both sleep induction and maintenance, but latency to sleep and the number of awakenings were back to baseline values after two weeks of triazolam use [42]. Importantly, early-morning insomnia associated with short-acting benzodiazepines triazolam and midazolam markedly worsened after 7 days of treatment [43]. However, conflicting studies with triazolam exist that did not show any tolerance development [44, 45]. Another study applying the longer-acting benzodiazepine temazepam (15 or 30 mg) for either 26

or 54 nights in 7-8 subjects with chronic insomnia found no development of drug tolerance due to long-term temazepam administration [46]. Flurazepam, which has a relatively long elimination half-life, was shown to be effective for initiating and maintaining sleep with intermediate and long-term use (over 4 weeks), even though daytime sedation diminished during prolonged use [47]. Thus, even though tolerance to the sedative effects quickly emerges in most studies, these effects seem to be most prominent with benzodiazepines with a short half-life. Tolerance could thus depend on the half life of the applied benzodiazepine. However, this may be an overgeneralization, as a review showed that tolerance in human subjects only marginally emerged after chronic treatment with the short-acting drugs midazolam and zolpidem, even though the short-acting drug triazolam was associated with tolerance [48]. A limitation of most studies is their relatively short duration of exposure. Another issue is that convincing evidence for improved sleep after long-term use is lacking [49], yet this may not be the sole result of tolerance but could also be attributable to a generalized lack of efficacy. In support, in human subjects, discontinuation of benzodiazepines did not decrease sleep quality compared to a group that stayed on benzodiazepines up to 52 weeks after cessation [50], or even increased sleep quality and slow wave sleep after discontinuation in insomnia patients [51].

3.2. Clinical Studies on Anticonvulsant Tolerance. The use of benzodiazepines over a longer period of time in epilepsy is limited due to the development of tolerance [52]. In line with preclinical studies [53–55], tolerance develops during the first several months in 30–50% of epilepsy patients treated with either clobazam or clonazepam [56]. Thus, benzodiazepines are only prescribed in acute epileptic seizures or in a status epilepticus. However, in certain cases, intermittent use may be indicated, which may reduce the likelihood of tolerance [57]. Chronic treatment in rodents with the α_1-preferential compound CL218, 572 resulted in loss of picrotoxin-induced seizures [58]. In contrast to classical benzodiazepines, partial $GABA_A$ receptor PAMs including bretazenil did not result in anticonvulsant tolerance in several preclinical studies [54, 59, 60]. However, to our knowledge, these drugs have not been tested for (continuing) anticonvulsant activity in humans, precluding firm conclusions on their tolerance-inducing effects in epilepsy patients.

3.3. Clinical Studies on Amnesic Tolerance. Most studies have found continued short-term memory impairment after acute administration of benzodiazepines in chronically treated subjects [38, 39, 61]. Also, no tolerance for memory-impairing effects of alprazolam was found during a 10-day acute treatment [41]. However, another study reported tolerance to the acute amnesic effects of alprazolam after chronic use [40]. A major concern is that loss of memory associated with benzodiazepine use may be lasting, even after treatment discontinuation [62, 63], although other studies reported improved cognitive functioning after discontinuation with increased speed and accuracy of information processing, improved reaction time and working memory [50, 64–66]. Collectively, clinical data do not support the existence of tolerance to benzodiazepine-induced cognitive impairments.

3.4. Clinical Studies on Anxiolytic Tolerance. If developing al all, tolerance to the anxiolytic effects seems to develop more slowly compared to tolerance to the hypnotic effects. In patients with panic disorder, neither anxiolytic tolerance nor daily dose increase was observed after 8 weeks of alprazolam treatment with continued efficacy [67]. This was confirmed by another study in panic disorder patients who already chronically took alprazolam. Here, no differences were found in cortisol responsivity or anxiolytic efficacy compared to alprazolam-naïve patients, independent of disease severity [40]. Another double-blind study allocated 180 chronically anxious outpatients to diazepam (15 to 40 mg/day) and found that prolonged diazepam treatment (6–22 weeks) did not result in tolerance to the anxiolytic effects of diazepam [68]. Furthermore, additional studies all show a continuing anxiolytic effect, at least for panic disorder [69–72], generalized anxiety disorder [73], and social phobia [74–76]. Although a declining anxiolytic efficacy after long-term use of benzodiazepines cannot be clearly established, it is important to remember that other disadvantages prevent benzodiazepines to chronically treat anxiety symptoms, such as continued memory impairment, accident risk, hip fractures, and withdrawal symptoms [7, 77]. In conclusion, there is no solid evidence from the existing literature that anxiolytic efficacy declines following chronic benzodiazepine use in humans.

3.5. Clinical Studies on Drug Reinforcement Tolerance. The relevant topic of benzodiazepine tolerance to the reinforcing effects of benzodiazepines was already discussed by Licata and Rowlett [9]. They concluded that tolerance to reinforcing effects of benzodiazepines appears unlikely, supported by studies in nonhuman primates in which midazolam and zolpidem maintained stable self-injection and physical dependence under conditions of chronic continuous availability [78, 79]. Also, in humans, tolerance to drug reinforcement could lead to dose escalation that would maintain the vicious cycle of tolerance and dependence. In clinical practice, the majority of patients do not escalate their dose, suggesting that drug reinforcement tolerance may not emerge [80].

3.6. Conclusion. In conclusion, tolerance develops relatively quickly for the sedative, hypnotic, and anticonvulsant actions of benzodiazepines. Tolerance to anxiolytic and amnesic effects most probably does not appear at all. The fact that benzodiazepine dosage may be hard to reduce after chronic use can be ascribed to physical dependence to avoid withdrawal symptoms rather than the development of tolerance.

With diverging rates and varying completeness of tolerance development, it may be speculated that either (i) different tolerance mechanisms exist depending on the benzodiazepine effect, or that (ii) a uniform mechanism accounts for tolerance but revolves around the subunit composition of the targeted $GABA_A$ receptor subtype and

the brain region involved. However, from the presented evidence it is difficult to conclude that benzodiazepines indeed produce a robust and reproducible tolerance for all (side) effects. It is clear however, that benzodiazepine tolerance is not a uniform process for all clinical effects and does not apply to all available benzodiazepines. However, it is not known which factors predict whether a certain benzodiazepine possesses the potential to produce tolerance. Unfortunately, many studies address the physical dependence of benzodiazepines and their abuse potential, but do not specifically investigate tolerance.

4. Mechanisms Underlying Tolerance

4.1. General. Decades of research into the molecular effects of long-term benzodiazepine treatment have already importantly advanced our understanding of tolerance and several excellent reviews on this topic have already been published [5, 11, 34, 77]. The general assumption is that chronic benzodiazepine use leads to compensating changes in the central nervous system. This way, the $GABA_A$ receptor may become less responsive to the continuing acute effects of benzodiazepines, either as a result of adaptations in the $GABA_A$ receptor itself, intracellular mechanisms, or changes in other neurotransmitter systems, such as the glutamatergic system. Although adaptive processes probably play an important role, it is important to realize that the development of tolerance is not uniform for all its actions, and differences between preclinical and clinical tolerance development may exist. Therefore, the possibility that not one but multiple adaptive mechanisms simultaneously coexist complicates research into benzodiazepine tolerance. Moreover, these adaptive changes could be limited to one or more specific brain areas. This makes it very challenging to single out one a priori unifying mechanism underlying tolerance. In support, a study in rats using 2-deoxyglucose quantitative autoradiography showed that during chronic diazepam treatment, heterogeneous tolerance to the diazepam-induced reduction of glucose utilization occurred in the brain, depending on treatment duration and brain region [6]. Whereas acute diazepam administration resulted in reductions in glucose utilization throughout the brain, 3 days of diazepam treatment led to tolerance in brain structures associated with sensory processing (parietal cortex, auditory cortex, cochlear nucleus) which was interpreted to correlate with reduced sedation. After 28-day diazepam treatment, tolerance to the depressant effect of diazepam on cerebral glucose occurred in the mamillary body, subiculum, and caudate nucleus, whereas changes in the frontal cortex approached significance. Of particular interest is the finding that none of the amygdaloid nuclei showed any blunting over time, in line with persistent anxiolytic effects of benzodiazepines.

Before taking a closer look at specific mechanisms that have been proposed to underlie benzodiazepine tolerance, it is important to note that pharmacokinetic factors probably do not play a major role in the development of tolerance [81]. In support, plasma levels after acute diazepam administration did not differ between chronically alprazolam-treated and untreated panic disorder patients, even though sedative and amnesic tolerance was observed [40]. The most obvious candidate to mediate the adaptive changes in cellular and synaptic function after chronic benzodiazepine treatment is the $GABA_A$ receptor. Therefore, we will first discuss the evidence supporting changes in the GABA system (including $GABA_A$ receptor coupling and GABA receptor expression) after chronic benzodiazepine exposure.

4.2. $GABA_A$ System Hypotheses

4.2.1. Mechanism 1: $GABA_A$ Receptor Uncoupling. One explanation for a loss of benzodiazepine function is a loss in $GABA_A$ receptor allosteric coupling. The $GABA_A$ receptor contains two GABA-binding sites and one benzodiazepine-binding site that are allosterically coupled, that is, binding to the benzodiazepine-binding site potentiates binding of GABA to the GABA-binding site (Figure 1). Benzodiazepines are generally referred to as positive allosteric modulators (PAMs) because their binding alters the $GABA_A$ receptor conformation with an increased capacity to bind GABA, leading to increased channel opening frequency, increased chloride influx, and, consequently, to hyperpolarization. $GABA_A$ receptor uncoupling is defined as a decreased ability of benzodiazepines to enhance GABA-induced IPSPs at the $GABA_A$ receptor. In terms of tolerance development, it has been hypothesized that chronic treatment affects the benzodiazepines' capacity to pharmacologically enhance the GABA response (i.e., tolerance leads to uncoupling). A decreased coupling may develop as a result of changed $GABA_A$ receptor subunit composition, alterations to the $GABA_A$ receptor itself (including phosphorylation) or its second messenger ligands, or any process affecting the conformational state of the $GABA_A$ receptor. The receptor uncoupling hypothesis is attractive as it does not assume any changes in subunit expression and ligand binding yet uses the knowledge on the specialized functions of the $GABA_A$ receptor and the different subunits. However, the uncoupling process is an aspecific process as it can be induced by exposure to different classes of $GABA_A$ receptor modulators acting at different modulatory sites, such as neurosteroids and barbiturates [82].

Already in 1984, an electrophysiological study indicated that allosteric coupling may play a role by showing a 50% decrease in the GABA enhancement of benzodiazepine-binding without significant changes in benzodiazepine-binding site density or affinity [83]. Also, more recent indications for reduced allosteric coupling were found after chronic treatment using transfected cell lines that express $GABA_A$ receptors or in neurons [84–94]. The mechanisms underlying possible differences in coupling remain poorly understood. If the $GABA_A$ receptor assembly process is modified, GABA receptor composition can be modified due to subunit replacements or altered expression in the receptor. This way, $GABA_A$ receptors with a different functionality could potentially possess reduced benzodiazepine sensitivity due to reduced $GABA_A$ receptor coupling. To our knowledge, no studies exist which have directly investigated $GABA_A$ receptor subunit composition after chronic

exposure. Another mechanism to affect receptor coupling is $GABA_A$ receptor phosphorylation. $GABA_A$ receptors are phosphorylated by various protein kinases and dephosphorylated by phosphatases [95]. Dynamic functional alterations in $GABA_A$ receptor phosphorylation status may directly affect the inhibitory synaptic strength, with changes in channel openings (or indirectly influence receptor trafficking). However, the precise effects of phosphorylation on neuronal $GABA_A$ receptor function are complex, even though key residues within the intracellular loop of the $GABA_A$ receptor seem of particular importance. Using whole-cell patch-clamp recordings of $GABA_A$ receptor IPSCs in hippocampal neurons, brain-region-dependent effects of activation of cAMP-dependent protein kinase A (PKA) or Ca^{2+}/phospholipid-dependent protein kinase C (PKC) were shown [96]. Also, PKA activity was found to be directly involved in changed $GABA_A$ receptor functioning in hippocampal pyramidal cells following chronic flurazepam treatment [97]. Probably, phosphorylation patterns rather than individual sites are of importance, supported by the finding that mutation to one PKA phosphorylation site is not involved in tolerance [90]. Using a point mutation genetic approach, transcriptional reduction was found in calcium-/calmodulin-dependent kinase IIα and MAP kinase phosphatase-1in control mice but not in α1(H101R) after acute administration of diazepam [98]. Unfortunately, no chronic treatment was included in these studies.

It remains to be seen whether changes in allosteric coupling are relevant to the development of tolerance *in vivo*. Because benzodiazepine tolerance gradually develops over days to weeks, this would suggest that structural changes take place, whereas posttranslational compensation would be expected to be directly manifest. In support, uncoupling seems to develop rapidly, with the classical benzodiazepine chlordiazepoxide (applied together with GABA) stimulating the rate and extent of desensitization produced in a single neuron within several seconds [99]. Also, the observed uncoupling after chronic benzodiazepine treatment is rapidly reversed by a brief exposure *in vivo* to the benzodiazepine antagonist flumazenil [83, 86].

4.2.2. Mechanism 2: Alterations in $GABA_A$ Receptor Subunit Expression. The most straightforward hypothesis to explain impaired sensitivity after chronic benzodiazepine exposure would be a general downregulation of $GABA_A$ receptors throughout the brain. Indeed, the process of tolerance requires $GABA_A$ receptors at least to some extent, as cell lines expressing one specific type of the $GABA_AR$ are susceptible to tolerance [86, 87, 90]. Because classical (nonselective) benzodiazepines bind to $GABA_A$ receptors that contain an α_1, α_2, α_3, or α_5 subunit, it could be expected that expression of receptors containing these α subunits (plus a γ_2 subunit) is changed. Of course, this would depend on the cellular and anatomical distribution of $GABA_A$ receptors. Already earlier in Section 2.1, the differentiated and unique distribution of GABAergic subunits in the CNS was discussed. With regard to the benzodiazepine-sensitive α subunits, the α_1 subunit is ubiquitously expressed in the entire brain, whereas the other α subunits (α_2, α_3 and α_5) display a more restricted pattern of expression (see Table 1). If receptor internalization simply downregulates $GABA_A$ receptor density, then a priori regional differentiation would be expected based on receptor distribution.

The processes that control the assembly, membrane trafficking, and synaptic accumulation of $GABA_A$ receptors are complex (for review, see [100]). In short, $GABA_A$ receptors are assembled from individual subunits out of the endoplasmic reticulum within minutes after their translation, with amino acid sequences in the N-terminus influencing the $GABA_A$ receptor subtype (Figure 2). Then, receptor trafficking to the plasma membrane takes place, facilitated by diverse helper $GABA_A$ receptor-associated proteins (among that GABARAP, BIG2, PRIP, gephyrin, and radixin). Ultimately, (clathrin-dependent) endocytosis occurs after receptor dephosphorylation, after which degradation or recycling may ensue (Figure 2). If prolonged activation of the GABA system leads to receptor downregulation, then this could be established by interfering at multiple steps of the dynamic $GABA_A$ receptor life cycle. These include decreased subunit mRNA transcription, subunit degradation in the endoplasmic reticulum (e.g., by ubiquitylation), decreased expression of $GABA_A$ receptor-associated helper proteins, and alterations in the endocytosis of specific $GABA_A$ receptor subtypes. The finding that the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D blocked the effects of chronic diazepam exposure in recombinant cells expressing $GABA_A$ receptors indicates that $GABA_A$ receptor synthesis is of at least some importance [87].

Up to now, a plethora of studies have tried to address whether chronic benzodiazepine treatment indeed affects $GABA_A$ receptor expression (and thus benzodiazepine binding sites) using compounds with different subtype selectivity profiles at different doses and varying treatment duration. A recent excellent review summarized all data on the regulation of $GABA_A$ receptor subunit after chronic benzodiazepine treatment that was mostly studied in rats [102]. It is beyond the scope of this review to repeat the meticulous work laid down in this paper. Of all subunits, α, β, and γ subunits have been mostly examined. This paper confirms that both for mRNA and protein subunit levels, the available evidence leads to a divergent and sometimes conflicting picture, although the majority of the studies essentially do not show any significant difference in subunit expression [102]. Furthermore, a lack of consistency appears for subunit changes in different specific brain areas. Moreover, the length and method of chronic treatment seem relevant since differences in $GABA_A$ receptor subunit mRNA levels after chronic diazepam treatment in rats can depend on whether diazepam is administered as daily systemic injections or via osmotic minipumps [103]. Binding studies also generally report no changes in benzodiazepine binding after chronic treatment [92, 93, 104]. Together, $GABA_A$ receptor expression (both mRNA and protein levels) is not consistently and robustly altered after various long-term treatment regimens. Thus, a general central downregulation or even consistent region-specific changes in $GABA_A$ receptor expression after chronic benzodiazepine use are not supported by the literature. Even though methodological differences (e.g., treatment regimen,

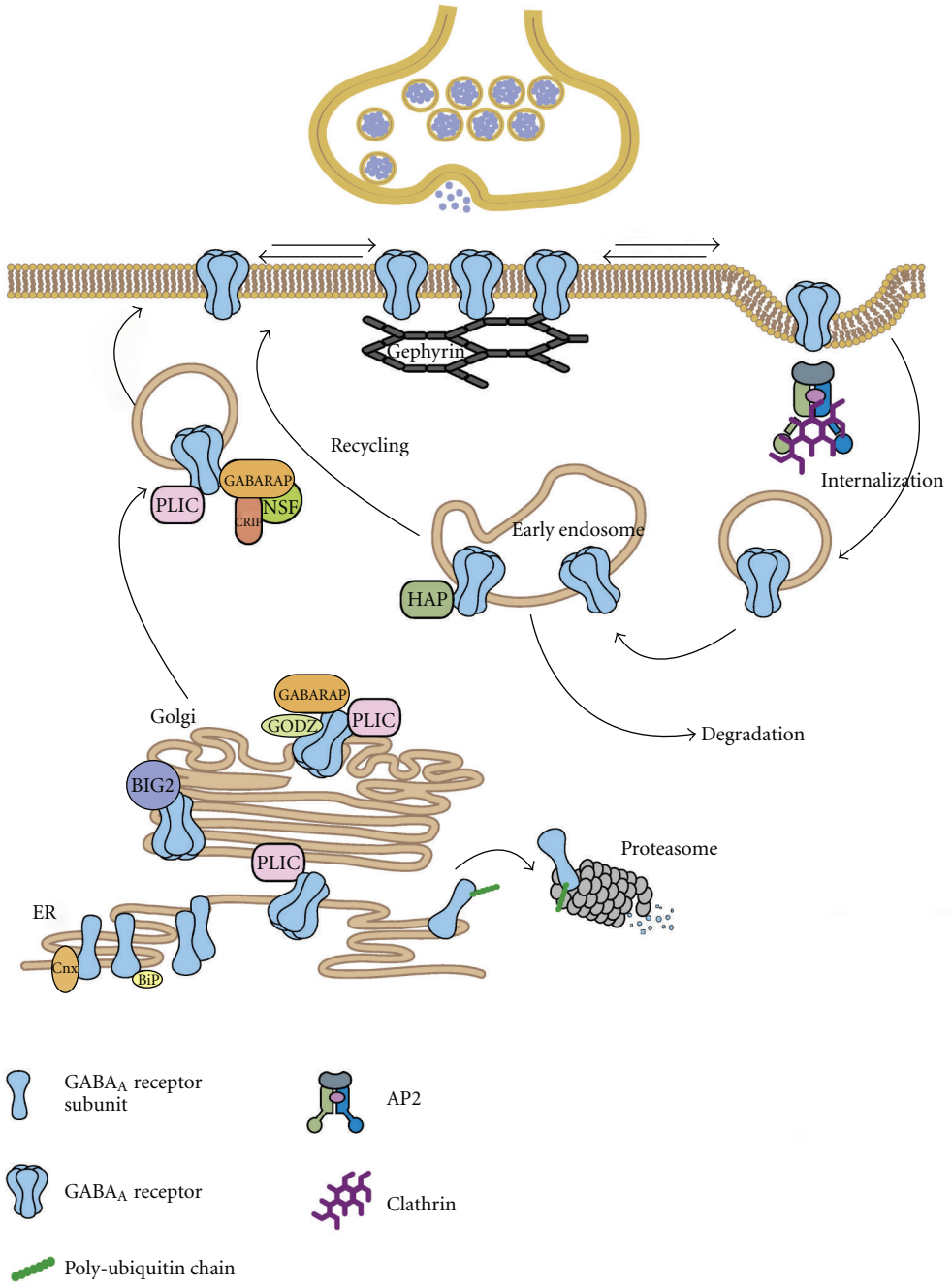

Figure 2: GABA$_A$ receptor trafficking and associated proteins. GABA$_A$ receptors are assembled from individual subunits in the endoplasmatic reticulum (ER) where the chaperones BiP and Calnexin assist in quality control. Unassembled GABA$_A$ receptor subunits that are to be targeted for ER-associated degradation are ubiquitinated and degraded in the proteasome. The ubiquitin-like protein PLIC can interact with GABA$_A$ receptors thereby inhibiting their targeting for proteasomal degradation. Assembled pentameric GABA$_A$ receptors exit the ER and bind the guanidine exchange factor brefeldin-A-inhibited GDP/GTP exchange factor 2 (BIG2) in the Golgi. Here they also interact with the palmitoylase transferase GODZ and Gamma-aminobutyric acid receptor-associated protein (GABARAP). GABARAP interacts with the NEM sensitive fusion (NSF) protein, as does the GABA$_A$ receptor β subunit, and this association may facilitate transport of the receptor complexes to the cell surface. GABA$_A$ receptors are inserted at extrasynaptic sites and can diffuse along the plasma membrane in and out of synaptic domains. At synapses they are stabilized by an interaction with the scaffolding protein Gephyrin. The interaction of the GABA$_A$ receptor intracellular loops with the μ2 subunit of the adaptin complex AP2 is important for GABA$_A$ receptor internalization. GABA$_A$ receptors are delivered by a clathrin-mediated pathway to early endosomes where they can be targeted for degradation in the lysosome or for recycling upon binding of Huntington-associated protein (HAP1). Reprinted by permission from Elsevier, reprinted from [101].

species, route of administration, and applied drug) may account for some conflicting findings, the results seem overall inconsistent. Moreover, molecular results are often not combined with behavioral tests, preventing a direct correlation between behavioral tolerance and molecular changes. Clinical studies applying *in vivo* binding or postmortem $GABA_A$ receptor expression after chronic benzodiazepine treatment are to the knowledge of the authors lacking.

Changes in rates of $GABA_A$ receptor endocytosis, receptor membrane insertion, intracellular trafficking, and association with helper $GABA_A$ receptor-associated proteins could still play a role, leading to a reduction in membrane surface receptors without affecting overall subunit protein expression (e.g., see [105]). Another interesting suggestion is that a possible loss of synaptic function after chronic exposure could be due to a shift to a perisynaptic or even an extrasynaptic localization of $GABA_A$ receptors, away from clustering of $GABA_A$ receptors at synapses (Figure 2) [106]. At least in alcohol research, such dynamic changes in plasticity at inhibitory synapses have been shown [107]. Moreover, it cannot be excluded that particular subunits play a role in the development of tolerance after chronic treatment in the absence a direct up- or downregulation. Using the previously mentioned α subunit point mutation mice, acutely administered diazepam still reduced locomotor activity in α5 (H105R) mice even after chronic 8-day diazepam treatment at a combined daily dose of 15 mg/kg [27]. This suggests that the α_1 subunit that mediates the sedative effects remains responsive, indicating that simultaneous activation of the α_1 and α_5 subunit may be necessary for tolerance to the locomotor-reducing effects of classical benzodiazepines. Specifically, it was hypothesized that increased phasic signaling would alter extrasynaptic tonic inhibition mediated by α_5-containing $GABA_A$ receptors, whereas a decrease in hippocampal α_5-specific binding was reported in diazepam-tolerant mice. Also, in contrast to α_1-, α_2-, and α_3-containing receptors, α_5-containing $GABA_A$ receptors are located extrasynaptically at the base of dendritic spines where they can modulate excitatory glutamatergic input. However, α1(H101R) mice are not sensitive to the acute sedative benzodiazepine effects, making a comparison to isolated α_1 subunit activation not possible. Moreover, only tolerance to the sedative effects of diazepam was reported. Thus, it may still be possible that tolerance to other benzodiazepine effects is mediated by other subunits.

4.3. Glutamate System Hypotheses

4.3.1. General. From the previous sections, we conclude that compensatory changes solely arising from the GABA system may at most partially explain the tolerance arising following chronic treatment with benzodiazepines. Glutamate is an excitatory neurotransmitter acting on glutamate receptors. Together with the GABA system, they constitute the two fast-acting and opposing neurotransmitter systems that can modulate synaptic plasticity. In support, close neuroanatomical connections exist between GABAergic and glutamatergic neurons [108, 109]. With a presence in at least 30–50% of all synapses in the CNS, inhibitory GABA and excitatory glutamate together coordinate the balance in the brain's excitability. Therefore, it is not surprising that as these two opposing and fast-acting neurotransmitter systems form a delicate balance, chronic (increased) activation of the GABAergic system during benzodiazepine treatment may pertubate glutamatergic transmission. The basis of benzodiazepine tolerance could then lie in sensitization of the glutamatergic system—a putative process that could account for the withdrawal symptoms after chronic benzodiazepine discontinuation [5, 110]. Such sensitization is reminiscent to adaptive glutamatergic processes as seen in kindling experiments, although it should be noted that kindling only occurs with intermittent and not after continuous treatment [111]. Glutamatergic sensitization could thus play a role in the development of tolerance as well as withdrawal symptoms upon cessation of treatment. Glutamatergic changes after benzodiazepine withdrawal will not be discussed here, but there are indications that the glutamatergic system plays a role in withdrawal states with accompanying increases in anxiety and seizure activity (for review see [5]). However, glutamate receptor mRNA and protein changes may be dynamic during withdrawal, with unchanged levels during the early phase of withdrawal but changes occurring several days later [112]. This consequently complicates the interpretation of withdrawal studies and their significance for our understanding of benzodiazepine tolerance.

Similar to the GABAergic system, the glutamate system is diverse and complex, generally being divided into ionotropic and metabotropic receptor types. Ionotropic glutamate receptors form a class of heteromeric ligand-gated cation channels that potentiate the influx of K^+, Na^+, or Ca^{2+} ions following glutamate binding. Three classes of the ionotropic glutamate receptor occur in het central nervous system: the NMDA receptor (N-methyl-D-aspartate), the AMPA receptor (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid), and the kainate receptor (for a recent review see [113]). Functional NMDA receptors contain two obligatory $GluN_1$ and two regulatory $GluN_{2/3}$ subunits and are vital for synaptic plasticity (for review, see [114]). Each GluN subunit contains extracellular loops where coagonists glycine or D-serine ($GluN_1$ and $GluN_3$ subunits) and glutamate ($GluN_2$ subunits) can bind [115]. Although the channel is blocked by Mg^{2+} ions, changes in membrane potential can make the channel permeable to Na^+, Ca^{2+}, and K^+ ions. The central distribution of $GluN_2$ subunits eventually ensures heterogeneity in the NMDA receptor system. AMPA receptors are widespread heterotetrameric ligand-gated ion channels composed of four types of subunits ($GluR_{1–4}$), and are crucial to long-term synaptic plasticity such as long-term potentiation (for review see [116]). Although glutamate possesses lower affinity for the AMPA receptor compared to NMDA receptors, faster excitation-inducing kinetics are present at the AMPA receptor. Relevant to this review, a study showed that AMPA receptor desensitization was caused by a rupture of a domain interface which allowed the ion channel to close, providing a simple yet elegant explanation [117]. Kainate receptors are made up of four subunits, $GluR_5$, $GluR_6$, $GluR_7$, KA_1, and KA_2, which are similar to AMPA and NMDA receptor

subunits and can be arranged in different ways to form a functional tetramer (for review, see [118]). Compared to NMDA and AMPA receptors, synaptic kainate receptors exhibit slow rise and decay properties.

4.3.2. Mechanism 3: Role of Ionotropic Glutamatergic Receptors. Several studies have addressed the compensatory glutamate sensitization hypothesis during chronic benzodiazepine exposure to account for the development of tolerance (as reviewed by [5, 110]).

In rodents, the development of tolerance to the sedative effects of the classical benzodiazepines diazepam and chlordiazepoxide was prevented by coadministration of the NMDA receptor antagonists CPP, dizocilpine, MK-801, and ketamine [119–121]. Also, lorazepam-induced tolerance to its acute anticonvulsant effects was partially prevented with simultaneous CPP treatment [122]. In contrast, the development of tolerance to the anxiolytic effects of diazepam in a social interaction test was not blocked by concomitant administration of dizocilpine [123]. This suggests that the mechanism underlying tolerance to the anxiolytic effects of diazepam is different from that underlying tolerance to the sedative effects. Increases in cortical mRNA of NMDA NR_1 and NR_{2B} subunits have been reported in rats tolerant to diazepam [124, 125], which were prevented by concomitant treatment with the NMDA receptor antagonist MK-801 [126]. However, another study showed decreases in hippocampal NR_{2B} subunits after chronic flurazepam treatment, even though the total amount of NMDA receptors was unchanged [127].

In support, after long-term (but not acute) lorazepam treatment, no differences were found in the affinity or density of NMDA receptors, even though increased *in vitro* glutamate release and NMDA-induced cGMP efflux in the hippocampus was reported [128]. Together, these data suggest that NMDA-dependent mechanisms contribute to the development of benzodiazepine tolerance. However, as anxiolytic tolerance was not blocked by NMDA receptor antagonism, the NMDA system could also play a differential role in tolerance depending on the specific behavioral effects [123]. Moreover, a straightforward glutamate sensitization may be an oversimplification, as tolerance to the sedative effects of lorazepam after 21-day treatment correlated with a decreased rather than an increased sensitivity for glutamate (using $^{[(3)H]}$ glutamate binding) [129].

Even though the AMPA receptor antagonist GYKI 52466 did not affect the development of tolerance to the sedative effects of diazepam [121], changes in AMPA receptor subunits have been reported to be altered after long-term benzodiazepine exposure [130]. Specifically, significant reductions of mGLuR1 (cortex and amygdala) and mGluR2 mRNA (amygdala) were reported in rats treated chronically with diazepam, even though the effects were complex and dependent on treatment route (subcutaneous or intraperitoneal injections). Adding to the complexity of the published data, another study did not show changes in hippocampal GluR1-3 subunit proteins following chronic flurazepam treatment, even though mEPSCs were found and nonspecific binding was increased using the AMPA receptor antagonist $^{[(3)H]}$ Ro48-8587 [131]. A genetic approach with $GluR_1$ knockout mice showed that after subchronic flurazepam treatment, these mice developed a reduced and incomplete tolerance to the muscle relaxation and sedative effects of flurazepam, even though acute flurazepam effects were comparable between knockout and wild-type mice [132].

With regard to glutamatergic kainate receptors, we found no pharmacological or genetic studies investigating the development of tolerance.

Together, the evidence does not support a universal and replicable glutamatergic component, even though there are indications that NMDA receptor blockade can prevent tolerance to at least some behavioral benzodiazepine effects. However, molecular data are diverse and sometimes inconsistent, which are reminiscent of the molecular changes in the GABA system after chronic benzodiazepine treatment (see Section 4.2.2).

4.4. Other Mechanisms

4.4.1. Mechanism 4: Transcriptional and Neurotrophic Factors. Although the hypothesis that downstream signaling events adjust in response to chronic exposure to benzodiazepines seems plausible, a surprising paucity of data exist in this field. It is tempting to speculate on the expression of diverse helper $GABA_A$ receptor-associated proteins (including GABARAP, BIG2, PRIP, gephyrin, and radixin) after long-term benzodiazepine use (Figure 2). In addition, changes in intracellularly located cAMP-response-element-binding protein (CREB) or calcium, vital in various second messenger systems, could be altered, and prolonged GABA concentrations in a neuronal culture have been shown to affect voltage-gated calcium channels [133]. However, until further studies provide additional proof for chronic benzodiazepine-induced downstream intracellular changes, the evidence that this process plays a role is inconclusive.

Neurotrophic proteins support neuronal survival, synaptic growth, and differentiation throughout the brain via tyrosine kinase receptors (Trk) and, with lower affinity, via p75 receptors (p75NTRs) [134]. Neurotrophic factors that have discovered so far include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), and nerve growth factor (NGF). Since they act as potent factors in regulating fast synaptic inhibition, adaptations leading to tolerance following chronic benzodiazepine treatment could in part be mediated via these neurotrophic factors. In support, BDNF (and NT-4) was found to acutely reduce postsynaptic $GABA_A$ receptor immunoreactivity via activation of TrkB receptors [135–139], even though one study reported an increase [140], and another study reports that chronic BDNF treatment potentiates GABAergic inhibition [141]. This reduced immunoreactivity was hypothesized to be caused by a reduction in $GABA_A$ receptor surface expression and was accompanied by reduced postsynaptic responses with the direct $GABA_A$ receptor agonist muscimol [142]. Mechanistically, BDNF-induced suppression of GABAergic signaling was hypothesized to stem from altered $GABA_A$ receptor composition, increased $GABA_A$ receptor

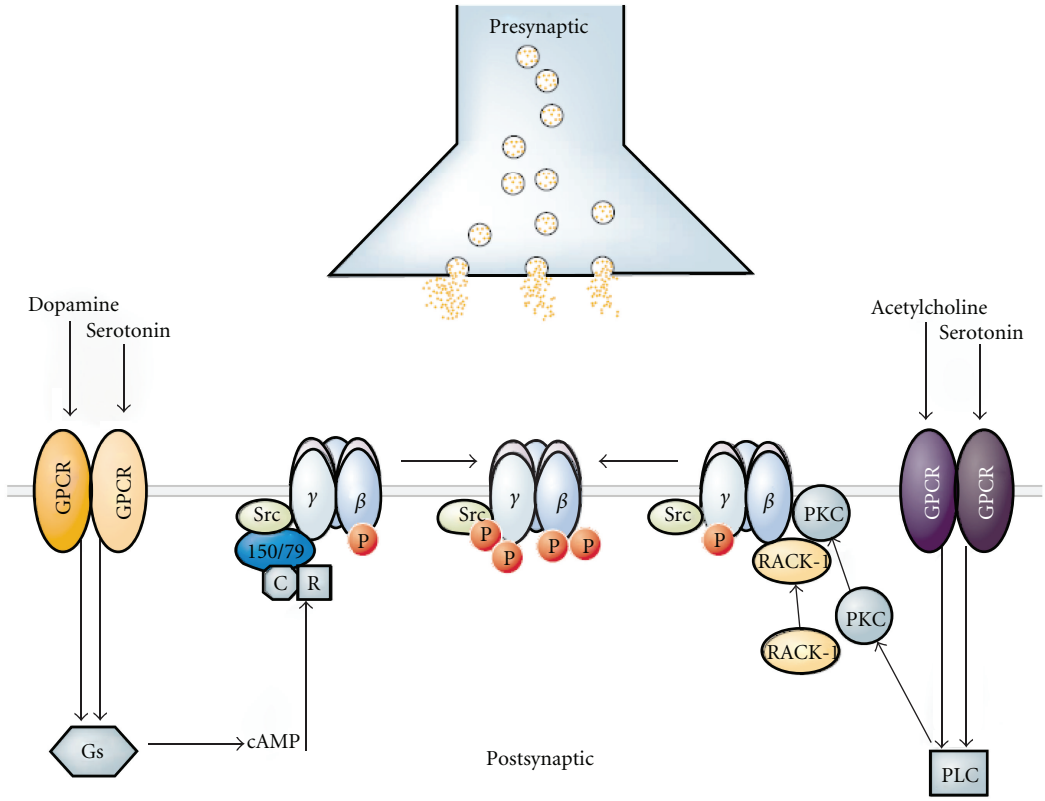

Figure 3: Functional crosstalk between G-protein coupled receptors (GPCRs) (which are present in the serotonin, dopamine, acetylcholine system) and $GABA_A$ receptors is facilitated through multiple protein kinases and scaffold proteins. $GABA_A$ receptor β and $\gamma 2$ subunits are phosphorylated (P) by PKA and PKC upon the activation of individual GPCRs for dopamine and serotonin. PKA phosphorylation of $GABA_A$ receptor $\beta 1$ and $\beta 3$ subunits is dependent upon AKAP150/79, which directly interacts with these receptor subunits. AKAP150/79 also binds inactive PKA composed of regulatory (R) and catalytic (C) subunits. In addition, PKC phosphorylates the receptor $\beta 1$–3 and $\gamma 2$ subunits. Upon the activation of the appropriate GPCR, PKC-mediated phosphorylation is facilitated by the direct (but independent) interaction of the receptor for activated C kinase (RACK-1) and the β isoform of PKC with the $GABA_A$ receptor $\beta 1$–3 subunits. RACK-1 facilitates functional regulation of $GABA_A$ receptors by controlling the activity of PKC associated with these proteins. The $GABA_A$ receptor $\gamma 2$ subunit is also phosphorylated by Src, and this kinase is capable of binding to receptor β and $\gamma 2$ subunits. Finally, the functional effects of phosphorylation are diverse and range from inhibitions to enhancements of $GABA_A$ receptor activity, dependent upon the receptor subunit composition. Reprinted by permission from Elsevier, reprinted from [95].

phosphorylation, decreased subunit synthesis, or increased postsynaptic receptor internalization or diffusion [139]. Interestingly, all these proposed mechanisms were already discussed in this paper. Thus, neurotrophin-induced changes may not be an independent mechanism, but be a player in a causal chain of events. Again, to our knowledge, no studies exist on the effects of chronic benzodiazepine treatment on neurotrophic expression and functionality.

4.4.2. Mechanism 5: Serotonin, Dopamine, Acetylcholine Systems. There is ample evidence that the serotonin, dopamine, and acetylcholine receptor systems can modulate the $GABA_A$ receptor functionality [143–146] (Figure 3). For example, the receptor for activated C kinase (RACK-1) potentiated PKC-dependent phosphorylation of $GABA_A$ receptors mediated by the activation of muscarinic acetylcholine receptors [145], and serotonergic neurotransmission inhibited GABAergic signaling via $GABA_A$ receptor PKC-dependent phosphorylation, again with involvement of RACK-1 [144]. Altogether, these neurotransmitter systems act via G-protein-coupled receptors to activate protein kinases (PKA and PKC) and scaffold proteins that may subsequently modulate $GABA_A$ receptor β and γ_2 subunit phosphorylation (Figure 3) [95].

However, studies investigating the role of the serotonin, dopamine, and acetylcholine system in response to

chronic benzodiazepine treatment are scarce. Three weeks of diazepam treatment (25 mg/day) in healthy male volunteers resulted in tolerance to the prolactin and growth hormone response induced by the 5-HT precursor L-tryptophan, even though sedative effects of L-tryptophan remained present [147]. Another study showed that chronic diazepam treatment resulted not only in diazepam tolerance but also in a very modest reduced efficacy of the 5-HT_{1A} receptor agonist 8-OH-DPAT to induce flat body posture and forepaw treading [148]. In contrast, only acute but not chronic diazepam treatment decreased basal extracellular dopamine levels in rats, even though both acute and chronic treatment regimens could reverse the stress-induced rise of cortical dopamine levels [149].

4.4.3. Mechanism 6: Neurosteroids. There is ample and convincing evidence that neurosteroids are endogenous allosteric regulators that interact with $GABA_A$ receptors to modulate both tonic (extrasynaptic) and phasic (synaptic) inhibition (for reviews, see [150, 151]). Also, acute or chronic neurosteroid treatment may change $GABA_A$ receptor subunit expression, especially extrasynaptic α_4 and δ subunits [151]. In light of the plasticity-inducing actions of neurosteroids on inhibitory signaling, long-term enhancement of the GABA system with benzodiazepines may in turn evoke changes in the neurosteroids system such as changes in neurosteroid synthesis and metabolism, although classical benzodiazepines may differ in their potency to cause such changes [152]. In support, ovariectomy attenuated the development of tolerance to the anticonvulsant actions of diazepam [153]. Moreover, co-administration of the neurosteroids allopregnanolone or pregnenolone (but not dehydroepiandrosterone) prevented the development of tolerance after chronic treatment with either triazolam and diazepam [154]. Adding to the complexity of the putative involvement of neurosteroids in benzodiazepine tolerance, factors such as $GABA_A$ receptor subunit composition, phosphorylation mechanisms, and ((extra)synaptic) localization—which are all factors that were already found to be involved in tolerance development—influence the specific dynamics of neurosteroid activity.

4.4.4. Conclusion. From our review of the literature on the various mechanisms that may underlie benzodiazepine tolerance, it occurs that there is a considerable variance in the published data. The heterogeneity of the data lies in the application of different methodologies, species, treatment regimens, and benzodiazepines. Specifically, we have considered classical benzodiazepines as a homogenous drug class since they all lead to a nonspecific enhancement of $GABA_A$ receptors that contain an α_1, α_2, α_3, or α_5 subunit. However, *in vivo* pharmacodynamic potency and pharmacokinetic half-life differences could greatly impact on tolerance processes [7]. In support, subchronic treatment with different classical benzodiazepines lead to differential propensity for FG7142-induced seizures in mice, with triazolam, clonazepam, and diazepam producing around seizures in around 80% of the mice, whereas alprazolam and midazolam did so in 60% of the animals and lorazepam in 40% of the animals [155]. Surprisingly, chlordiazepoxide did not lead to any precipitated seizures, even though a comparable $GABA_A$ receptor occupancy was obtained. Therefore, the assumption that classical benzodiazepines act as a homogeneous class probably complicates the interpretation of the current literature.

Altogether, it appears that none of the proposed putative mechanisms can sufficiently explain tolerance development. Thus, multiple mechanisms may (synergistically) coexist, or an additional yet undiscovered mechanism may be present. However, the complex and adaptive nature of the GABA system and the existing heterogeneous literature on benzodiazepine tolerance suggest that one unifying tolerance mechanism may be a vast oversimplication. In any case, the proposed tolerance mechanisms are not completely independent, exemplified by the fact that neurotrophic factors and neurosteroids are influenced by $GABA_A$ receptor composition and phosphorylation status, which are themselves proposed to be involved in benzodiazepine tolerance. Unfortunately, the present literature does not consistently support a clear recommendation in terms of a pharmacological $GABA_A$ receptor profile (e.g., subunit preference) to aid in the development of novel and more selective benzodiazepines that lack tolerance development and are suitable for long-term treatment.

5. Tolerance to Novel Subtype-Selective Benzodiazepines

Here, we will review the evidence for tolerance development with novel $GABA_A$ receptor subtype selective compounds that provide the direct opportunity to evaluate their roles in tolerance. With the development of subunit-selective benzodiazepines, it has become possible to dissect the different effects of classical benzodiazepines (see Section 2.2 and Table 2). However, declining efficacy over time is a complex process which may not be easily attributed to one specific α subunit. Still, if novel drugs possess reduced propensity to lead to tolerance development, this will be greatly welcomed from a clinical perspective. Continuing efficacy with these drugs would advance the clinical use of drugs acting at the $GABA_A$ receptor benzodiazepine site. Unfortunately, not many studies have directly addressed tolerance development using these novel compounds. Recent data from our laboratory suggest that no tolerance develops to the acute hypothermic, anxiolytic, or sedative effect of diazepam in mice treated for 28 days with the $GABA_A$-α_2/α_3 selective compound TPA023 (Table 2) [156], indicating that chronic activation of $GABA_A$-α_2/α_3 receptors does not lead to anxiolytic tolerance after acute diazepam challenge (unpublished data). Also, in contrast to morphine, no analgesic tolerance occurred in rats after a 9-day treatment with the $\alpha_{2/3}$ subtype $GABA_A$ receptor positive allosteric modulator L838,417 using a model of neuropathic pain [157]. From these data, it seems that tolerance development after chronic administration of $GABA_A$-α_2/α_3 subtype selective drugs may not develop, or, alternatively, that tolerance to diazepam's sedative actions needs concomitant activation of $GABA_A$-α_1/$GABA_A$-α_5 receptors. In support of the latter

hypothesis, ligands that do not bind to the α_5 subunit such as zolpidem have a reduced tendency to engender tolerance [158, 159], supported by studies in which chronic treatment with zolpidem (but not midazolam) did not produce any tolerance to sedative and anticonvulsant effects in mice and rats [160–162].

In addition to studies directly assessing tolerance, several studies have investigated the precipitated withdrawal after (sub) chronic treatment with subtype-selective compounds. Compounds with selective efficacy at α_2, α_3, and α_5 $GABA_A$ receptor subtypes were shown to lead to differential seizures susceptibility in mice in response to the inverse agonist FG-7142 [155]. Chronic treatment with zolpidem, as well as the selective compounds L-838,417 (partial agonist at $\alpha 2$ $GABA_A$, $\alpha 3GABA_A$, and $\alpha 5GABA_A$ receptors) and SL651498 (full agonist at $\alpha 2GABA_A$ and $\alpha 3GABA_A$ receptors, partial agonist at $\alpha 1GABA_A$ and $\alpha 5GABA_A$ receptors), did not result in seizures following FG-7142 administration [31, 155] (Table 2). Similarly, chronic treatment with TPA023 (partial agonist at $\alpha 2GABA_A$, $\alpha 3GABA_A$, and $\alpha 5GABA_A$ receptors) also did not result in FG-7142-induced seizures in mice [156]. However, because these studies do not specifically address tolerance development, the rather general conclusion from these studies is that partial or selective modulation of the $GABA_A$ receptor results in a reduced liability for physical dependence. Thus, it is important to note that, even though zolpidem does not seem to engender any obvious tolerance development, zolpidem can lead to withdrawal symptoms that are comparable to those seen after chronic classical benzodiazepine treatment [29, 77]. Thus, tolerance and withdrawal symptoms may constitute separate entities in benzodiazepine dependence. In support, one study demonstrated that marked withdrawal symptoms appeared upon abrupt discontinuation of chronic clorazepate treatment in dogs, even though tolerance was present to a rather limited extent [163].

Together, it can be concluded that so far, α_2/α_3 subtype selective compounds have neither been found to lead to tolerance nor withdrawal symptoms. This would constitute a significant improvement over currently used benzodiazepines, even though the anxiolytic profile of these compounds remains to be determined [164], and abuse liability may still be present [8]. However, interpretations should be made with caution since chronic treatment with nonselective partial positive allosteric modulators such as bretazenil did neither result in anticonvulsant tolerance [54, 59, 60] nor in FG-7142-precipitated seizures [155]. These studies implicate that the potency of classical and subtype-selective compounds, in addition to or despite subtype selectivity, may also be of importance in the development of tolerance. It could be also hypothesized that low efficacy at the α_1 subunit, rather than selectivity or reduced efficacy at α_2/α_3 subtypes, may be the causal mechanism preventing tolerance development. Also, the clinical anxiolytic efficacy of α_2/α_3 subtype selective compounds has not yet been established. In addition to a specific efficacy profile, tolerance development may also depend on a compound's *affinity* at certain $GABA_A$ receptor subtypes. This way, tolerance processes may be different with affinity-selective compounds such as zolpidem compared to efficacy-selective compounds such as TPA023. Circumstantial evidence stems from the fact that α_1-preferential affinity-selective compounds such as zolpidem produce physical dependence [165], even though the compound TPA123 that possesses 23% efficacy at the α_1 subunit (but is not affinity selective) did also result in physical dependence [8]. However, based on the currently available evidence, no definite conclusions can be drawn regarding the subtype involved in tolerance. Also, it is not possible to distinguish tolerance processes in selective binding (affinity) and selective activation (efficacy).

6. Conclusion

In the present paper, we summarized the rather inconsistent data regarding changes in several neurotransmitter systems to explain the development of tolerance. Specifically, we addressed possible changes at the level of (i) the $GABA_A$ receptor (subunit expression and receptor coupling), (ii) intracellular changes stemming from transcriptional and neurotrophic factors, (iii) ionotropic glutamate receptors, (iv) other neurotransmitters (serotonin, dopamine, and acetylcholine systems), and (v) the neurosteroid system. From the large variance in the studies, it appears that either different (simultaneous) tolerance mechanisms occur depending on the benzodiazepine effect, or that one tolerance-inducing mechanism depends on the activated $GABA_A$ receptor subtypes. This is not unlikely, given that tolerance is a heterogeneous process that occurs at different rates for the various effects and also depends on the profile of the (subtype selective) benzodiazepine. Adaptations could then occur on different time scales depending on the receptor subtype and brain region involved. In line with this hypothesis, tolerance develops relatively quickly for the sedative and anticonvulsant actions of benzodiazepines, whereas tolerance to anxiolytic and amnesic effects most probably do not develop at all. It is intriguing that anxiolytic effects of classical benzodiazepines may not decline during prolonged treatment. In addition to subtype selectivity, additional factors may be important for a (subtype-selective) benzodiazepine to cause tolerance, including $GABA_A$ receptor potency (efficacy) and *in vivo* receptor occupancy over time. The finding that partial agonists with an overall but comparable lower efficacy at all α subunits of the $GABA_A$ receptor such as bretazenil did not result in anticonvulsant tolerance raises the possibility that chronic clinical use of these compounds is associated with a lower tolerance.

An important question is how the development of tolerance of benzodiazepines could be reduced. One interesting suggestion could be—rather than intermittent use that can be defined by an individual—to develop benzodiazepine dosing schedules with varying daily doses including placebos. This could result in continued clinical efficacy (obviously depending on the indication) and utilize the placebo effect. The other possibility to reduce tolerance is the currently developing and promising body of literature on subtype-selective $GABA_A$ receptor PAMs. From the literature we reviewed, it appears that α_2/α_3 subtype selective compounds do not lead to tolerance or withdrawal symptoms. However,

the underlying mechanism (reduced α_1 efficacy or a generally reduced efficacy profile) is unknown. Also, it is presently unclear whether this lack of tolerance also applies to α_1- and α_5-selective GABAergic positive allosteric modulators, although a broad and unspecific tolerance resulting from selective (and often low potency) compounds seems unlikely.

In conclusion, the development of tolerance following chronic benzodiazepine treatment is a complex process in which multiple processes may simultaneously act to cause varying rates of tolerance depending on the studied effect and the administered drug. There is no convincing evidence that subtype-selective compounds acting at the benzodiazepine site lead to tolerance at a level comparable to classical benzodiazepines. If this is indeed the case, one consequence may be that such subtype-selective compounds are unlikely to engender clinical tolerance, which would be a clinically significant improvement over classical benzodiazepines.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2012, Article ID 708428, 8 pages
doi:10.1155/2012/708428

Review Article

Perisynaptic GABA Receptors: The Overzealous Protector

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Received 13 April 2011; Accepted 12 December 2011

Academic Editor: John Atack

An attempt to find pharmacological therapies to treat stroke patients and minimize the extent of cell death has seen the failure of dozens of clinical trials. As a result, stroke/cerebral ischemia is the leading cause of lasting adult disability. Stroke-induced cell death occurs due to an excess release of glutamate. As a consequence to this, a compensatory increased release of GABA occurs that results in the subsequent internalization of synaptic $GABA_A$ receptors and spillover onto perisynaptic $GABA_A$ receptors, resulting in increased tonic inhibition. Recent studies show that the brain can engage in a limited process of neural repair after stroke. Changes in cortical sensory and motor maps and alterations in axonal structure are dependent on patterned neuronal activity. It has been assumed that changes in neuronal excitability underlie processes of neural repair and remapping of cortical sensory and motor representations. Indeed, recent evidence suggests that local inhibitory and excitatory currents are altered after stroke and modulation of these networks to enhance excitability during the repair phase can facilitate functional recovery after stroke. More specifically, dampening tonic GABA inhibition can afford an early and robust improvement in functional recovery after stroke.

1. γ-Aminobutyric Acid (GABA)

GABA is the major inhibitory neurotransmitter within the mammalian brain. Twenty to 50% of all synapses within the CNS use GABA as a neurotransmitter, mediating both fast and slow inhibitory synaptic transmission [1]. GABA is an endogenous ligand for the $GABA_A$, $GABA_B$, and $GABA_C$ receptors [2], and these receptor subtypes have been classified according to differences in both structure and pharmacology. $GABA_A$Rs are ligand-gated chloride channels [2, 3] formed from 5 subunits arranged around a central ion pore. At least nineteen mammalian genes encoding for the various $GABA_A$R subunits exist: $\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, φ, π, and $\rho_{1–3}$, with slice variants also contributing to variations in receptor functions [4–9]. The most common subunit combinations are believed to be composed of 2α, 2β, and γ, with the γ-subunit being able to be substituted for either an ε- or a δ-subunit [7–9].

Depolarization of inhibitory interneurons produces a phasic release of GABA and inhibition of postsynaptic neurons. Extrasynaptic $GABA_A$R's respond to ambient levels of GABA present in the extracellular space to regulate baseline pyramidal neuron excitability and show reduced desensitization remaining active for long periods of time [10]. Tonic $GABA_A$R's in the hippocampus and cortex contain either α5 or δ-subunits [6, 10]. Reduced activity of α5 or δ-subunits enhances pyramidal neuron firing to afferent inputs [10–12], enhances neuronal network excitability [13], and facilitates LTP and cognitive performance [14–17]. GABA transporters modulate the level of tonic $GABA_A$R activity [18] with the uptake of GABA into neurons and astrocytes for recycling. Low GABA concentrations activate extrasynaptic $GABA_A$R's, leading to persistent or tonic inhibition [19, 20]. Synaptic and extrasynaptic $GABA_A$R's exhibit distinct pharmacological and biophysical properties that differentially influence brain physiology and behavior [19].

Synaptic $GABA_A$R's are composed of $\alpha_{1–3}$, $\beta_{1–3}$, and $\gamma_{1–3}$, subunits, and the site of action for a variety of clinically important drugs, such as benzodiazepines, neurosteroids, and anesthetics. Where as extrasynaptic $GABA_A$R's are composed of subunit combinations containing $\alpha_{4–6}$,

$\beta_{1–3}$, and γ_2- or δ-subunits. Of these receptors, the δ-containing $GABA_AR$'s coassembled as $\alpha_4\beta\delta$—located in the cortex, hippocampus and thalamus—or $\alpha_6\beta\delta$—located in the cerebellum—that are emerging as unique and fundamental players in GABAergic neurotransmission [19]. In addition to δ-containing $GABA_AR$'s having a functional role in the cortex, the α5-containing $GABA_AR$'s coassembled primarily as $\alpha_5\beta\gamma_2$ have also been implicated in poststroke repair [21]. Even though the expression of the α5-subunit is low in the cortex compared to the δ-subunit, greater functional improvements in motor recovery are seen following modulation of the α5-subunit [21]. The pharmacology of these extrasynaptic receptors is inconsistent between research groups [22] and has been hampered by the lack of selective agents to probe function in recombinant, native, and whole animal systems [23]. Conflicting data is also present with respect for the ability of these receptors to desensitize [19, 24]. Determining the composition and pharmacology of this receptor will enable the development of much needed therapies for use in stroke.

1.1. Disability in Stroke. Stroke is the leading cause of death and long-term disability in adults worldwide. Stroke-induced sensory and motor loss of limb function, in particular, prevents patients from returning to work and accounts for the statistic that almost one-third of stroke survivors become institutionalized after having a stroke [25–28]. Recent studies have shown that the brain has a limited capacity to repair after stroke. In both humans and animals, neural repair after stroke has been shown to involve remapping of cognitive functions and sprouting of new connections in tissue adjacent to the stroke site, the peri-infarct cortex [29, 30]. However, mechanisms associated with poststroke neural repair and recovery have not been well characterized, and it has been assumed that changes in cortical representational maps underlying the recovery involve changes in neuronal excitability. Consistent with this, animal studies suggest that therapies associated with rehabilitation can promote plasticity changes in tissue that survives the stroke [31].

Functional recovery within the peri-infarct cortex involves changes in neuronal excitability. Clinical studies using direct current stimulation of the peri-infarct cortex, with protocols that boost local neuronal excitability, have been shown to improve use of the affected limb in stroke patients [32, 33]. Furthermore, forced use or task-specific repetition of the affected limb have also been shown to activate the peri-infarct cortex and improve functional recovery [34]. Studies suggest that decreases in γ-aminobutyric acid GABA activity within the motor cortex could facilitate structural changes [35] and promote recovery of motor function [36]. Alterations in neuronal excitability underlie fundamental changes in information transfer in neuronal circuits [37] such as long-term potentiation and depression (LTP and LTD) as well as the unmasking of quiescent synaptic connections and remodeling of cortical maps [38]. Furthermore, changes in LTP and cortical map formation occur within the peri-infarct cortex adjacent to the stroke [29]. These data suggest a critical role for modulating cortical excitability as a means for promoting functional recovery after stroke.

1.2. Brain Excitability in Learning, Memory, and Repair. The processes of neurorehabilitation involve physical, occupational, and cognitive therapies [27, 28]. Further changes in poststroke cortical plasticity play a critical role in mediating repair mechanisms. While these modalities clearly promote functional recovery, no drug treatments exist that promote poststroke brain repair and recovery. Recent evidence suggests that suppression of either cortical tonic GABA inhibition or stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents can promote poststroke function gain [21, 39]. This ability to regain function relies heavily on the ability to learn or relearn after stroke and likely follows classical activity-dependent processes associated with motor learning and memory [40, 41]. In addition to these behavioral links, stroke recovery and classical learning and memory pathways share similar molecular and cellular links. For instance, genes that are important for learning and memory are also elevated during periods of poststroke repair and include membrane-associated phosphoproteins GAP43 and MARCKS, the transcription factor c-jun, and the cell adhesion molecule L1 [42].

Modulation of learning and memory pathways have previously been shown to promote functional recovery and poststroke axonal sprouting following administration of pharmacological agents such as amphetamines and phosphodiesterase type-4 inhibitors that boost cAMP/CREB signaling and learning and memory function [43, 44]. These data indicate that manipulating learning and memory pathways can offer a novel means for promoting recovery. As with stroke recovery, the processes of learning and memory can be enhanced by manipulations that increase neuronal excitability, which has also been shown to promote function recovery [21]. Significant data is accumulating indicating an imbalance in inhibitory and excitatory pathways after stroke, and modulation of these pathways by either enhancing glutamate-mediated transmission or dampening the tonic form of GABA can facilitate functional recovery [21, 39, 45–48]. α5$GABA_AR$ negative allosteric modulators are part of a broad class of drugs that boost learning and memory function by influencing key elements in neuronal memory storage, such as LTP [14, 16]. α5$GABA_AR$ negative allosteric modulators, and indeed any mechanism that dampens tonic GABA signaling, could significantly improve poststroke recovery [21]. This suggests that the similarities between neuronal mechanisms of learning and memory and those of functional recovery after stroke extend to common treatment strategies for both.

Most strategies that promote functional recovery after stroke, such as axonal sprouting, neurogenesis, or angiogenesis, focus or rely on inducing structural changes in the brain as a means to promote functional recovery after stroke [49–53]. In order to promote structural change in the brain, however, these treatments take time to develop a

functional effect. Blocking tonic GABA inhibition induces a rapid improvement in behavioral recovery in the absence of any change in axonal sprouting within the peri-infarct cortex [21]. This data suggests that treatments that focus on inducing molecular memory systems after stroke may have the advantage of promoting synaptic plasticity in peri-infarct cortex rapidly and without altering the tissue reorganization that normally occurs after stroke. These therapies are highly translatable into the clinic due to their timing of drug administration, 3–7 days after stroke in rodents, and with the early effects seen with functional recovery, will aid in the huge social and economical burdens seen after stroke.

1.3. Attenuating GABAA Receptor Function in Neural Repair after Stroke. As with stroke recovery, the processes of learning and memory can be enhanced by manipulations that increase neuronal excitability. However, unlike the stroke recovery field, basic science studies in learning and memory have defined specific cellular pathways that lead to enhanced neuronal excitability and improved function.

Recent work has shown that enhanced neuronal excitability occurs following the dampening of the baseline level of inhibition in neurons. This baseline inhibition is in part set by a tonic, always present, degree of inhibitory signaling from the major inhibitory neurotransmitter, GABA. Unlike the phasic nature of synaptically released GABA, the action of GABA via extrasynaptic receptors is to tonically suppress neuronal excitability and to help regulate neuronal action potential firing. These extrasynaptic GABA receptors consist of $\alpha 5$ and δ-subunit containing $GABA_AR$'s. Recent evidence using $\alpha 5GABA_AR$ "knock-out", and point-mutated mice have clearly shown that the $\alpha 5$-subunit plays a key role in cognitive processing [15, 17]. In addition, *in vitro* and *in vivo* work has shown that $\alpha 5GABA_AR$ negative allosteric modulators can enhance cognition within the Morris water maze, enhance hippocampal LTP and do not have any proconvulsant effects [14, 16]. Using pharmacological and genetic manipulations of extrasynaptic $GABA_AR$'s, we have shown marked improvements in functional recovery when starting treatments from 3 days after the stroke [21]. These data are consistent for offering a potential role for extrasynaptic $GABA_AR$'s in processes involving synaptic plasticity and learning and memory and more recently poststroke recovery.

Neuronal inhibition and network function is disturbed in peri-infarct tissue during periods of cortical plasticity, re-mapping, and recovery. The increase in tonic inhibition in cortical pyramidal neurons reported by Clarkson and colleagues [21] occurs at precisely the same time as cortical map plasticity and recovery [54]. Behavioral recovery in stroke is closely correlated with functional plasticity in peri-infarct and connected cortical regions. In human stroke patients, an expansion in motor representation maps is seen in tissue adjacent to or connected to stroke [29, 55]. In animal models, when stroke damages primary motor or somatosensory areas, motor and sensory representations remap in peri-infarct cortex [54, 56]. These processes of recovery identify plasticity in the cortical circuits in peri-infarct cortex as key elements in functional recovery.

2. GABA and Cerebral Ischemia

A large body of work has been devoted to developing and exploring neuroprotectants that act to block glutamate-mediated neurotransmission in animal models of cerebral ischemia [57, 58]. Increased inhibitory neurotransmission associated with GABA has been shown to normalize the balance of glutamate-mediated excitation. Therefore, pharmacological enhancement of $GABA_AR$ neurotransmission provides an alternative means for neuroprotection. Indeed, over recent years, changes in GABA function following cerebral ischemia and possible protective benefits of GABAergic drugs have been extensively assessed [59–65]. Even though it has been proposed that enhancing GABA transmission may elicit protection against cerebral ischemia [60–62, 65], the exact mechanisms that are associated with these neuroprotectants have, as yet, not been fully elucidated and increasing GABA function may be protective during cerebral ischemia for different reasons [59–65]. However, even though GABA agonists have shown great promise in animal model, these compounds have failed to translate into the clinic [66, 67]. The failure of these compounds highlights the need to firstly establish better preclinical rodent models of stroke that better mimic what occurs in humans. Secondly, the use of subunit specific GABA compounds is more likely to show an effect, due to them having less side effects, such as drug-induced hypothermia and sedation. However, even with recent developments in this area, studies are lacking. The need to assess subunit-specific GABA compounds to help understand what is happening after stroke in terms of GABA function is highlighted with clinical reports showing that zolpidem, an $\alpha 1$ subunit $GABA_AR$ modulator, can result in transiently improves in aphasia in chronic stroke survivors [68].

During situations of cerebral ischemia, it has been shown that the extracellular concentrations of GABA increase (approx. 50 fold compared to basal levels) to the micromolar range [59, 69] and remain elevated for at least 30 minutes during periods of reperfusion. Prolonged exposure of the $GABA_ARs$ to high concentrations of GABA agonists *in vitro* has routinely been shown to become desensitized and/or downregulated [70–72]. Similarly, the $GABA_AR$ is also downregulated in the gerbil hippocampus following transient cerebral ischemia [63]. In this model, receptor downregulation was shown to be via internalization, as there was a rapid decrease in binding of the hydrophilic ligand [3H]-SR-95531, but not the hydrophobic ligand [3H]-flunitrazepam [63]. This increase in extracellular GABA is likely to result in the spill over onto peri-synaptic $GABA_AR$'s resulting in an increase in tonic inhibition. Indeed, recent evidence showing an increase in tonic inhibition after stroke supports this notion [21]. This increase in tonic inhibition is most likely a safety mechanism imposed by the brain as a means to minimize neuronal damage. However, as this

increase in tonic inhibition persists for at least 2 weeks after the stroke, this safety mechanism which is likely to have either wrong or no feedback mechanism has been formed to compensate for such a change in tonic GABA.

3. Poststroke Tonic Inhibition

Changes in neuronal excitability, loss of GABAergic inhibition, enhanced glutamatergic transmission, and synaptic plasticity all contribute to neuronal reorganization after stroke. Studies that promote an increase in local brain excitability result in improved function [21, 34, 39, 45] and suggest that decreasing GABA activity within the brain could facilitate structural changes that promote functional recovery [21, 34, 45]. In particular, this enhancement of neuronal excitability involves dampening baseline levels of inhibition.

Tonic or continuous signaling from GABA sets baseline inhibition. GABA acts via extrasynaptic $GABA_AR$'s to tonically suppress neuronal excitability and regulate neuronal action potential firing. Therefore, in order to facilitate functional recovery, an increase in brain excitability is required to overcome this hypofunctionalism [34]. Recently Clarkson and colleagues have demonstrated marked improvements in poststroke functional recovery using pharmacological manipulations of extrasynaptic $GABA_AR$'s, implicating $\alpha 5$ or δ-containing $GABA_AR$'s as novel targets for developing agents to help stroke sufferers.

GABA has been shown to mediate both fast and slow inhibitory synaptic transmission [1]. During development, however, the $GABA_ARs$ have been shown to mediate excitation as well as play an important role in neural migration and synaptogenesis [73, 74]. During situations of cerebral ischemia, extracellular concentrations of GABA are significantly elevated [59, 69], resulting in $GABA_A$ receptor desensitization and/or downregulation [63, 71]. This is supported by immunohistochemical and autoradiographic data showing decreased expression of $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\gamma 2$ subunits following photothrombotic stroke and freeze-lesion-induced cortical injury [75–77].

Recent work has shown that epileptogenesis results in the suppression of functionally active $\alpha 5GABA_ARs$ and results in an increase/substitution of other $GABA_AR$'s with a subsequent increase in rather than suppression of tonic inhibitory currents [78]. A similar compensatory increase in $\alpha 4\delta$-mediated tonic currents has been seen in the $\alpha 5$ knockout mice within region CA1 of the hippocampus [11]. Extracellular GABA concentrations and thus tonic inhibition have been shown to increase as the excitatory drive increases resulting in the modulation of neuronal excitability and prevention of neuronal saturation [79]. Consistent with these findings, Clarkson and colleagues reported an increase in GABA tonic inhibitory currents from 3–14 days poststroke in layer II cortical pyramidal neurons [21]. This poststroke increase in tonic inhibition may act as a compensatory mechanism to prevent further neuronal injury. However, this prolonged increase in tonic inhibition during the repair phase is acting as a hindrance by preventing cortical expansion and improvements in functional recovery. This is supported by findings by Clarkson and colleagues who show that both pharmacological and genetic modulation of tonic inhibition, dampening either $\alpha 5$ or δ-mediated increase in tonic GABA currents, results in early and marked improvements in functional recovery [21].

Understanding the profile for which cortical plasticity occurs, altered after a stroke, is critical for fully determining when to start treatments and with what therapeutic compound to use. Based on our findings, we have clearly shown that dampening of tonic GABA currently from 3 days results in robust functional improvements of motor recovery [21]. These improvements, however, may not be the same if treatments are started weeks after stroke onset as previously shown in humans using zolpidem, which was shown to transiently improve aphasia in chronic stroke survivors [68]. The $\alpha 1$ and $\beta 2$ $GABA_AR$ subunits are densely localized within the cortex and coassembly with the $\gamma 2$-subunit accounts for about 40% of all $GABA_ARs$ within the cortex [80]. Assembly of $GABA_ARs$ containing $\alpha 1\beta 2\gamma 2$ has been shown to be enriched at synaptic sites throughout the cortex [81] and involved in changes in synaptic plasticity. However, studies have also shown that the δ subunit can coassemble with $\alpha 1$ subunits to form functional recombinant receptors[82, 83]. Furthermore, immunoprecipitation studies have shown that δ subunits can associate with $\alpha 1$ subunits [84], and $GABA_AR\alpha 1$ subunits have also been found extrasynaptically [85, 86] consistent with the typical localization of δ-containing $GABA_ARs^{8181}$. These data could suggest an alternative method for why zolpidem was having an effect in chronic stroke patients to alleviate the burden of aphasia. However, further studies are needed, as one previous study would suggest that the $\gamma 2$-subunit is required in order for zolpidem to have an effect [87].

4. Dampening Cortical Inhibition Alters Cortical Responsiveness

Disinhibition of cortical connections within the peri-infarct or regions associated with the peri-infarct cortex have been argued as either occurring as a direct consequence of the stroke or as a potential compensatory mechanism related to the recovery [88]. This argument has come about based on a number of observations such as local blockage of GABAergic inhibition unmasking preexisting horizontal connections within the rat motor cortex [38]; LTP of adult rat motor cortex horizontal connections is dependent on GABA disinhibition during theta burst stimulation, unlike other regions such as the hippocampus or somatosensory cortex [35]; and finally modulation of GABA has been shown to be involved in learning in healthy humans as shown using imaging studies showing a correlation between a decrease in GABA concentration in motor cortex and motor skill learning [89]. Consistent with the notion that cortical disinhibition is occurring as a compensatory mechanism, Clarkson and colleagues have shown a robust and persistent increase in tonic inhibition in the peri-infarct cortex after stroke and blockade of this tonic inhibition at the time of stroke with the

extrasynaptic $GABA_AR$ negative allosteric modulator, L655-708, exacerbated the lesion [21]. Further to this, Clarkson and colleagues showed for the first time that delayed treatment L655-708, which has previously been shown to induce LTP [14], provides an early and robust reversal in behavioral deficits [21]. Given the early behavioral effects seen and the lack of effect on sprouting of new connections, cortical disinhibition following L655-708-treatment seems a logical argument. To support the notion that dampening GABA activity is having a beneficial effect, no improvement in motor function was observed after stroke following administration of the GABA agonist, muscimol [21]. This is backed by clinical studies illustrating the reemergence of stroke symptoms following administration of the GABA agonist midazolam in chronic stroke patients that have shown significant improvements in function [90]. The peri-infarct cortex exhibits neuronal metabolic dysfunction over a one-month period [91], which would indicate a therapeutic time window for blockade of tonic GABA signaling of at least one month after stroke. Consistent with this is the fact, when L655-708 treatment is discontinued after a two-week period of administration after stroke, a slight rebound effect/reversal in functional recovery is observed compared to animals that received treatment for the six-week period [21].

5. Conclusions

Therapies that promote functional recovery after stroke are limited to physical rehabilitation measures. While specific measures, such as constraint-induced therapies, promote recovery of motor function, no pharmacological therapies are available that aid in recovery. Functional recovery after stroke follows psychological learning rules [41] that indicate learning and memory principles may underlie behavioral recovery. At the cellular level, learning and memory are mediated by specific excitatory neuronal responses, such as LTP, and are potentiated by drugs that facilitate aspects of excitatory neuronal signaling [13], such as tonic $GABA_AR$ antagonists [10]. Recent data shows that stroke alters the balance of excitatory and inhibitory inputs to neurons in the peri-infarct cortex, by increasing inhibitory tone. This altered excitatory balance occurs through a decrease in the normal cellular uptake of GABA. Dampening GABA-mediated tonic inhibition restores the excitatory/inhibitory balance in peri-infarct motor cortex *ex vivo* and promotes recovery of motor function *in vivo*. These effects occur through blockade of $\alpha 5$ or δ-containing $GABA_AR$'s. This data indicates a novel role for tonic $GABA_AR$ function in promoting poststroke recovery most likely via cortical disinhibition [38, 92, 93] and suggests a new avenue for pharmacological treatment of neurorehabilitation in stroke. This early effect on stroke recovery opens the possibility for treatments that block tonic GABA signaling and may be used in conjunction with later-acting stroke repair therapies in a combinatorial manner. More generally, tonic GABA signaling has a biphasic role in stroke. Early tonic GABA signaling limits stroke size, later tonic GABA signaling limits stroke recovery. These data identify a promising molecular system for future stroke recovery therapies and implicate molecular memory systems as likely key players in recovery from stroke.

Acknowledgments

This paper was completed during tenure of a Repatriation Fellowship from the New Zealand Neurological Foundation and the Sir Charles Hercus Fellowship from the Health Research Council of New Zealand.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2012, Article ID 134523, 10 pages
doi:10.1155/2012/134523

Review Article

An Overview of the CNS-Pharmacodynamic Profiles of Nonselective and Selective GABA Agonists

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Received 9 July 2011; Revised 2 December 2011; Accepted 7 December 2011

Academic Editor: Keith Wafford

Various $\alpha_{2,3}$ subtype selective partial GABA-A agonists are in development to treat anxiety disorders. These compounds are expected to be anxiolytic with fewer undesirable side effects, compared to nonselective GABA-A agonists like benzodiazepines. Several $\alpha_{2,3}$ subtype selective and nonselective GABA-A agonists have been examined in healthy volunteers, using a battery addressing different brain domains. Data from five placebo-controlled double-blind studies were pooled. Lorazepam 2 mg was the comparator in three studies. Three $\alpha_{2,3}$-selective GABAA agonists (i.e., TPA023, TPACMP2, SL65.1498), one α_1-selective GABAA agonists (zolpidem), and another full agonist (alprazolam) were examined. Pharmacological selectivity was assessed by determination of regression lines for the change from baseline of saccadic-peak-velocity- (ΔSPV-) relative effect, relative to changes in different pharmacodynamic endpoints (ΔPD). SPV was chosen for its sensitivity to the anxiolysis of benzodiazepines. Slopes of the ΔSPV-ΔPD relations were consistently lower with the $\alpha_{2,3}$ selective GABA-A agonists than with lorazepam, indicating that their PD effects are less than their SPV-effects. The ΔSPV-ΔPD relations of lorazepam were comparable to alprazolam. Zolpidem showed relatively higher impairments in ΔPD relative to ΔSPV, but did not significantly differ from lorazepam. These PD results support the pharmacological selectivity of the $\alpha_{2,3}$-selective GABA-A agonists, implying an improved therapeutic window.

1. Introduction

Anxiety is a psychological and physiological state with somatic, emotional, cognitive, and behavioral components [1], which dominates thinking and leads to disturbance of daily functioning. Serotonergic antidepressants, either selective serotonin reuptake inhibitors (SSRIs) or serotonin-norepinephrine reuptake inhibitors (SNRIs), are currently prescribed as the 1st-line treatment for several anxiety disorders. However, the slow onset of therapeutic effect and the presence of sexual side effects prevent these drugs from more extensive use and lead to lack of treatment compliance [2]. Moreover, SSRIs/SNRIs cause transient increase of anxiety during the first few weeks of administration. All these clinical experiences provide space for the use of benzodiazepines (BZDs) in acute anxiety episodes.

Benzodiazepines are the most commonly prescribed anxiolytic drugs, although treatment guidelines generally limit their use to several weeks to prevent the occurrence of tolerance and dependence. Benzodiazepines are allosteric modulators of the $GABA_A$ receptors that affect the central nervous system (CNS) as full GABAergic agonists [3]. As a consequence, these drugs have detrimental effects on alertness, memory, postural stability, and muscle tone. In loss-of-function studies conducted in point-mutated mice [4], different subtypes of $GABA_A$ receptors have been found responsible for the specific aspects of benzodiazepine pharmacology: (1) α_1-containing receptors are associated with sedative effects of benzodiazepines [5, 6]; (2) α_2/α_3-containing receptors are related to anxiolysis and analgesia [7, 8]; (3) α_5-receptors are associated with cognition [9, 10]. BZDs exert their CNS actions in a concentration-related manner [11]. The anxiolytic, hypnotic, muscle relaxant, and amnesic effects of BZDs generally appear concomitantly, and the onset and duration of action of the compounds correlate closely with their pharmacokinetic properties. The effect profile of BZDs has been attributed to their non-selective agonism at the α_1, α_2, α_3, and α_5 subunit-containing $GABA_A$ receptors. To improve the pharmacological and functional selectivity, novel GABAergic anxioselective compounds are

Table 1: *In vitro* pharmacological property of the GABAergic compounds.

Compound	α_1		α_2		α_3		α_5		α_1/α_2-ratio
	K_i (nM)	Efficacy° (%)	K_i (nM)	Efficacy° (%)	K_i (nM)	Efficacy° (%)	K_i (nM)	Efficacy° (%)	
TPA023* [26]	0.27	0#	0.31	11	0.19	21	0.41	5	0
TPACMP2* [13]	0.22	18	0.40	23	0.21	45	0.23	18	0.78
SL65.1498# [28]	17	45	73	115	80	83	215	48	0.39
Zolpidem	20 [29]	75§ [30]	400 [29]	78§ [30]	400 [29]	80§	5000 [29]	9§ [30]	0.96

° Relative efficacy is defined as the extent of the potentiation of GABA-A EC20-equivalent current produced by the compound compared to that produced by a nonselective full agonist (chlordiazepoxide/diazepam).
* Mean values of 3 experiments in Xenopus oocytes with human recombinant $\alpha\beta3\gamma2$ receptors; efficacy relative to chlordiazepoxide.
# Mean values of 3 experiments in hek293 cells with recombinant rat receptors $\alpha\beta2\gamma2$; efficacy relative to chlordiazepoxide.
§ Mean values of 3 experiments in Xenopus oocytes with human recombinant $\alpha\beta2\gamma2$ receptor; efficacy relative to diazepam.

Table 2: Component tests of the Neurocart battery and the related CNS domains.

Neurocart test	Targeted function	Related CNS areas
Saccadic eye movement	Neurophysiologic function	Superior colliculus, substantia nigra, amygdala
Smooth pursuit	Neurophysiologic function	Midbrain
Adaptive tracking	Visuomotor coordination	Neocortex, basal nuclei, brain stem, cerebellum
Body sway	Balance	Cerebellum, brain stem
Visual verbal learning test (VVLT)	Memory	Hippocampus
VAS Bond and Lader	Alertness, mood, calmness	Cortex, prefrontal cortex
VAS Bowdle	Feeling high, internal and external perception	Cortex, prefrontal cortex, amygdala

evaluated using recombinant human $GABA_A$ receptors during preclinical development. The GABAergic effect profile of a compound is characterized by the affinity of the ligand for the receptor and by the *in vitro* efficacy of the compound at each $GABA_A$ receptor subtype. In the past years, several partial $GABA_A$ agonists have been developed, which have a relatively high *in vitro* efficacy at α_2/α_3 subtypes compared with α_1 or α_5 subtypes. Such α_2/α_3 subtype-selective partial GABA agonists are anticipated to have favorable therapeutic effect and to be less sedating or cognition impairing (Table 1).

Based on nonclinical investigations with *in vitro* assays and animal models of anxiety, the human pharmacology of novel GABAergic agents is approached through sequential clinical studies regarding pharmacokinetics, receptor occupancy, and pharmacodynamics (PD) in healthy volunteers. Direct links have been proposed between plasma drug concentration and receptor occupancy [4], as well as between plasma drug concentration and pharmacodynamic parameters [12–15]. Such pharmacokinetic/pharmacodynamic (PK/PD) relationships warrant the assessment of surrogate biomarkers in healthy volunteers treated with single doses of selective novel GABAergic compound(s).

More than 170 pharmacodynamic tests or test variants have been developed to assess the CNS effects of benzodiazepines [11]. De Visser et al. analyzed the interstudy consistence, sensitivity, and pharmacological specificity of the frequently used biomarkers. Saccadic peak velocity (SPV) and visual analogue scale of alertness ($VAS_{alertness}$) were identified as the most sensitive parameters for benzodiazepines. Both tests showed consistent effects to a variety of benzodiazepines at different doses.

During the past fifteen years, the Centre for Human Drug Research (CHDR) has established a selection of computerized neuropsycho-pharmacodynamic tests called the Neurocart battery. The components of this battery target a variety of neurophysiological and/or neuropsychological domains (Table 2). Of this battery, adaptive tracking, saccadic eye movements, and body sway were proved sensitive to the sedating effects of sleep deprivation [16], as well as benzodiazepines and other GABAergic drugs. In the recent years, the Neurocart battery was used in a series of phase I studies to assess CNS pharmacodynamics of partial $\alpha_{2,3}$ subtype selective $GABA_A$ agonists. Both nonselective and/or selective $GABA_A$ agonists were administered as single oral dose to healthy volunteers. Clear distinctions of effect profile were observed in these trials [12–14]. The objective of this paper was to characterize the pharmacodynamic effect profiles of novel anxioselective $GABA_A$ agonists and identify suitable biomarkers to distinguish $\alpha_{2,3}$ subtype-specific $GABA_A$ agonists from full $GABA_A$ agonists like benzodiazepines.

2. Methods

Five clinical studies, all of which are published [12–15, 17], were conducted at the CHDR in healthy volunteers after approval from the Ethics Review Board of Leiden University Medical Centre. All subjects provided written inform consent for study participation. Each trial was designed as single-dose, cross-over or parallel-armed, randomized, double-blind, placebo- and/or positive-controlled study. The subjects took single oral doses of a selective GABAergic compound, placebo-, and/or a nonselective benzodiazepine. Three studies used lorazepam 2 mg as a positive control, whereas in the studies with zolpidem 10 mg and alprazolam 1 mg, these drugs were the only GABAergic study medications. Data of all studies came

from the same research center and were pooled from the studies-specific electronic databases kept by the center. *In vitro* pharmacological parameters of novel compounds were extracted from the Investigator's Brochures and published articles. These parameters provide reliable information about the subtype selectivity of each compound, but it is more difficult to compare the pharmacological properties between the drugs. Due to the diversity of cell types and $GABA_A$ receptor homologies used in the whole-cell patch clamping assays, the links between *in vitro* pharmacology and human *in vivo* effects are considered less quantitative and semiquantitative comparisons are preferred.

2.1. Treatments. Three novel drugs designed to be $\alpha_{2,3}$ subtype selective were dosed in three of the above-mentioned studies (for each dose group, the number of study participants is provided in parentheses): TPA023 0.5 mg, 1.5 mg ($n = 12$) [12]; TPACPM2 (MK0343) 0.25 mg, 0.75 mg ($n = 12$) [13]; SL65.1498 2.5 mg, 7.5 mg, and 25 mg ($n = 20$) [14]. Zolpidem is a hypnotic with a high affinity for α_1-subtypes, and alprazolam is a nonselective GABAergic anx-anxiolyticiolytic. Zolpidem 10 mg ($N = 14$) [15] and alprazolam 1 mg ($N = 20$) were administered in another two studies, respectively.

2.2. Pharmacodynamic Assessments

2.2.1. Saccadic Eye Movement. Saccadic eye movements are very sensitive to a variety of mostly CNS-depressant drugs [18, 19]. Saccadic peak velocity has been shown to be closely related to the anxiolytic properties of benzodiazepines [4]. Since partial $\alpha_{2,3}$-subtype-selective $GABA_A$ agonists are developed to be anxiolytic, it was expected that these compounds would reduce saccadic peak velocity, similar to what is typically observed with benzodiazepines. Therefore, saccadic peak velocity was used as a biomarker for the anxiolytic properties of the $GABA_A$ agonists, to which all other pharmacodynamics effects were compared in this meta-analysis. Recording and analysis of saccadic eye movements was conducted with a microcomputer-based system for sampling and analysis of eye movements. The program for signal collection and the AD converter were from Cambridge Electronic Design (CED Ltd., Cambridge, UK), the amplifiers were supplied by either Nihon Kohden (Nihon Kohden, Life Scope EC, Tokyo, Japan) or Grass (Grass-Telefactor, An Astro-Med, Inc. Product Group, Braintree, USA), and the sampling and analysis scripts were developed at CHDR (Leiden, The Netherlands).

2.2.2. Smooth Pursuit. The same systems as used for saccadic eye movements were also used for measuring smooth pursuit. For smooth pursuit eye movements, the target moves sinusoidally at frequencies ranging from 0.3 to 1.1 Hz, in steps of 0.1 Hz. The amplitude of target displacement corresponds to 22.5 degrees eyeball rotation to both sides. Four cycles were recorded for each stimulus frequency. The method has been validated at CHDR by Van Steveninck based on the work of Bittencourt et al. [20] and the original description of Baloh et al. [21].

2.2.3. Visual Analogue Scales (VASs). Visual analogue scales as originally described by Norris [22] were used previously to quantify subjective effects of benzodiazepines [19]. From the set of sixteen scales, three composite factors were derived as described by Bond and Lader [23], corresponding to alertness, mood, and calmness. These factors were used to quantify subjective drug effects.

2.2.4. Body Sway. The body sway meter measures body movements in a single plane, providing a measure of postural stability. Body sway was measured with an apparatus similar to the Wright ataxiameter, which integrates the amplitude of unidirectional body movement transferred through a string attached to the subject's waist. Two-minute measurements were made in the anteroposterior direction with eyes open and closed, with the subject standing comfortably on a firm surface with their feet slightly apart. The method has been used before to demonstrate postural instability due to benzodiazepines [24, 25].

2.2.5. Adaptive Tracking. The adaptive tracking test as developed by Hobbs and Strutt was used, according to specifications of Atack et al. [26]. The adaptive tracking test is a pursuit-tracking task. A circle of known dimensions moves randomly across a screen. The test subject must try to keep a dot inside the moving circle by operating a joystick. If this effort was successful, the speed of the moving circle increases. Conversely, the velocity was reduced if the test subject cannot maintain the dot inside the circle. The adaptive tracking test is a measure of visuomotor coordination that has proved to be very sensitive of various psychoactive drugs [27].

Table 3 summarizes the pharmacodynamic tests used in the different studies.

2.3. Statistical Analysis. Individual graphs are generated for each pharmacodynamic variable (y-axis) versus SPV change from baseline (x-axis). Summary graphs are generated with lorazepam and one other treatment per graph, for all GABAergic treatments.

A regression analysis of change from baseline of body sway (ΔSway), tracking (ΔTrack), VAS alertness ($\Delta VAS_{alertness}$), or VAS calmness ($\Delta VAS_{calmness}$) against the change from baseline of SPV (ΔSPV) was performed with a mixed effect model on the available individual data. The fixed factor was the GABAergic treatment and treatment by saccadic peak velocity, while the random factors were subject slope and intercept. The values of body sway were analyzed after log-transformation, while the other parameters were taken without transformation. The estimates of the slopes of the linear relations of these ΔSPV-relative effect profiles were compared between each dose of subtype-selective $GABA_A$ agonists and lorazepam. The estimates of slopes, their estimated difference, and the P values were tabulated. Thereafter, summary plots were generated, combined with the population regression line as calculated in the regression.

All statistical analyses were carried out with SAS for Windows v9.1.3 (SAS institute, inc., Cary, NC, USA).

Table 3: Use of pharmacodynamic tests in each study.

Study	CHDR99112	CHDR0102	CHDR0105	CHDR0614	CHDR0407
compound	TPA023	TPACMP2	SL65.1498	Alprazolam	Zolpidem
comparator	Lorazepam	Lorazepam	Lorazepam	NA	NA
SEM	Done	Done	Done	Done	Done
Sway	Done	Done	Done	Done	Done
VAS BL	Done	Done	Done	Done	Done
Smooth	ND	ND	Done	Done	Done
Track	ND	ND	ND	Done	Done

ND: not done; NA: not applicable; SEM: saccadic eye movement; Smooth: smooth pursuit; Sway: body sway; VAS BL: VAS Bond and Lader; Track: adaptive tracking.

Table 4: Results of the linear model for saccadic peak velocity change from baseline and log body sway change from baseline by treatment with treatment by SPV change from baseline as interaction.

Treatment	ΔSPV-relative relation	Item	Estimate of treatment	Estimate of lorazepam	P value
TPA023 1.5 mg	ΔSway-ΔSPV	Slope	−0.00048	−0.00305	<0.0001
		Intercept	−0.01316	0.1292	<0.0001
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.03312	0.126	0.0001
		Intercept	0.4551	−4.4739	0.0021
TPACMP2 0.75 mg	ΔSway-ΔSPV	Slope	−0.00027	−0.00305	<0.0001
		Intercept	0.03784	0.1292	0.0009
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.09884	0.126	0.2525
		Intercept	−1.4465	−4.4739	0.0397
SL65.1498 25 mg	ΔSway-ΔSPV	Slope	−0.00128	−0.00305	0.0003
		Intercept	0.0222	0.1292	<0.0001
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.04193	0.126	0.0009
		Intercept	0.2453	−4.4739	<0.0001
	ΔSmooth-ΔSPV	Slope	0.01554	0.1099	<0.0001
		Intercept	−1.4483	−6.2553	<0.0001
Alprazolam 1 mg	ΔSway-ΔSPV	Slope	−0.00204	−0.00305	0.0667
		Intercept	0.001788	0.1292	<0.0001
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.0734	0.126	0.0763
		Intercept	−0.628	−4.4739	0.0254
	ΔTrack-ΔSPV	Slope	0.0747	0.0572	0.1545
		Intercept	0.3023	−4.0742	<0.0001
	ΔSmooth-ΔSPV	Slope	0.08077	0.1099	0.2808
		Intercept	−1.4025	−6.2553	0.0002
Zolpidem 10 mg	ΔSway-ΔSPV	Slope	−0.0033	−0.00305	0.7336
		Intercept	0.06014	0.1292	0.0127
	$\Delta VAS_{alertness}$-ΔSPV	Slope	0.1526	0.126	0.5231
		Intercept	−3.2697	−4.4739	0.5219
	ΔTrack-ΔSPV	Slope	0.0489	0.0572	0.6240
		Intercept	−0.9123	−4.0742	<0.0001
	ΔSmooth-ΔSPV	Slope	0.09771	0.1099	0.7412
		Intercept	−3.8439	−6.2553	0.0815

3. Results

3.1. ΔSPV-ΔSway Relation (Δ = Change from Baseline). Average changes from baseline of body sway against SPV within the investigational time course (i.e., 6 hours after dose) were plotted by study. Figure 1 demonstrates clear distinctions between the ΔSPV-relative effect profile of lorazepam 2 mg and most doses of the $\alpha_{2,3}$-subtype selective compounds (i.e., TPA023 1.5 mg, TPACMP2 0.75 mg). The full $GABA_A$ agonist alprazolam is similar to lorazepam. The slope of the ΔSPV-ΔSway plots for zolpidem is slightly steeper than for lorazepam.

As was revealed by the statistical analysis using the mixed linear model (Table 4), the estimated differences

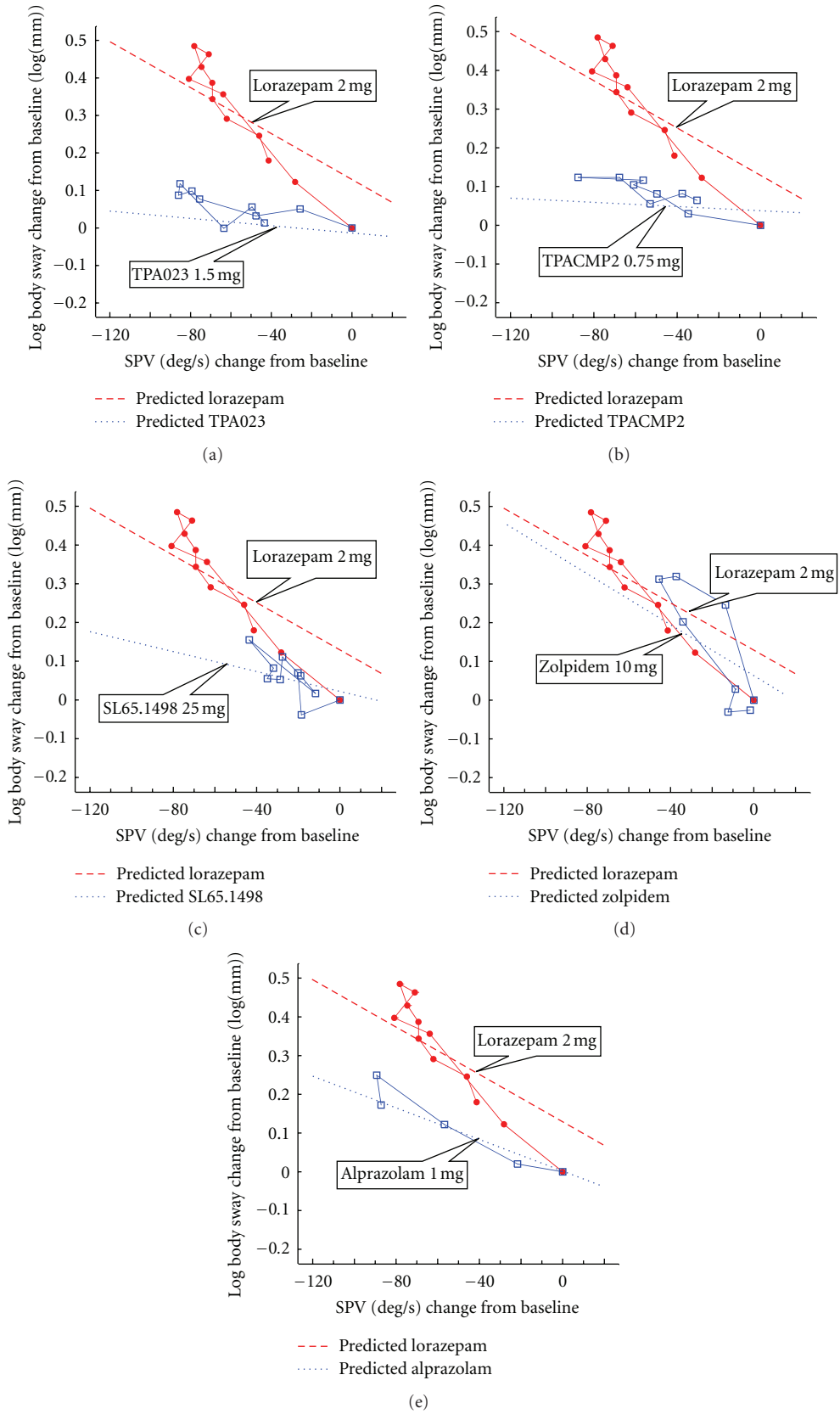

Figure 1: ΔLogSway (log mm)-ΔSPV (deg/sec) relative effect profile of TPA023 1.5 mg, TPACMP2 0.75 mg, SL65.1498 25 mg, zolpidem 10 mg, and alprazolam 1 mg versus lorazepam 2 mg, respectively. (Blue open square: investigational compound; red closed circle: lorazepam 2 mg; blue dot line: the comparator drug; red dash line: lorazepam 2 mg.)

of the slope of regression lines are statistically significant between lorazepam and the $\alpha_{2,3}$ subtype selective partial GABAergic treatment of TPA023 1.5 mg, TPACMP2 0.75 mg, and SL65.1498 25 mg. There is no statistically significant difference between the slopes for lorazepam and alprazolam, and the difference with zolpidem suggested by the average plots (Figure 1) is not confirmed by the model (Table 4).

3.2. ΔSPV-ΔVAS$_{alertness}$ Relation. Figure 2 plots the average values of $\Delta VAS_{alertness}$ versus ΔSPV obtained from individual subjects per study. As was found for the ΔSPV-ΔSway relations, a similar difference to lorazepam was observed with novel subtype selective GABAergic compounds. The slopes of the regression line of the ΔSPV-ΔSway relation for TPA023 1.5 mg and SL65.1498 25 mg are statistically shallower than the slope for lorazepam, respectively. No statistical differences can be demonstrated for TPACMP2 0.75 mg, alprazolam 1 mg, or zolpidem 10 mg.

3.3. ΔSPV-ΔSmooth Relation. Figure 3 and Table 4 provide the ΔSPV-relative effect profiles and the slopes and intercept for smooth pursuit after alprazolam, zolpidem, and SL65.1498. Smooth pursuit was not determined with the other partial agonists. Statistically significant differences are found in the slope of regression lines with SL65.2498 25 mg. Zolpidem and alprazolam show comparable slopes to lorazepam.

3.4. ΔSPV-ΔPD Relations versus In Vitro Pharmacological Properties. This analysis surmises that comparisons of ΔSPV-ΔPD profiles represent the underlying pharmacological characteristics of subtype selective and nonselective $GABA_A$ agonists. A further corroboration of this approach could be provided by a comparison of ΔSPV-ΔPD profiles with the underlying pharmacological properties. This should be possible in principle, but the quantitative preclinical information provided in Table 1 was derived from different sources which in themselves were incomparable, despite the fact that all programs used oocyte-clamp assays to characterize the different GABAergic compounds. Some of these differences could be diminished by calculation of the ratio of relative efficacy on the α_1 $GABA_A$ subunit to that on the α_2 subunit, as a benchmark of α_2-specificity of the GABAergic compounds. This calculated ratio is provided in Table 1. Although the number of compounds in this overview is too small for any meaningful statistical evaluation, it is interesting that the four compounds for which this could be calculated showed a close relationship between α_1/α_2-efficacy ratios and ΔSPV-ΔVAS alertness ratios with borderline statistical significance ($r^2 = 0.86$, two-sided $P = 0.0727$). Due to the absence of *in vitro* pharmacological data and the difference of experimental settings of the trail with alprazolam, alprazolam was not included into the present analysis.

4. Discussion

This analysis was performed to explore the central nervous system (CNS) effects of various GABAergic agents and characterize the pharmacodynamic effect profiles of these compounds in healthy volunteers and correlate such profiles to their pharmacological properties.

A battery of CNS pharmacodynamic tests was administered to healthy volunteers who were dosed with GABAergic compound(s). The composition of the CNS battery was based on the sensitivity of the measurements to nonselective GABAergic treatments, and on the coverage of a wide range of different CNS domains (Table 2). This approach enabled us to identify unique effect profiles for pharmacologically distinct GABAergic treatments, including (1) traditional, pharmacologically nonselective, full GABAergic compounds at their clinical dose(s) (i.e., lorazepam 2 mg and alprazolam 1 mg), (2) a marketed GABAergic compound with high α_1-subtype affinity (i.e., zolpidem 10 mg), and (3) several novel, $\alpha_{2,3}$-subtype selective GABAergic compounds at different investigational doses.

The new class of partial subtype selective GABA agonists was expected to be anxiolytic but less sedating and cognition impairing, as indicated by the preclinical *in vitro* and *in vivo* data. The anxiolytic effects of nonselective GABAergic agonists are accompanied by somnolence, impaired locomotion, and cognitive disturbance. These clinical side effects are reflected by the pharmacodynamics effects of lorazepam or alprazolam on $VAS_{alertness}$ (measure of subjective sedation), body sway (measure of postural instability), and adaptive tracking (measure of visuomotor coordination). Memory testing was not performed frequently and consistently enough to allow a comparative analysis among the different compounds. However, the original publication of the TPA023-study provides indications that the partial subtype selective GABA agonist has fewer cognitive effects than the partial subtype selective GABA agonist. In this study, lorazepam 2 mg showed clear memory reductions, which did not occur with a dose of TPA023 1.5 mg that caused comparable SPV reductions [12].

Saccadic peak velocity (SPV) has previously been shown to be closely related to the anxiolytic doses of benzodiazepines [11], and SPV was therefore used as a reference parameter. As expected, SPV showed significant responses to almost every GABAergic compound investigated in these six studies [12–14]. In contrast to lorazepam or alprazolam, which influenced each output parameter of the saccadic eye movement test (i.e., SPV, saccadic reaction time, and inaccuracy), the α_1-(zolpidem) or $\alpha_{2,3}$-subtype selective GABAergic compounds (TPA023, TPACMP2, SL65.1498) only affected SPV.

At their highest investigational dose, the effect size of TPA023 and TPACMP2 on SPV was comparable to the effects observed with lorazepam or alprazolam, whereas the effect of SL65.1498 was only marginally significant on SPV. In almost all these cases, the impact on other CNS effects was lower. This by itself is an indication of pharmacological selectivity, but a comparison based merely on overall or maximum effects could obscure some of the more subtle pharmacological differences (like the findings of SL65.1498 study) when the pharmacodynamic biomarker is less sensitive to the drug or if the dose of a drug

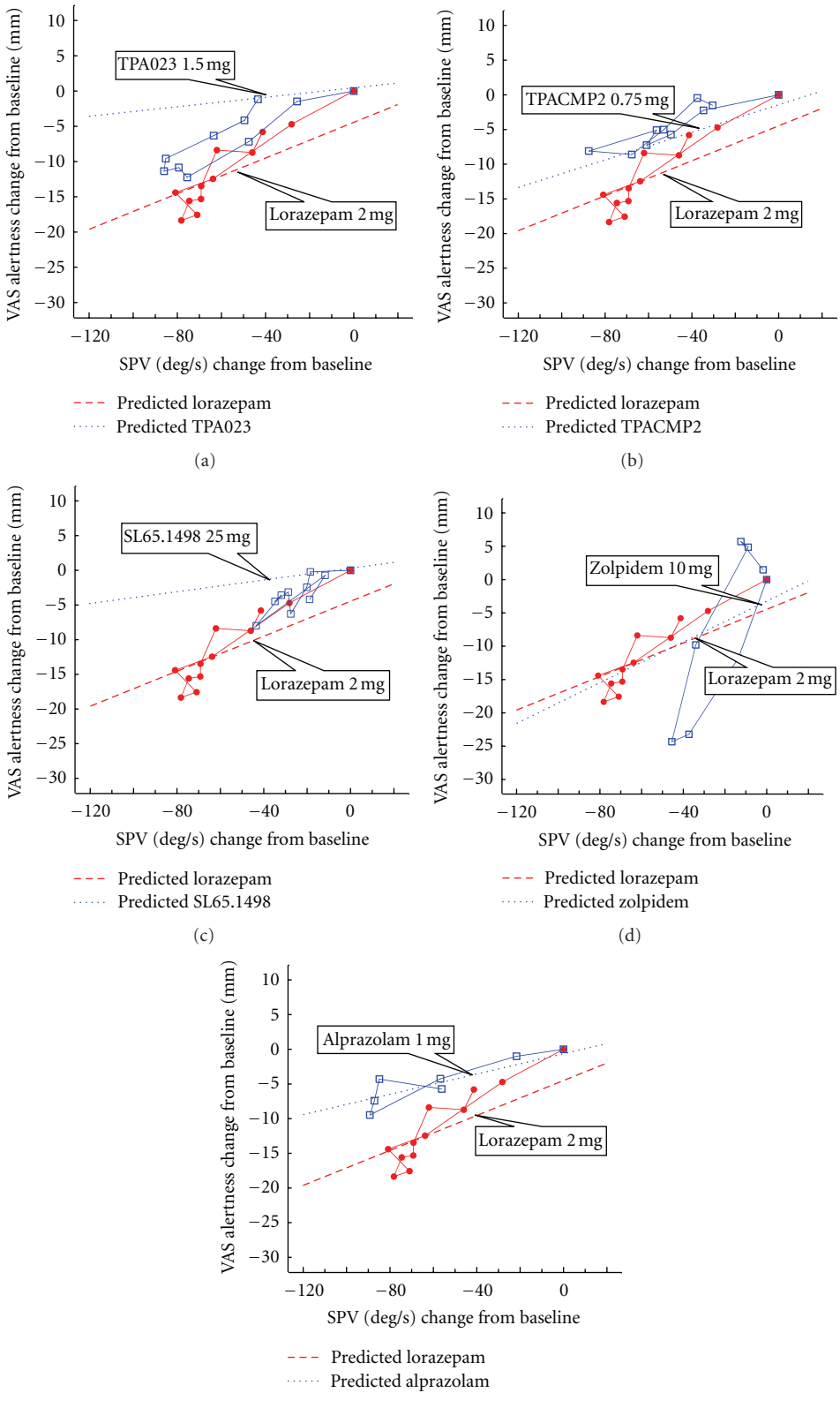

FIGURE 2: $\Delta VAS_{alertness}$-ΔSPV relative effect profile of TPA023 1.5 mg, TPACMP2 0.75 mg, SL65.1498 25 mg, zolpidem 10 mg, and alprazolam 1 mg versus lorazepam 2 mg, respectively. (Blue open square: investigational compound; red closed circle: lorazepam 2 mg; blue dot line: the comparator drug, red dash line; lorazepam 2 mg.)

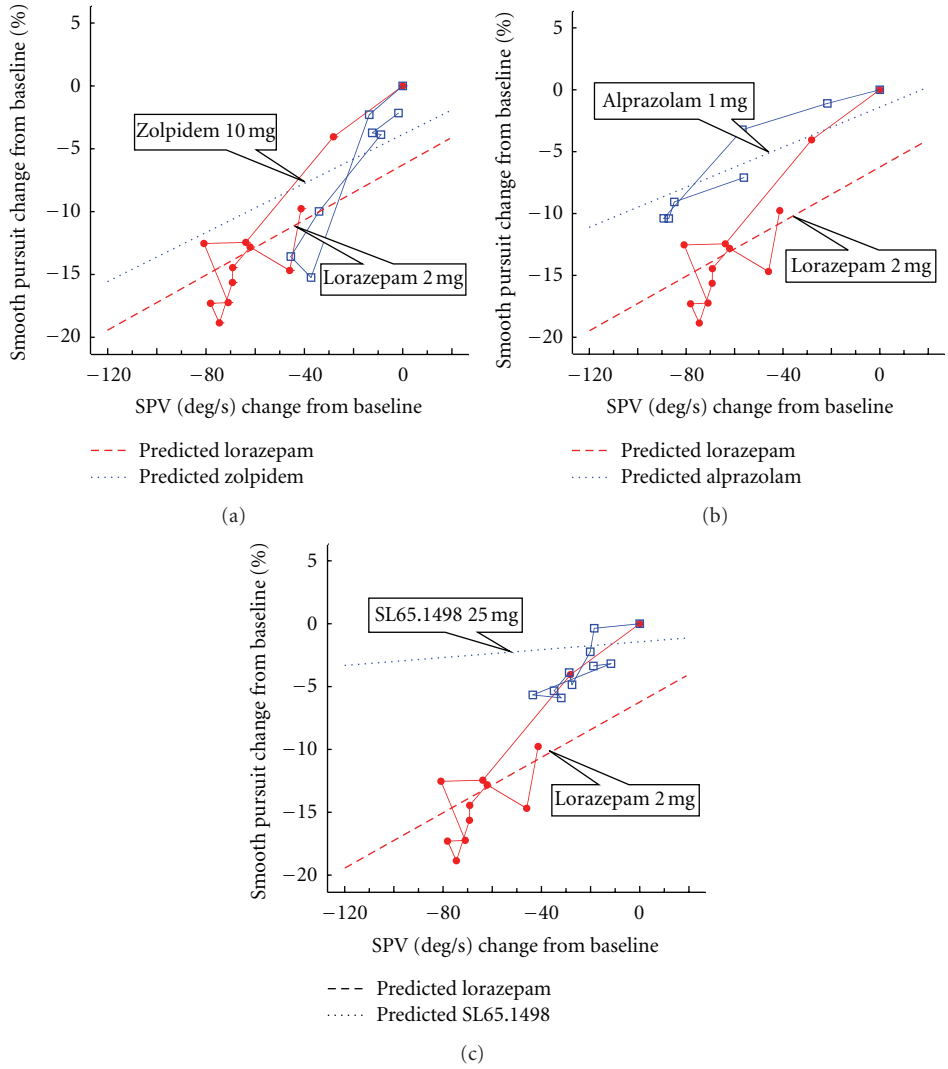

FIGURE 3: ΔSmooth-ΔSPV relative effect profile of SL65.1498 25 mg, zolpidem 10 mg, and alprazolam 1 mg versus lorazepam 2 mg, respectively. (Blue open square: investigational compound; red closed circle: lorazepam 2 mg; blue dot line: the comparator drug, red dash line; lorazepam 2 mg.)

is subtherapeutic. The relationships between the ΔSPV-effects and other pharmacodynamic (ΔPD) effects provide a complete profile of the differential effects, at each time point after drug administration. These outputs reflect the degree of $\alpha_{2,3}$ selectivity and may therefore also be indicators for anxioselectivity. Based on these perceptions, a GABAergic compound with "flat" regression lines in the ΔSPV-relative plotting graphs would show anxiolysis with reduced off-target effects in clinical settings. For most of the novel compounds described in this overview, there are no clinical reports of anxiolytic effects or improved tolerability. However, a recent article on TPA023, the oldest compound in this meta-analysis, reported reduced anxiety in a preliminary clinical trial at doses that were also used in our pharmacodynamic studies [4]. No detailed comparative information is available on the therapeutic window in these clinical trials.

We found that the ΔSPV-relative effect profiles of $\alpha_{2,3}$ subtype-specific GABAergic compounds are similar among each other but different from lorazepam 2 mg. The absolute slopes of the regression lines for the ΔSPV-ΔPD relations are generally lower with the selective $GABA_A$ agonists than with the benzodiazepines. The results of alprazolam were comparable to lorazepam, which provides additional confidence that the analyses reflect pharmacological differences as well as similarities. Zolpidem seemed to be the only major exception, since this α_1 subtype-selective GABAergic compound produced considerably steeper average slopes for certain ΔSPV-relative profiles than lorazepam or alprazolam, whereas the statistical population model did not reveal statistically significant differences between zolpidem and the benzodiazepines. This could reflect a limitation of the population model for ΔSPV-ΔPD relationships, which was chosen to be simple and unbiased, but necessarily had to ignore some rather complex individual response relationships. The analyses were based on linear slope estimates without a fixed intercept. In reality, however, all individual data points started at a fixed intercept (at $T = 0$, when ΔSPV and

ΔPD were both zero), and, in many cases, the ΔSPV-ΔPD relationships were not linear, and zolpidem even formed loops when the SPV effect displayed a different time course than the PD effect. In almost all other cases, however, the statistical analyses and the graphical representations of the average relationships provide accurate representations of the individual plots.

This meta-analysis indicates that comparisons of ΔSPV-ΔPD profiles are able to identify pharmacological differences between subtype selective and nonselective $GABA_A$ agonists. A comparison of ΔSPV-ΔPD profiles with the underlying pharmacological properties was refuted by the very small number of compounds for which this could be compared. Nonetheless, strong relationships (with an R-value of 0.93) between the α_1/α_2-ratios of the four compounds for which this could be determined and their ΔSPV-$\Delta VAS_{alertness}$ ratios. Clearly this remains to be confirmed with larger numbers of compounds. Still, the consistent ΔSPV-relative profiles of the selective GABAergic compounds suggest potential links between the preclinical profiles and the ΔSPV-relative pharmacodynamics profiles of these compounds. Moreover, TPACMP2 showed a distinct ΔSPV-$\Delta VAS_{alertness}$ relation but shared a similar ΔSPV-ΔSway relation with the other $\alpha_{2,3}$-subtype-selective GABAergic agonists. The relatively large amount of sedation with TPACMP2 could reflect the relatively high ratio of α_1/α_2-efficacy of TPMCMP2 compared to the other compounds. Similarly, the large efficacy of zolpidem is compatible with its steep ΔSPV-$\Delta VAS_{alertness}$ ratio and the strong hypnosedative effect of this z-hypnotic in the clinic.

5. Conclusion

TPA023, TPACMP2, and SL65.1498 are members of the novel experimental drug family of $\alpha_{2,3}$-subtype selective receptor agonists. *In vitro* pharmacological properties of these compounds indicate higher binding affinity and relative efficacy at the $\alpha_{2,3}$-subunits. *In vivo* preclinical studies with animal models translated such pharmacological properties into potential of anxiolysis and relatively reduced off-target effects in comparison with nonselective full GABAergic agonists like benzodiazepines.

The Neurocart battery is a collection of validated tests amenable to the effects of various CNS-acting drugs. Components of this battery were shown to be sensitive to different rapid-onset CNS effects of the benzodiazepines, in which reduction of saccadic peak velocity displays features of a GABAergic anxiolytic biomarker, whereas impairments of body sway, adaptive tracking, and memory are translated to effects that are less desirable for an anxiolytic drug. Most novel GABAergic compounds showed dose-dependent responses to saccadic peak velocity but did not affect the other CNS effects to the same extent, indicative of the pharmacoselectivity of these new compounds. Moreover, the ΔSPV-relative effect profiles provide information about dose potency and effect specificity. This battery is suitable to not only present the general depressive effects of benzodiazepines but also demonstrate the pharmacological selectivity and specificity of the novel GABAergic compounds. Comparative effect profiling as used in these studies can provide clear indications for the pharmacological selectivity and specificity of novel GABAergic compounds in healthy volunteers. This is a valuable approach for the early drug development of this new drug class, which will hopefully contribute novel anxiolytics with an improved therapeutic window to patients with anxiety disorders.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2011, Article ID 608912, 12 pages
doi:10.1155/2011/608912

Research Article

A Comparison of the α2/3/5 Selective Positive Allosteric Modulators L-838,417 and TPA023 in Preclinical Models of Inflammatory and Neuropathic Pain

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Received 15 March 2011; Accepted 28 July 2011

Academic Editor: John Atack

$GABA_A$ receptors containing α2/3 subunits are current targets in the battle to develop new pain medications, as they are expressed in the spinal cord where increasing inhibitory drive should result in analgesia. However, this approach is prone to a range of side effects including sedation, cognitive impairment, and abuse as a consequence of the widespread influence of GABA. The ability to make subtype selective low-efficacy benzodiazepine compounds, which potentiate the action of GABA at specific α subunits, has the potential to reduce this side effect profile. In this study, we have investigated the effects of the medium-efficacy positive allosteric modulator (PAM) L-838,417 and the low-efficacy PAM TPA023 in a number of preclinical inflammatory and neuropathic pain models. We conclude that either the higher level of efficacy at α2/3 or efficacy at α5 is required for compounds to have a significant analgesic effect in a range of models, and, therefore, although the side-effect profile of compounds can be reduced compared to typical benzodiazepines, it is unlikely that it can be completely eliminated.

1. Introduction

GABA and glycine are the main inhibitory neurotransmitters in the CNS. GABA mediates its effects through both $GABA_A$ receptors which are ligand-gated ion-channels and $GABA_B$ receptors which are GPCRs. $GABA_A$ receptors are heteropentameric, and the majority of those present in the CNS contain two α, two β, and a single γ subunit [1]. Benzodiazepines are allosteric ligands, that is, they exhibit no intrinsic activity of their own, but potentiate or inhibit the effects of GABA at receptors that contain either an α1, 2, 3, or 5 subunit [2]. GABA activation of $GABA_A$ receptors leads to the opening of their integrated chloride channels. Chloride influx inhibits transmitter release from primary afferent terminals and hyperpolarizes spinal cord neurones, decreasing the probability of firing.

Inhibitory neurotransmission, in the spinal cord, is of great importance in pain transmission, and enhancement of inhibition leads to analgesia. Clinically, Ziconotide, an N-type calcium channel blocker which inhibits neurotransmitter release in the spinal cord, was recently approved for severe chronic pain [3]. However, its use is severely limited by CNS side effects, so there is a need for better tolerated medications. It was only recently that $GABA_A$ receptors as targets for pain have gained some support from preclinical evidence, with the use of both point mutant diazepam insensitive $GABA_A$ mice and subtype selective compounds. In particular, positive modulation of GABA action at α2 and α3 $GABA_A$ containing receptors, in the spinal cord, results in pain relief [4]. This study looked at a combination of diazepam efficacy in point mutant mice and the efficacy of the α2/3/5 selective positive allosteric modulator (PAM) L-838,417 [5] in a rat chronic constriction injury (CCI) model to draw this conclusion. An additional study has added weight to the evidence, as NS11394 (which is also α2/3/5 selective [6]) is also analgesic in preclinical models of inflammatory and neuropathic pain [7].

Compounds, such as TPA023 [8], which has no $\alpha 1$ activity, low levels of $\alpha 2/3$ efficacy, and minimal activity at $\alpha 5$ subunits, have been shown to be anxiolytic, both preclinically and clinically [9]. However, so far all the preclinical pain studies published have used compounds with higher efficacy than TPA023. Studies using cognitive enhancing $\alpha 5$ specific NAMS suggest that PAM activity at $\alpha 5$ may be impair cognition [10]; therefore, avoidance of this activity would be an advantage in an analgesic. Furthermore, although $\alpha 1$ is likely the primary mediator of the addictive properties of benzodiazepines [11], decreasing the level of $\alpha 2/3$ activity may reduce the abuse potential of the compound [12]. We were therefore interested in whether a compound showing a lower level of $\alpha 2/3$ efficacy and minimal activity at $\alpha 1/5$, with little indication of acute clinical side effects [13] would have efficacy in preclinical pain models. For that reason, in this study, we have compared and contrasted the analgesic profile of L-838,417 ($\alpha 1$ 2%, $\alpha 2$ 43%, $\alpha 3$ 43%, $\alpha 5$ 39% compared to chlordiazepoxide [5]) and TPA023 ($\alpha 1$ 0%, $\alpha 2$ 11%, $\alpha 3$ 21%, $\alpha 5$ 5% compared to chlordiazepoxide [8]) in a wide range of preclinical neuropathic and inflammatory pain models. In addition, we have measured changes in qEEG beta frequency as a potential biomarker of *in vivo* pharmacology. We find that the lower-efficacy compound TPA023 does not exhibit a broad analgesic profile across the spectrum of preclinical pain models and that this corresponds to nonsignificant changes in the qEEG beta frequency. However, the higher-efficacy compound L-838,417 significantly reverses allodynia in the majority of the pain models tested and significantly increases qEEG beta frequency.

2. Materials and Methods

2.1. Animals. All experiments were conducted in accordance with the United Kingdom (UK) Home Office Animals (Scientific Procedures) Act (1986) and were subject to local ethical review. Experiments were performed using male Sprague Dawley rats in the light period of a twelve-hour light/dark cycle. Animals were acclimatised to the facility for at least five days prior to commencing studies, were group-housed, unless otherwise stated, and had access to food and water *ad libitum*. All surgical procedures were conducted in aseptic conditions.

2.2. Receptor Occupancy (RO) Studies. RO was determined using a separate cohort of animals, and these values correlated to pharmacokinetic (PK) data obtained in the studies described below. In RO studies, rats received either vehicle control, L-838,417, or TPA023 (0.3, 1, or 10 mg/kg) *p.o.* Nonspecific binding was determined in a separate group of animals by administering 5 mg/kg bretazenil *i.p.*, with a thirty-minute pretreatment time. At three mins prior to the cull, all rats were dosed *i.v.* with 10 μCi/kg [^{3}H] Ro 15-1788 (flumazenil). Following euthanasia, the whole brain was removed and homogenised in 10 vol of ice-cold buffer (10 mM potassium phosphate/100 mM potassium chloride buffer, pH 7.4, 4°C) using a polytron homogeniser (setting 6 for 10 s). Three 300 μL aliquots of homogenate were filtered over 0.5% v/v polyethyleneimine-(PEI-) soaked (Sigma, Poole, UK) GF/B filters (Whatman, Maidstone, Kent) to separate the bound radioactivity from the free radioactivity [14] and washed twice in 5 mL ice-cold buffer. Filters were then placed in vials, scintillation fluid added and radioactivity counted using a 3100TR TriCarb beta liquid scintillation counter (Perkin-Elmer, Cambridge, UK). Plasma samples were also collected for PK analysis. The receptor occupancy values of L-838,417 and TPA023 were determined by calculating the reduction in specific binding in drug-treated rats relative to vehicle controls. Typically, vehicle levels of radioactivity were around 2000 dpm, and non-specific (bretazenil treated) levels were around 50 dpm.

2.3. Behavioural Studies. All behavioural studies were conducted in a double-blind fashion. Animals were allocated to treatment groups according to their baseline scores, in order to balance groups. Plasma samples were taken in all studies for PK analysis and to extrapolate brain RO levels.

2.4. Complete Freund's Adjuvant-(CFA-) Induced Thermal Hyperalgesia. Following training to the testing procedure, rats (150–200 g, Charles River, UK) received an intraplantar injection of 100 μg (in 100 μL) CFA suspension (Sigma, Poole, UK) to the right hind paw. Behavioural studies were conducted twenty four hours later. Following acclimatisation to the testing chamber, a mobile infrared heat source (Ugo Basile, Italy) was applied directly below the plantar surface of the contralateral hind paw (for a maximum of 20 s) and paw withdrawal latency time (PWL, s) measured, using a modified method of Hargreaves et al. [15]. Three separate readings were taken and an average value calculated. This procedure was then repeated on the ipsilateral hind paw. Animals were considered to be hyperalgesic if the ipsilateral PWL value was 5 s or less. Animals were then allocated to treatment groups and received either vehicle, test compound or 100 mg/kg ibuprofen *p.o.* PWL was assessed again at one hour postdose.

2.5. Tibial Nerve Transection-(TNT-) Induced Static Allodynia. Tibial nerve transection was conducted using the methods previously described by Lee et al. [16]. Rats (175–200 g, Charles River, UK) were anaesthetised via an induction chamber using 2% isoflurane (Abbott, Maidenhead, UK) in oxygen. Once anaesthetised, animals were transferred to a nose cone and a homeothermic blanket system for surgery (Harvard Apparatus, Edenbridge, UK). The right common sciatic nerve was exposed via blunt dissection through the biceps femoris. The tibial nerve was tightly ligated using two ligatures placed 3 mm apart, 5 mm distal to the sciatic trifurcation. The tibial nerve was then cut and laid back in its original position. The incision was closed, and animals recovered in heated boxes before being returned to their homecages. Two weeks postsurgery, animals were habituated to test arenas and von Frey filaments (Stoelting, Wood Dale, USA) over a three-day training period. Following this training period, static allodynia was assessed using the up-down method described by Chaplan et al. [17]. In brief,

von Frey filaments ranging from 0.4 to 15.0 g were applied to the plantar surface of the ipsilateral hind paw, starting with a 2.0 g filament. Filaments were then presented in an ascending or descending pattern, depending on the animal's responses, according to published methods. Each von Frey filament was applied until a withdrawal response was obtained, up to a maximum of six seconds. This was repeated on the contralateral hind paw. Animals were considered to be allodynic if the ipsilateral 50% paw withdrawal threshold (50% PWT) value calculated using this paradigm was 4.0 g or less. Animals were then allocated to treatment groups and received either vehicle, test compound or pregablin 20 mg/kg *p.o.* Static allodynia was assessed again at one hour postdose. Studies were conducted four to eight weeks postsurgery.

2.6. Chronic Constriction Injury-(CCI-) Induced Static Allodynia. Rats (175–200 g Charles River, UK) were anaesthetised and the sciatic nerve exposed as described above. CCI surgery was conducted as previously described by Bennett and Xie [18]. In brief, proximal to the sciatic trifurcation, approximately 7 mm of nerve was freed from surrounding tissue via blunt dissection and four loose ligations applied (4–0 silk), each approximately 1 mm apart. The incision was closed, and animals recovered in heated boxes before being returned to their homecages. Two weeks postsurgery, animals were habituated to test arenas and von Frey filaments as described above. Following this training period, static allodynia was assessed using the up-down method described above. Animals were considered to be allodynic if the ipsilateral 50% PWT value was 4.0 g or less. Animals were then allocated to treatment groups and received either vehicle, test compound, or pregablin 20 mg/kg *p.o.* Static allodynia was assessed again at one hour postdose. Studies were conducted at two to four weeks postsurgery.

2.7. Spinal Nerve Ligation-(SNL-) Induced Static Allodynia (Performed at Aptuit, Edinburgh, UK). Rats (200–300 g, Harlan, UK) were anaesthetised using isoflurane in oxygen. The left L_6 transverse process was removed and the L_5 and L_6 spinal nerves tightly ligated (6–0 silk) [19]. The incision was closed, and animals recovered before being returned to their homecages. Animals were acclimatised to the testing procedure prior to SNL surgery and retested twice after surgery as part of the training procedure. Following this training period, static allodynia was assessed two to three weeks postsurgery by applying a range of von Frey filaments from 2.0 to 26.0 g in ascending order. Each filament was applied 8–10 times at a frequency of 1 Hz. Both the contralateral and ipsilateral hind paws were assessed. Animals were considered to be allodynic if the ipsilateral paw withdrawal threshold (PWT) was 5.0 g or less. Animals were then allocated to treatment groups and received either vehicle, test compound, or pregablin 50 mg/kg *p.o.* Static allodynia was assessed again at one hour postdose.

2.8. Electrophysiology Studies in CCI Animals. Following CCI surgery and subsequent assessment of static allodynia, a cohort of animals were used for *in vivo* electrophysiology studies (2.5–4.5 weeks postsurgery). Animals were anaesthetised as described above. Surgery was conducted under 2.5–3.5% isoflurane. The jugular vein and carotid artery were cannulated and laminectomy performed in the lumbar enlargement region. The spinal cord was exposed, the dura removed, and the cord covered in mineral oil (Sigma, Poole, UK) at 37°C throughout the remainder of the study. Following surgery, isoflurane was decreased to 1.8–2.5% and blood pressure monitored via the carotid artery cannula. Extracellular, single-unit recordings were made using 5 MΩ fine tungsten electrodes (A-M Systems Inc., Sequim, USA) from wide dynamic range (WDR) neurones with a receptive field on the plantar surface of the ipsilateral hind paw. Neurones were characterised by intensity-dependent firing to a range of cutaneous stimuli. Action potentials were preamplified (Neurolog NL100AK headstage), amplified (Neurolog NL104A), and filtered (Neurolog NL125) (Digitimer, Welwyn Garden City, UK), and recordings digitized using a Power 1401 (CED, Cambridge, UK). Data were recorded and analysed using Spike 2 (CED, Cambridge, UK). Once identified, a noxious mechanical pinch stimulus was applied to the centre of the cell's receptive field, via an 8 cm Glover bulldog clamp (503236, WPI, Stevenage, UK). This stimulus was applied for five seconds at 10 min intervals and evoked responses recorded. The number of evoked potentials in 1–5 seconds of each stimulus application was calculated. Once stability of response was achieved, stimuli were continued in the presence of either L-838,417 or vehicle. Solutions were infused via the jugular vein at 4 mL/kg/hr over thirty mins. Blood samples were taken from the carotid artery for PK analysis. Studies were conducted in a randomised fashion.

2.9. Taqman Analysis of Tissue from TNT Animals. Fourteen days after surgery (and following confirmation of the development of static allodynia), a cohort of TNT-injured animals was used for analysis of KCC2 mRNA levels in the dorsal horn of the ipsilateral spinal cord. Comparative tissues were also taken from the contralateral side and from a group of sham-operated animals. Tissues were lysed using RLT buffer before extracting and purifying RNA using an RNAeasy microkit (Qiagen, Crawley, UK). The RNA quantity (A260) and purity (260/280 ratio) were assessed using spectrophotometry, and the integrity was checked using an Agilent 2100 Bioanalyser (Agilent, Winnersh, UK). Following this, a two-step amplification process was carried out, before conducting Taqman analysis.

2.10. Electroencephalogram (EEG) Studies. Rats (250 g, Charles River, UK) were anaesthetised using isoflurane anaesthesia as described above and implanted intraperitoneally with radio telemetric transmitters (TL11M2 F40-EET, Data Sciences International, St. Paul, Minn, USA) and with cortical EEG electrodes (stainless steel screw electrodes). These were implanted epidurally over the left parietal cortex (2.0 mm anterior and 2.0 mm lateral to lambda) and over the left frontal cortex (2.0 mm anterior and 2.0 mm lateral to bregma) for a frontal-parietal EEG recording [20].

The cortical electrodes and accompanying leads were secured to the skull by covering with dental acrylic. Animals recovered in heated boxes before being returned to their homecages (from this point animals were single-housed). EEG studies were conducted a minimum of two weeks after surgery. At the beginning of the light phase, animals received either L-838,417, TPA023, or vehicle control *p.o.* in a four-way cross-over design, so that all animals received all of the treatments, thus enabling within animal comparisons. EEG data were then immediately recorded, sampling continuously at 500 Hz for four hours with Data Sciences International hardware and Data Acquisition Gold version 3.01 software (Data Sciences International, St. Paul, Minn, USA). Data were analysed using Spike 6 (CED, Cambridge, UK). For the EEG analysis, consecutive 12-s epochs were subjected to a Fast Fourier Transform and the EEG power density within four frequency bands (δ 1–4 Hz; θ 6–9 Hz; α 8–13 Hz; β 13–40 Hz; γ 40–80 Hz) was calculated. Spectral analysis was performed on raw data files, which were sampled as for sleep data (512 Hz, Hanning window). Epochs containing artefacts were excluded from analysis, but otherwise, data were integrated for each frequency band, as defined above, and mean values were computed for each.

2.11. Quantification of the Plasma Concentrations of L-838,417 and TPA023. Quantification of L-838,417 and TPA023 in plasma was carried out using liquid chromatography-mass spectrometry (LC-MS) over a number of occasions. A typical system consisted of a binary pump (Agilent 1100 series), autoinjector (CTC PAL), and API4000 triple quadrapole mass spectrometer (Sciex). Typical HPLC conditions used a Monolith C18 column with a binary solvent system consisting of solvent mix (A) 0.027% v/v formic acid and 10 mM ammonium formate in 90 : 10 water : methanol and solvent mix (B) 0.027% v/v formic acid and 10 mM ammonium formate in 90 : 10 methanol : water. The flow rate was 1200 uL/min with the following gradient system: 0-0.1 min 0% B, increasing to 100% B at 0.45 min and holding until 2 min, returning to 0% B at 2.1 min and holding until 2.5 min. Flow was diverted to waste for the first min and after 2.4 min of each injection. The analytes were extracted from a 50 μL plasma sample following the addition of 10 μL of 1 μg/mL internal standard solution (the two compounds were used as internal standards for each other), 300 μL of pH 10 borate buffer, and 1000 μL of methyl t-butyl ether (MTBE) before vortex mixing. Samples were then centrifuged at 13,000 rpm for 15 min at 4°C, before transfer of 800 μL aliquots of the MTBE layer to a fresh 96-well plate which were then evaporated to dryness under N_2 at 40°C. The samples were then reconstituted with 100 μL of the mobile phase B, and up to 45 μL injections were made on to the LC-MS system described. The compounds were monitored using selective reaction monitoring with Q1/Q3 transitions of 400.0/96.0 and 396.0/110.0 for L-838,417 and TPA023, respectively. The retention times of L-838,417 and TPA023 were 1.8 and 1.9 min, respectively. The concentration range of the standard curves was typically 0.5–1000 ng/mL and linear regression equations of the standard curve required correlation coefficient of >0.97 for acceptance.

2.12. Drugs. For RO, behavioural and EEG studies L-838,417 (L-838) and TPA023 (Pfizer, Sandwich, UK) and pregablin (Parke-Davis, Cambridge, UK) were formulated as a suspension in 0.5% methyl cellulose (Sigma, Poole, UK) vehicle. Ibuprofen (Sigma, Poole, UK) was dissolved in saline. For electrophysiology studies, L-838,417 was formulated as a solution in 18% glycerol formal (Sigma, Poole, UK), 17% solutol HS (BASF, Germany) and 65% saline vehicle. For RO studies, bretazenil was formulated as a solution in 70% polyethylene glycol (PEG) 300 (Sigma, Poole, UK), 30% saline vehicle.

2.13. Statistical Analysis. Data are expressed as means ± sem, unless stated. Statistical analysis of behavioural data and KCC2 mRNA levels was conducted using a One-Way Analysis of Variance test, with the exception of SNL data where a nonparametric Mann-Whitney test was used. Statistical analysis of electrophysiology data was conducted using a two-sided *t*-test. EEG data were analysed using a Restricted Maximum Likelihood (REML) analysis, followed by Fisher's post hoc analysis. In each case, treatment groups were compared to time-matched vehicle control groups.

3. Results

The aim of this study was to determine whether $GABA_A$ α2/3 selective, positive allosteric modulators with varying efficacies *in vitro* would affect changes in *in vivo*, in preclinical pain models. In order to fully interpret and compare the data generated with the two compounds used, we first determined the brain $GABA_A$ receptor occupancy of both L-838,417 and TPA023 and correlated this to nonprotein bound plasma drug levels (Figure 1). In terms of Occ50 values, TPA023 was approximately 0.3 mg/kg and L-838,417 was approximately 1 mg/kg, these data are very similar to those published by Merck [21, 22]. However, as equivalent doses did not always result in equivalent plasma exposures, in subsequent studies plasma samples were always taken for PK analysis and drug levels correlated to brain $GABA_A$ receptor RO values determined from the results described above.

In terms of effects in preclinical pain models, we first examined the effects of these two modulators in a CFA-induced model of inflammatory pain. In this model, L-838,417 significantly increased PWL at 1 mg/kg (7.8 ± 1.2 s, $P < 0.01$) and 10 mg/kg (8.5 ± 0.6 s, $P < 0.01$), but was not efficacious at 0.3 mg/kg *p.o.*, when compared to vehicle control (4.9 ± 0.4 s) (Figure 2(a)). Free plasma drug levels at these doses corresponded to 64, 90 and 44% brain $GABA_A$ RO, respectively. These data suggested a link between brain RO and *in vivo* efficacy in this inflammatory pain model. In the same model, no statistically significant effect was observed with TPA023 at doses up to 10 mg/kg *p.o.* (Figure 2(b)), corresponding to 98% brain RO. Therefore, it appeared that *in vitro* efficacy, in addition to *in vivo* RO,

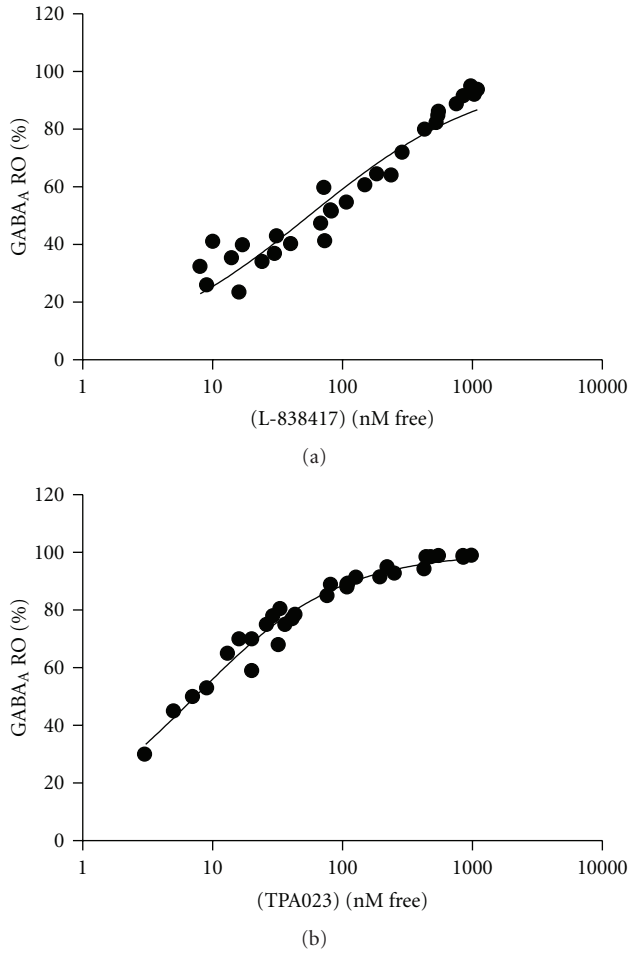

Figure 1: GABA$_A$ receptor occupancy of (a) L-838,417 and (b) TPA023 in whole rat brain compared to drug concentrations in the plasma (nM free). Each datapoint represents an individual animal.

was an important factor in determining the *in vivo* efficacy of GABA$_A$ modulators in pain models.

These compounds were then assessed in a model of neuropathic pain. Surprisingly, in contrast to the results obtained in the above inflammatory model, no significant effect was observed with either compound in the TNT neuropathic model of static allodynia (Figure 3), where the top doses correspond to 97% RO for TPA023 and 100% RO for L-838,417. This was also in contrast to data previously published by other authors in the CCI model of neuropathic pain [4].

In order to further investigate this discrepancy between our data and that reported by others, both compounds were then tested in a CCI model. L-838,417 significantly increased 50% PWT in this model at 10 mg/kg *p.o.* (9.0 ± 1.2 s, $P < 0.05$) compared to vehicle (3.6 ± 0.9 s) (Figure 4(a)). This dose corresponded to 94% RO. A trend for an increase in 50% PWT was also observed at 30 mg/kg *p.o.* Although this effect was not statistically significant, this value was not significantly different from that obtained at 10 mg/kg ($P = 0.074$). TPA023 also significantly increased 50% PWT in CCI animals at 1 mg/kg *p.o.* (9.1 ± 1.8 s, $P < 0.05$) with respect to vehicle control (3.6 ± 0.7 s), but this was not observed at any other dose (Figure 4(b)). This dose corresponded to 98% RO.

These apparent differences in the effects of GABA$_A$ allosteric modulators in models of neuropathic pain were studied further by testing both compounds in an SNL model. L-838,417 significantly increased PWT in SNL animals at 10 and 30 mg/kg *p.o.* (14.4 ± 2.6 g and 12.9 ± 3.1 g, resp., $P < 0.05$ relative to vehicle control 4.1 ± 2.3 g) (Figure 5(a)). These doses corresponded to 60 and 100% RO, respectively. TPA023 was also efficacious in this model of static allodynia at 10 mg/kg *p.o.* (9.9 ± 1.8 g, $P < 0.05$) versus vehicle control (4.9 ± 2.4 g) (Figure 5(b)). This dose corresponded to 100% RO. These results suggested that when a compound with lower *in vitro* efficacy is able to exert an effect in *in vivo* pain models, it may be necessary to achieve higher brain RO levels than with a higher efficacy compound.

Following initial behavioural assessment, L-838,417 was also tested in CCI-injured animals in an *in vivo* electrophysiology study. The aim of this study was to determine whether GABA$_A$ receptor modulation was affecting spinal nerve firing or reflex behaviours. Pinch-evoked firing of WDR neurones in CCI-injured animals was significantly decreased thirty mins after beginning *i.v.* administration of L-838,417 (58.0 ± 8.4% preinfusion number of action potentials, $P < 0.05$), relative to vehicle control (88.0 ± 7.1% preinfusion number of action potentials) (Figure 6). Drug levels at this timepoint corresponded to 86% RO. Due to the considerable number of animals utilised in these studies, we did not test TPA023 due to the weaker effect seen in the CCI behavioural study.

Other groups have reported that GABA activity can be excitatory in certain conditions due to decreased levels of the potassium chloride cotransporter KCC2 [23]. In our studies, the only experimental model in which no efficacy was observed with either GABA$_A$ modulator was the TNT model of neuropathic pain. We therefore examined KCC2 mRNA levels in this model using Taqman analysis. We found no significant differences in dorsal horn KCC2 levels in sham, ipsilateral TNT, and contralateral TNT tissues (Figure 7). As we observed a significant effect of GABA$_A$ PAMS in the CCI and SNL model, we did not investigate any potential changes in KCC2 levels in tissues from these models.

Finally, we studied the effects of L-838,417 and TPA023 in a quantitative EEG model, to assess the functional activation in a translatable pharmacology biomarker assay. L-838,417 dose-dependently increased the power in the beta frequency relative to vehicle control over a four-hour period (Figure 8). There appeared to be a trend towards an increase in this value with TPA023, but this was not statistically significant. PK samples could not be taken during these studies for technical reasons, so we were able to correlate these data exactly with RO. However, the three doses of each compound were targeting 50, 75 and >100% RO, respectively, and, based on experience, we are confident that we achieved 100% RO at the highest dose.

4. Discussion

In this paper, we have investigated the level of α2/3/5 efficacy required for GABA$_A$ PAMS to exhibit efficacy in

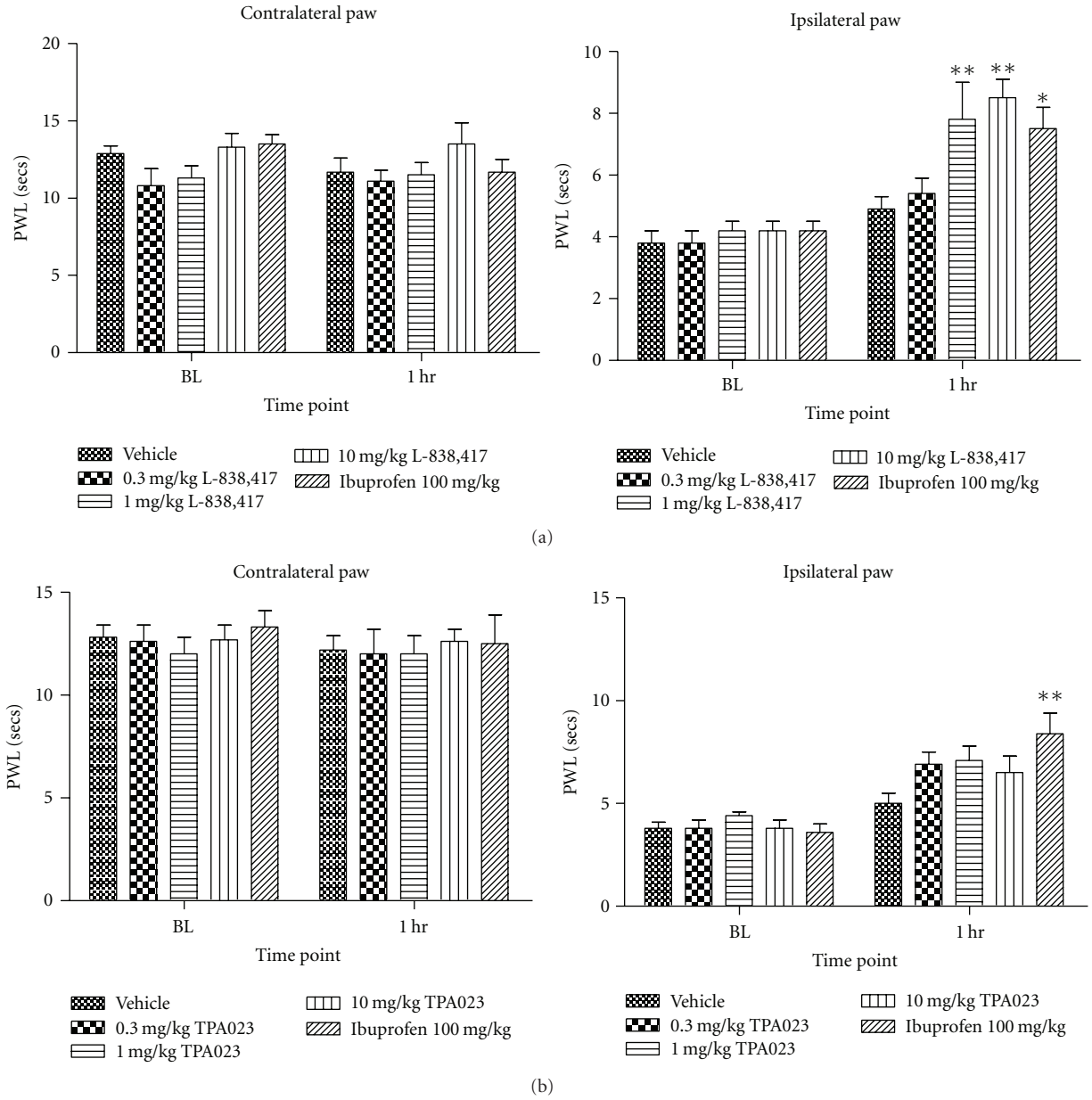

Figure 2: The effects of (a) L-838,417 and (b) TPA023 on complete Freund's adjuvant-induced thermal hyperalgesia in the rat. Data are means ± sem. n = 7-8. Data are expressed as paw withdrawal latency (PWL). BL: baseline. ($* = P < 0.05$, $** = P < 0.01$, ANOVA compared to vehicle-treated group).

preclinical pain models. We have shown that L-838,417, which exhibits moderate $\alpha 2/3/5$ efficacy, exhibits a significant effect in the majority (4/5) of preclinical pain models in which it was tested. Conversely, a lower-efficacy $\alpha 2/3$ compound, TPA023, with minimal $\alpha 5$ activity, only exhibited a significant analgesic effect in two out of the four preclinical pain models investigated.

Furthermore, we have revealed that, even considering that we reached ~100% RO in all studies, the effect of the same compound across similar preclinical neuropathic pain models differs considerably. L-838,417 was able to reverse the deficit in paw withdrawal induced by either CCI or SNL, but not TNT surgery, despite behavioural testing at similar timepoints after surgery across the different models. It has been proposed that changes in chloride homeostasis, due to decreases in expression of the potassium chloride co-transporter KCC2, may cause GABA transmission to become excitatory rather than inhibitory [23, 24]. This has led to much debate, as to whether $GABA_A$ PAMS will be of utility in the treatment of neuropathic pain. Due to the lack of effect of L-838,417 in our TNT model, we did investigate whether there were any changes in KCC2 expression 14 days after surgery (maximal allodynia), we found no changes in either mRNA or protein expression (data not shown) in either the ipsilateral or contralateral dorsal horn. It is possible that a deficit had normalised by this time point, as observed by

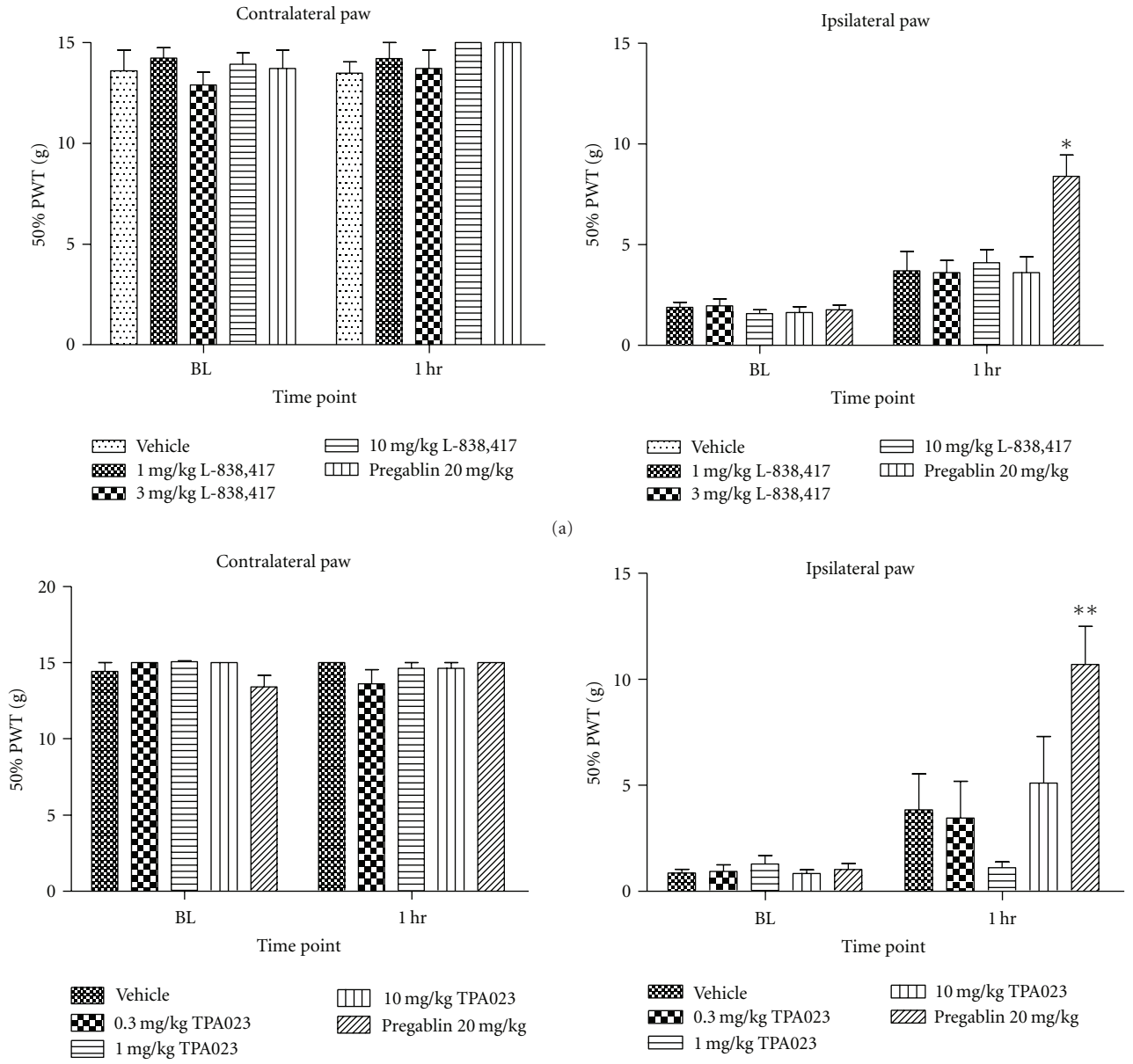

Figure 3: The effects of (a) L-838,417 and (b) TPA023 on tibial nerve transection-induced static allodynia in the rat. Data are means ± sem. $n = 22$. Data are expressed as 50% paw withdrawal threshold (PWT). BL: baseline. ($** = P < 0.01$, ANOVA compared to vehicle-treated group).

G. Miletic and V. Miletic [25], but in this case, any change in chloride homeostasis should have also normalised, so we conclude that a change in KCC2 expression was not responsible for the lack of $GABA_A$ PAM efficacy observed in the TNT model. Additionally, in human clinical studies, midazolam has been shown to have significant analgesic properties when given i.t in posthepatic neuralgia patients [26], suggesting that in the clinical neuropathic population, there is unlikely to be a reversal in chloride homeostasis. We do not have a conclusive explanation for the different effect of $GABA_A$ PAMS across the neuropathic models, as differences between them are not well understood. The CCI model is reported to be more sensitive to mechanical stimuli than the SNL model [27], but there has not been a comprehensive study comparing which models respond best to which pharmacological approaches. It could be the case that in the TNT model, that there is a lack of inhibitory drive, and, therefore, there is no benefit in enhancing it. Often with comparing data between laboratories, it may be that slight differences in surgery, time-point after surgery tested, or even the genetic background of the animals used makes more difference to outcome, than which model is used. In our opinion, it is therefore difficult to interpret results from preclinical neuropathic models when assessing novel targets.

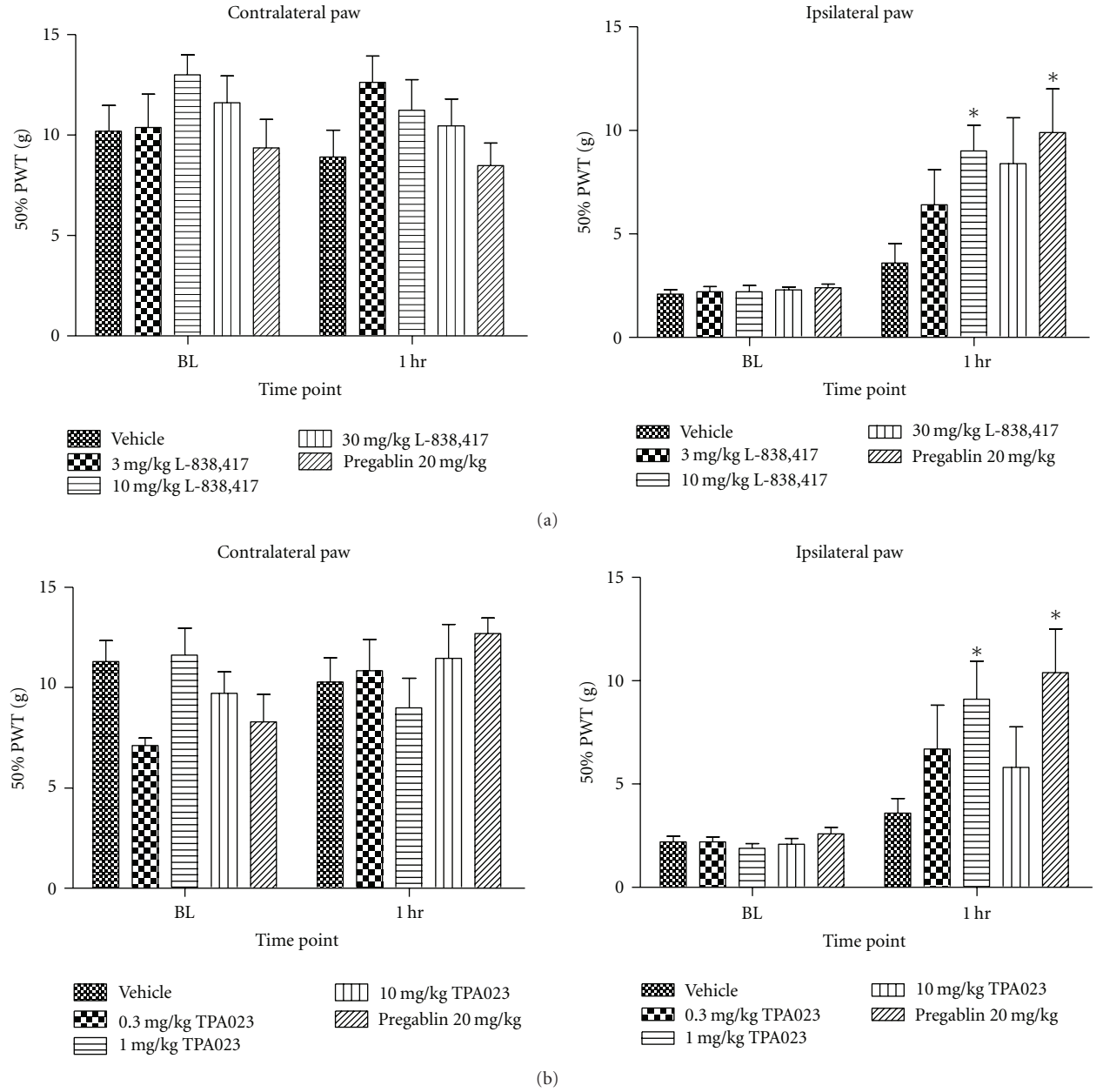

Figure 4: The effects of (a) L-838,417and (b) TPA023 on chronic constriction injury-induced static allodynia in the rat. Data are means ± sem. $n = 6$. Data are expressed as 50% paw withdrawal threshold (PWT). BL: baseline. ($* = P < 0.05$, ANOVA compared to vehicle-treated group).

Additionally, it is not believed that preclinical pain models accurately represent human clinical pain [28], so their value when used alone is limited.

As well as the above issues, in behavioural models, it can be difficult to separate out side effects from a true analgesic response. In particular, sedation can lead to a decrease in paw-withdrawal latency in evoked endpoints in preclinical models (internal data). Although data is always collected on the contralateral paw to try and gather globalised behavioural changes data and minimise any misinterpretation of the ipsilateral data, because of the difference in baseline between the two paws, it is possible that there may still be some side effect interference. Indeed, reviewing the published data on L-838,417, we consider that, although no statistics are given, it appears that there is a deficit in the dark phase of the fear-potentiated startle test [5]. Furthermore, we have also observed that zolpidem-trained rats generalise slightly when given L-838,417 (data not shown). Both of these observations suggest that L-838,417 may not completely lack *in vivo* activity at the α1subunit. In addition, although rotorod performance is less impaired by diazepam in the α1 KI mice, there is still a deficit in performance at high doses [5], pointing towards a motor-impairment effect, possibly muscle relaxation, mediated through one of the other α subunits. Additionally, TPA023B which has a similar *in vitro* profile to L-838,417 reportedly causes effects such as flaccid

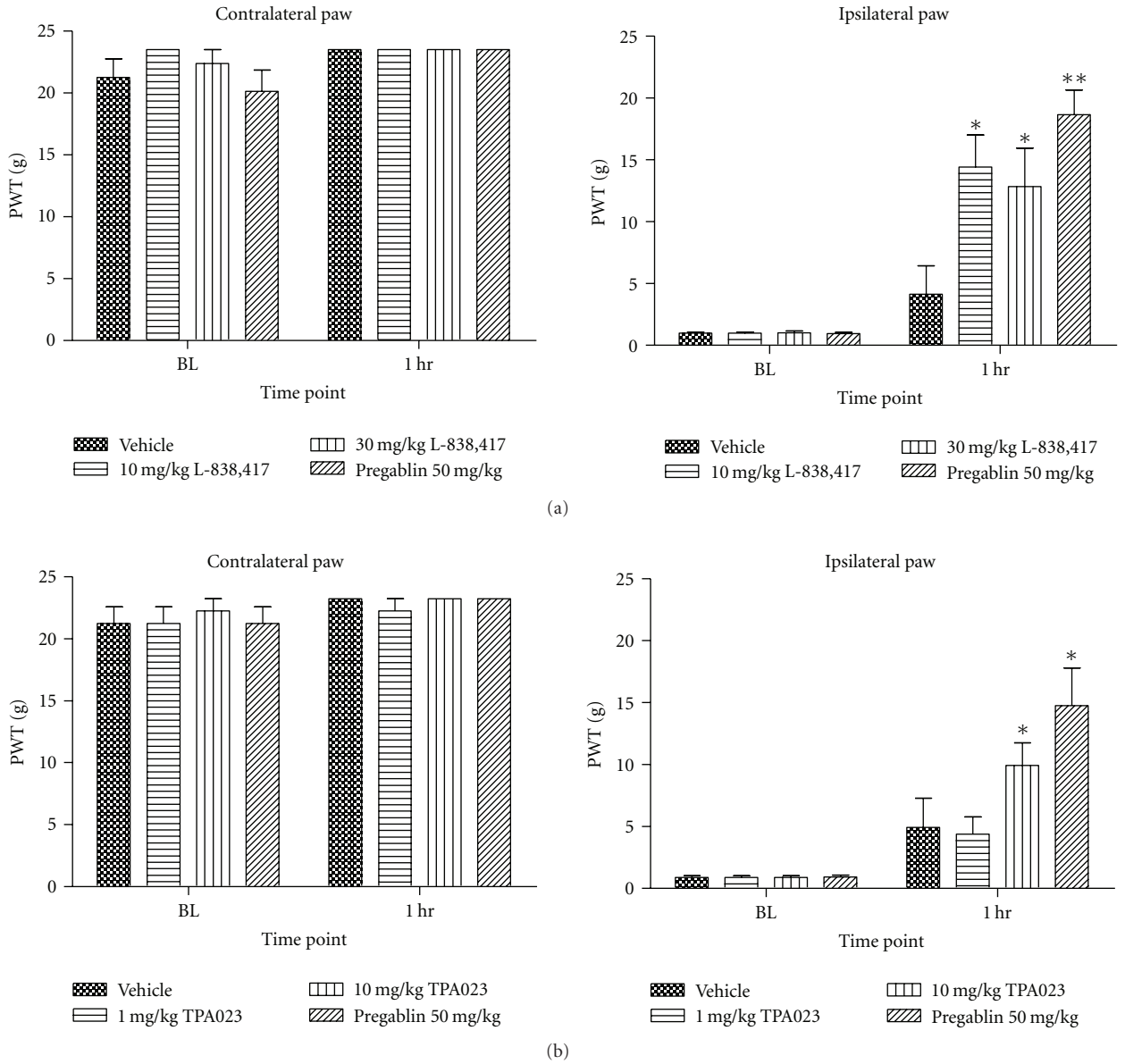

Figure 5: The effects of (a) L-838,417 and (b) TPA023 on spinal nerve ligation-induced static allodynia in the rat. Data are means ± sem. $n = 10$. Data are expressed as paw withdrawal threshold (PWT). BL: baseline. ($* = P < 0.05$, $** = P < 0.01$ compared to vehicle-treated group. Mann-Whitney nonparametric test).

body tone in conscious animals [29]. We consider, however, that the data obtained in the CCI-wide dynamic range (WDR) study provide unequivocal evidence that $GABA_A$ PAMS do significantly affect pain signalling. This is because recordings are from individual spinal WDR neurones, which respond to a range of sensory stimuli. Increasing intensities of stimulation cause increasing cell firing, with maximal firing caused by noxious stimuli. These cells signal pain (intensity and location) to the brain. The CCI-WDR assay is designed to measure the effects of compounds on activity evoked in WDR neurones by peripheral stimuli that are in the noxious range, and L-838,417 was able to reduce the amount of nerve firing caused by a pinch stimulus in these animals.

With regards to receptor occupancy (RO), even the higher activity compound L-838,417 required at least 60% RO in preclinical models to exhibit significant efficacy. Indeed, the SNL model appeared to be the most sensitive of the neuropathic models with regards detecting an analgesic effect, with L-838,417 requiring 60% RO and TPA023 requiring 100%, which correlates well with their different *in vitro* profiles. Conversely, in the CFA-thermal inflammatory model although L-838,417 again had an analgesic effect at 60% RO, TPA023 was ineffective in this model. How receptor occupancy requirements will translate from the preclinical to clinical setting is difficult to estimate. The optimistic viewpoint would be that similar to the sedative and anxiolytic properties of classical benzodiazepines, such as zolpidem

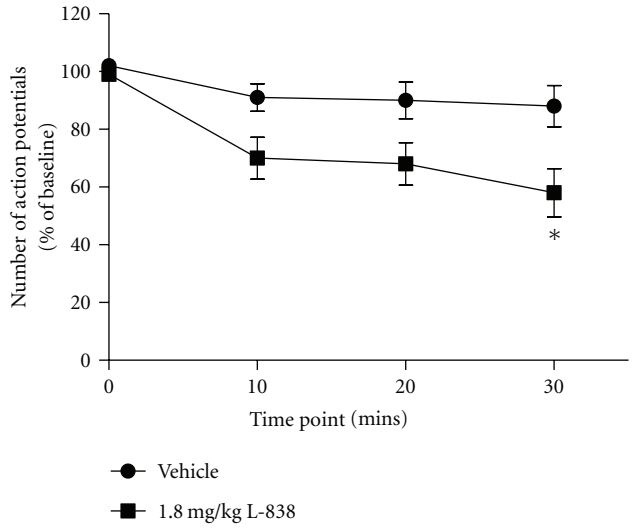

Figure 6: The effects of L-838,417 on pinch-evoked wide dynamic range (WDR) neurone firing in the chronic constriction injured rat. Data are means ± sem. $n = 6$–8. ($* = P < 0.05$ compared to vehicle-treated group, t-test.)

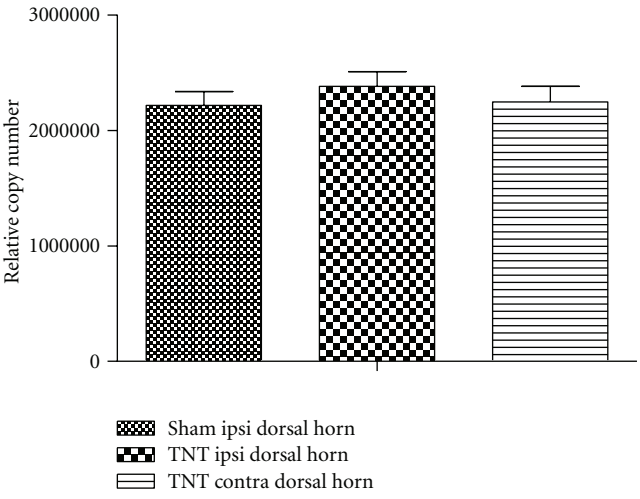

Figure 7: KCC2 mRNA levels in the dorsal horn of the rat spinal cord following either chronic constriction injury (CCI) or sham surgery. Data are means ± sem. $n = 18$. Ipsi: ipsilateral. Contra: contralateral.

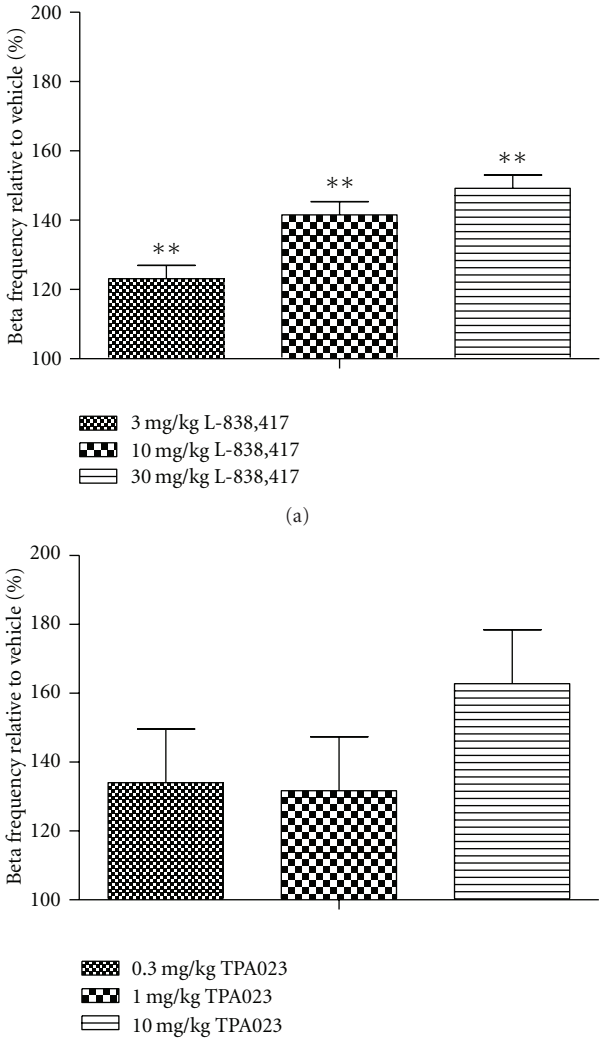

Figure 8: The effects of (a) L-838,417 and (b) TPA023 on quantitative electroencephalogram (EEG) in the telemetered rat. Data are expressed as the total power in the beta frequency of the EEG signal over a four-hour period, as a percentage of vehicle control. Data are means ± sed. $n = 6$. Studies were four-period crossovers, and therefore means and SED's are adjusted for period and rat effects. $** = P < 0.01$ compared to vehicle-treated group. Restricted maximum likelihood (REML) analysis, followed by Fisher's post hoc analysis.

and lorazepam, that lower occupancy is required in the clinical setting to achieve significant effects [30, 31], but this hypothesis needs clinical data to support it. In phase 1 safety studies, TPA023 was shown to be well tolerated up to approximately 60% receptor occupancy [9], in addition TPA023B which has a similar *in vitro* profile to L-838,417 was also well tolerated up to approximately 60% receptor occupancy [32]. This was an acute study, however, and, at higher doses, the reported side effect profile of TPA023B appears to be worse than that of TPA023, with clear sedative and ataxic effects.

Overall, our data cannot differentiate whether it is the higher $\alpha 2/3$ activity exhibited by L-838,417 compared to TPA023 that leads to a more robust analgesic profile in preclinical pain models, or whether the difference is due to the $\alpha 5$ activity present in L-838,417. Both of the functionally selective $GABA_A$ compounds currently published in the literature which exhibit a significant effect in preclinical pain models do show reasonable activity at $\alpha 5$ [4, 7]. In addition, in knockin mice, the efficacy of diazepam against mechanical and heat allodynia was significantly reduced in the $\alpha 5$ KI's after CCI surgery [4], suggesting that this subunit does play some role in mediating the analgesic effects of these compounds. Hopefully, advances in medicinal chemistry and the production of more selective compounds will allow this question to be answered in the near future.

Finally, in order to have a translatable measure of $\alpha 2$ activity *in vivo*, we also investigated the changes in qEEG beta frequency produced by L-838,417 and TPA023. Preclinically, the beta frequency is considered to be a marker of $\alpha 2$ activity, as qEEG studies have shown that the change in diazepam-induced beta frequency remains unaffected in $\alpha 1$ or $\alpha 3$ knockin mice [33–35], but was reduced in mice with diazpam-insensitive $\alpha 2$ subunits. In these mice, $\alpha 2$ subunits were functional (i.e., they respond normally to GABA) but are diazepam insensitive. Clinical qEEG changes have been reported for a number of benzodiazepine compounds [36–38], although the clinical data supporting a correlation between beta frequency and $\alpha 2$ has not, at this time, been generated. Our preclinical qEEG data correlate well with the reported *in vitro* activity of the two compounds and to some extent mimic the effects observed in our preclinical pain models, with L-838,417 exhibiting a significant change in beta frequency in a dose-related fashion, but TPA023 exhibiting a nonsignificant trend to increase. We propose that changes in qEEG beta frequency may be an appropriate pharmacological biomarker for $\alpha 2$ selective $GABA_A$ PAMS.

5. Conclusions

We conclude that $GABA_A$ functionally selective PAMSs are likely to have broad utility in treating clinical pain. We consider, however, it is unlikely that a low-efficacy compound such as TPA023 will show sufficient efficacy in the clinic. The balance of an increasing side effect profile and efficacy will therefore have to be carefully considered when taking compounds into clinical testing. We also recommend the use of qEEG as an early marker of pharmacology in the clinical setting.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2011, Article ID 578273, 10 pages
doi:10.1155/2011/578273

Review Article

Central and Peripheral $GABA_A$ Receptor Regulation of the Heart Rate Depends on the Conscious State of the Animal

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Received 8 June 2011; Accepted 5 September 2011

Academic Editor: Keith Wafford

Intuitively one might expect that activation of GABAergic inhibitory neurons results in bradycardia. In conscious animals the opposite effect is however observed. GABAergic neurons in nucleus ambiguus hold the ability to control the activity of the parasympathetic vagus nerve that innervates the heart. Upon GABA activation the vagus nerve will be inhibited leaving less parasympathetic impact on the heart. The picture is however blurred in the presence of anaesthesia where both the concentration and type of anaesthetics can result in different effects on the cardiovascular system. This paper reviews cardiovascular outcomes of GABA activation and includes own experiments on anaesthetized animals and isolated hearts. In conclusion, the impact of changes in GABAergic input is very difficult to predict in these settings, emphasizing the need for experiments performed in conscious animals when aiming at determining the cardiovascular effects of compounds acting on GABAergic neurons.

1. Introduction

Gamma-aminobutyric acid (GABA)ergic neurons are the major contributor to neuronal inhibition in the brain. The activity of GABAergic interneurons has profound impact on spike timing control, neuronal rhythms, and the activity pattern of neuronal circuits. The correct tonus for these neurons are therefore critical for a number of different conditions such as attention, perception, consciousness, working memory, sensorimotor processing, and schizophrenia [1–3]. Activation of GABAergic neurons is also applied for myorelaxation, anxiolytic treatment, sedation, and anaesthetics [4–6]. In addition to CNS effects GABAergic neurons also have a significant impact on the cardiovascular system. From this perspective GABAergic neurons can affect both electrical and hemodynamic parameters. CNS-controlled chronotropic effects on the heart can happen via effects on nucleus ambiguus that will subsequently affect the vagal tonus and thereby heart rate [7]. Additionally it has been suggested that GABA could have a direct effect on cardiac tissue [8]. In addition to direct or indirect effect on cardiac electrical parameters GABAergic input will also affect blood vessels and thereby participate in the control of vascular tonus and blood pressure, which reflexively has effects on heart rate [9].

GABA receptors come in two different families: Ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. A description of the G-protein-coupled $GABA_B$ receptors is beyond the scope of the present paper.

$GABA_A$ receptors are ligand-gated ion channels with chloride conductance. The functional channel is typically heteropentameric in structure even though homomeric ρ1, 2, and 3 receptors have been described [10]. A number of different subunits exist, and the exact subunit composition will determine the electrophysiological properties of the channel, thereby ultimately the phenotypic output in an *in vivo* situation [11]. The majority of $GABA_A$ receptors contain α, β, and γ subunits but δ, ε, θ, and ρ can also be present [12]. Ligand binding to $GABA_A$ receptor has been intensively studied, and detailed information is available. The GABA binding site is located at the interface between the α and the β subunit, and a number of important amino acids in the binding pocket have been identified [13]. The effect of positive allosteric modulators is well described for $GABA_A$ receptors exemplified by benzodiazepine. The binding site is

here located between the α-γ interface [14]. Antagonist are considered to bind in a pocket that is partly overlapping with the agonist site, with the difference being that antagonist can extend further into the solvent accessible cavity [15]. Pore blockers, in the form of picrotoxin, are also valuable experimental tools for addressing the effects of $GABA_A$ receptors, albeit this toxin has no therapeutic use due to induction of convulsions.

The present paper gives an overview of the known literature of GABAergic effects on the cardiovascular system with emphasis on the cardiac vagal neurons because of their major importance in controlling the chronotropic state of the heart. Supportive information will be given for experiments performed on isolated hearts and anaesthetized *in vivo* experiments under influence of different concentrations of isoflurane.

2. Parasympathetic Control of Heart Rate

Cardiac cells hold the ability to initiate action potentials. The sinoatrial node has the highest automaticity, and consequently the normal heart beat originates from here. The heart activity is however regulated by the intrinsic cardiac neurons, by hormones and by the sympathetic and parasympathetic branches of the autonomic nervous system [16]. The parasympathetic cardioinhibitory effects are opposed by the facilitatory sympathetic effects. In both animals and humans the parasympathetic tonus dominates over the sympathetic at rest [17–19]. Cardiac preganglionic neurons whose cell bodies are primarily found in the nucleus ambiguus and some in the dorsal motor nucleus of the vagus are responsible for this tonic control of heart rate. They are also important for the reflex and respiratory regulation of heart rate seen in response to baroreceptor activation and inspiration [20]. Via the vagal nerve, the axons from these nuclei reach the intrinsic cardiac ganglia located in the fat pads adjacent to the right atrium [16]. From here postganglionic neurons innervate the sinoatrial node, ultimately leading to activation of cholinergic G-protein-coupled receptors (M_2) and heart rate reduction. This happens primarily through activation of the acetylcholine-activated K^+ channel GIRK, but also by M_2 receptor-induced inhibition of the adenylyl cyclase, which ultimately leads to reduced HCN channel opening probability [21, 22] (Figure 1). The preganglionic cardiac vagal neurons do not hold any intrinsic pacemaker activity and are consequently intrinsically silent [23]. Therefore, they must rely on synaptic input mediated by both ionotropic and G-protein-coupled receptors for controlling their firing [24–26]. Excitatory inputs to the cardiac vagal neurons include both glutamatergic inputs predominantly from the nucleus tractus solitarius, and pre- and postsynaptic cholinergic nicotine receptors that can excite the cardiac vagal neurons (for review see [27]). With respect to the inhibitory GABAergic input to the cardiac vagal preganglionic neurons, it was found that inhibition of $GABA_A$ receptor activity by microinjection of bicuculline into nucleus ambiguus resulted in dose-related reduction of heart rate and that the effect was reversed by the $GABA_A$ receptor agonist muscimol [7].

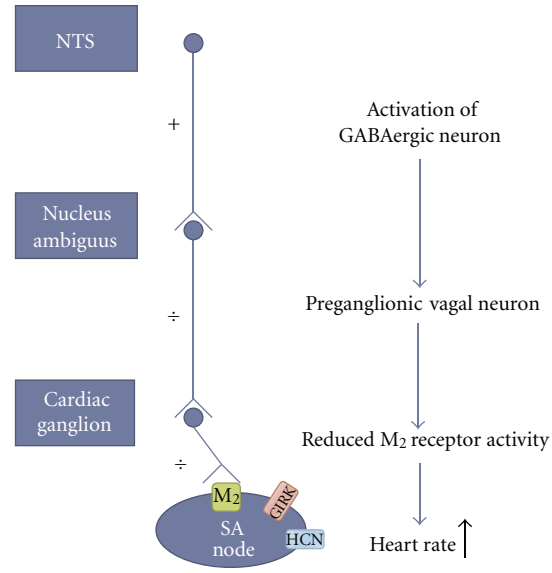

Figure 1: Model of how activation of GABAergic input to the cardiac vagal neurons increases heart rate. GABAergic neurons from the nucleus tractus solitarius inhibits the preganglionic cardiac vagal neurons, which leads to reduced postganglionic vagal input to the heart. Consequently, the muscarinic acetylcholine receptor (M_2) activity is reduced. Because the G_i protein no longer inhibits the production of cAMP by the adenylyl cyclase, HCN channel activity is increased. In addition the G protein-coupled inwardly rectifying potassium channel (GIRK) is no longer activated. Together this will cause the heart rate to increase.

This demonstrates the importance of GABAergic input to the nucleus ambiguus in setting the tonic heart rate level. The phasic inhibition of cardiac vagal neurons by activation of $GABA_A$ receptors in the nucleus ambiguus is important for the respiratory sinus arrhythmia where the heart rate slows during inspiration and for the baroreflex where changes in arterial pressure reflexively cause homeostatic changes in heart rate [28].

Upon synaptic release of GABA multiple types of postsynaptic GABAergic receptors on the parasympathetic cardiac neurons in the nucleus ambiguus are activated. *In vitro* electrophysiological studies found that the phasic inhibitory currents observed after release of GABA could be blocked by gabazine whereas gabazine-insensitive but picrotoxin-sensitive receptors were responsible for the tonic inhibitory synaptic currents [30]. This phenomenon is well known in other brain areas [31]. When the tonic currents were blocked the membrane potential depolarized and increased the firing activity of the cardiac vagal neurons. How the tonic GABA current is activated in the nucleus ambiguus is unknown, but a role for the GAT-1 GABA transporter is unlikely as inhibition of this did not augment the magnitude of the tonic GABAergic current in the vagal neurons [30]. Other mechanisms such as spontaneous opening of constitutive active GABA channels could be responsible for the tonic GABAergic current. Bouairi et al. further demonstrated that application of the benzodiazepine, flunitrazepam, increased the decay time of the phasic IPSC and augmented the tonic

current [30]. This would decrease the input resistance of the neuron and serve as a "sink" for any excitatory inputs [30]. These findings also provide a hint to the molecular composition of the cardiac vagal neuron $GABA_A$ receptor. The positive allosteric modulation by benzodiazepines requires the presence of a γ-subunit, with the binding site located at the α-γ interface [14]. In the brain stem $\alpha 1$ and $\alpha 3$ are more strongly expressed than $\alpha 2$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ (for review see [32]). However, the molecular composition of the $GABA_A$ receptors in the cardiac vagal neurons needs to be determined.

The sources of GABAergic neurotransmission to the cardiac vagal neurons in the nucleus ambiguus have been investigated. Both with respect to the tonic and phasic inhibitory GABAergic inputs they have been suggested to in part originate from the nucleus tractus solitaries (NTS) [33]. Electrical stimulation of the NTS produces a GABAergic current in the cardiac vagal neurons that can be blocked by bicuculline [33]. An excitatory monosynaptic glutamatergic pathway from NTS that activates NMDA and non-NMDA postsynaptic receptors in cardiac vagal neurons has also been identified [26]. By photo-uncaging glutamate in the near vicinity of GABAergic neurons and simultaneously recording from cardiac vagal neurons, Frank et al. could identify and map GABAergic neurons projecting to the cardiac vagal neurons in the nucleus ambiguus. Using this technique they identified areas in the nucleus tractus solitarius and in the close proximity to the nucleus ambiguus that when stimulated by uncaging of glutamate evoked a GABAergic inhibitory response in the cardiac vagal neurons [34].

The nucleus tractus solitarius is important for integrating the autonomic nervous system functions and so also for cardiovascular and respiratory regulation and reflexes. The NTS receives afferent input from the cranial nerves and hence information from a variety of organs and visceral regions, including sensory information from chemoreceptors and arterial baroreceptors [35, 36]. These sensory information is important for the baroreflex, where increase in blood pressure causes afferent baroreflex activity, that activates neurons in the NTS and possibly via the excitatory glutamatergic pathway evokes an increase in the cardiac vagal neuron activity and a compensatory decrease in heart rate (for review see [20, 37]). Respiration also influences the cardiac vagal neuron output through reflex mechanisms. One of these reflex mechanisms is the respiratory sinus arrhythmia, which describes the changes in the heart rate with respiration. From ECG recordings this can be observed as a shortening of the R-R interval (increased heart rate) during inspiration and a prolongation during expiration. This phenomenon has been suggested to save cardiac energy by effectively reducing the number of heartbeats during expiration, providing an efficient ventilation/perfusion matching [38]. Respiratory sinus arrhythmia is primarily mediated by altering the firing pattern of the cardiac vagal neurons. They are silent during inspiration and active during expiration. This rhythm is not achieved by changes in excitatory pathways projecting to the cardiac vagal neurons but rather by the activation of inhibitory pathways during inspiration which increases the GABAergic and glycinergic input to the cardiac vagal neurons, thereby lowering the vagal tone on the heart during inspiration [20, 39]. Respiratory sinus arrhythmia which has been observed in many different mammals is blunted, diminished or even reverted in anaesthetised animals depending on the anaesthetics used [40]. This demonstrates the importance of understanding how general anaesthetics work, especially when investigating the parasympathetic control of heart rate or when novel compounds are investigated for cardiovascular safety liability.

3. Effects of General Anaesthetics on the Heart

Many general anaesthetics have been found to affect cardiovascular reflexes by interfering with the cardioinhibitory vagal neurons in the nucleus ambiguus. Pentobarbital is often used for induction of anaesthesia and is known to cause respiratory depression, blunted baroreflex, and increased heart rate [41]. These effects are primarily related to the pentobarbital induced potentiation of the spontaneous postsynaptic inhibitory currents in the cardiac vagal neurons, whereby the cardioinhibitory parasympathetic input to the heart is decreased, and heart rate is increased [42]. Similarly, propofol, which is also known to potentiate $GABA_A$ currents, augments the GABAergic input to the cardiac vagal neurons by increasing both phasic and tonic $GABA_A$ receptor currents. This evokes an increase in heart rate [43, 44]. At supratherapeutic propofol concentrations inhibition of the GABAergic neurotransmission to the nucleus ambiguus is observed with a subsequent reduction in heart rate [44]. Isoflurane is known to decrease blood pressure, evoke respiratory inhibition, and to cause variable changes in heart rate depending on the depth of anaesthesia [45, 46]. Furthermore, studies have demonstrated that the baroreceptor reflex was not depressed significantly until 2.6% isoflurane (2X minimum alveolar concentration (MAC)) [47]. Other reflexes such as the respiratory sinus arrhythmia are however compromised during isoflurane exposure at clinically relevant concentrations [48]. Isoflurane increases heterologously expressed $GABA_A$ receptor currents with the effect peaking around 1 MAC. The concentration response curve is bell shaped, and eventually isoflurane produces an inhibition of the $GABA_A$ steady-state current as the isoflurane concentration increases. In order to lower the concentration of isoflurane needed to achieve the desired anaesthetic depth, the volatile anaesthetic is often supplemented with nitrous oxide (N_2O). From electrophysiological recordings of heterologously expressed $GABA_A$ receptors addition of N_2O results in an augmentation of the potentiating effects of isoflurane [49]. The enhancement of $GABA_A$ receptor currents by isoflurane results in prolonged inhibitory postsynaptic currents, increased Cl^- influx, and reduced excitability. In addition, recordings from cardiac vagal neurons in the nucleus ambiguus demonstrated that isoflurane also enhanced the tonic GABAergic current [43]. Taken together this augmentation of the GABAergic input to the cardiac vagal neurons would result in reduced vagal excitability. This would shift the parasympathetic/sympathetic balance resulting in tachycardia. However, Wang also measured a reduced frequency of GABAergic IPSC after exposure to isoflurane, which would

lessen the inhibition of the parasympathetic output from the vagal nerve, resulting in less tachycardia [43, 50]. Other anaesthetics do not induce tachycardia. The synthetic opiate fentanyl produces bradycardia partly via inhibition of the GABAergic pathways to the cardiac vagal neurons in the nucleus ambiguus [51].

With respect to cardiovascular safety pharmacology, knowledge about the profound effect of anaesthetics on the heart and vasculature is important when designing and interpreting studies of novel compounds and their possible detrimental effects on the cardiovascular system. Considering that GABA is the main inhibitory neurotransmitter in the central nervous system and the importance of the GABAergic system in determining the firing activity of the vagal cardiac neurons, it is maybe of no surprise that drugs modulating $GABA_A$ receptor activity will influence cardiac parameters. However, the outcome of such modulations can be hard to interpret and predict due to the complexity of these integrative systems and reflexes.

4. Cardiovascular Effects of Positive Allosteric Modulators of $GABA_A$ Receptors

Benzodiazepines are positive allosteric modulators of $GABA_A$ receptors. The cardiovascular effects of benzodiazepines have been investigated in both animals and humans. Animal studies have found that benzodiazepines result in lowered blood pressure and variable effects on heart rate. Findings of reduced heart rates in anaesthetized animals were explained by reduction of sympathetic outflow [52, 53]. In anaesthetised rats two benzodiazepine tested produced tachycardia, and this effect was attenuated by pretreatment with atropine [54], suggesting an important role of the parasympathetic nervous system. This role is also stressed by a study demonstrating that application of the benzodiazepine, flunitrazepam, increased the GABAergic input to the cardiac vagal neurons, which lowered their excitability and consequently reduced the parasympathetic outflow to the heart [30]. Differences in species, the anaesthetics, and the depth of anaesthesia used might help to explain these varying effects on heart rate. Because of the profound effects of general anaesthetics on cardiac regulation, the effects of positive allosteric modulators of $GABA_A$ receptors on conscious animals are important. In conscious trained dogs low doses of diazepam and bromazepam (p.o.) had no influence on heart rate, but a rapid onset positive chronotropic effect was observed at higher doses (10 mg/kg p.o.). The onset was rapid and could not be reverted by the beta-adrenoceptor blocking agents, indicating that the rapid heart rate was not solely a result of increased sympathetic outflow to the heart [55]. Using radiotelemetry devices diazepam (6 mg/kg i.p.) was found to increase the heart rate in conscious rats [56]. Using a similar setup another $GABA_A$ receptor potentiator JM-1232 was also found to increase heart rate, and this effect was prevented by pretreatment with atropine or propranolol. This indicates an involvement of both branches of the autonomic nervous system. The authors suggest that the tachycardia could therefore be a consequence of GABAergic inhibition of the vagal nerve output and/or baroreflex activation due to hypotension [57].

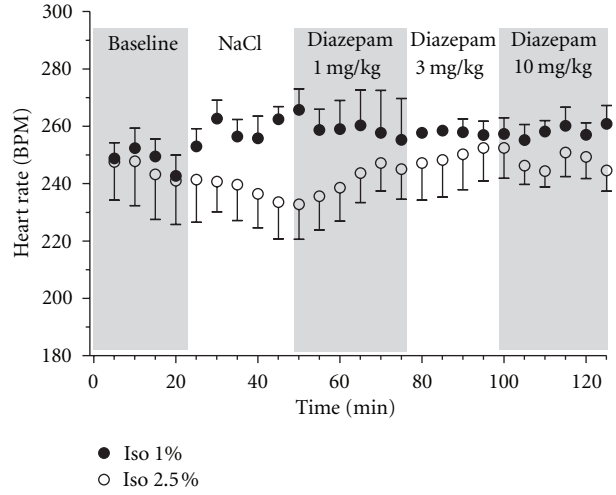

Figure 2: Effect of diazepam on heart rate in artificially ventilated (1% isoflurane, filled circles; 2.5% isoflurane, open circles; $O_2 : N_2O$ 1 : 1) female guinea pigs (538 ± 27 g). The animals were placed on heated mats and the temperature was monitored and kept constant at 37 ± 1°C throughout the experiment. Electrocardiographic (ECG) recordings were obtained using 2 electrodes placed in the subcutaneous layer of the forelimbs (left and right), and 1 electrode placed in the subcutaneous layer of the left hind limb. ECG recordings were analysed using Chart ADinstrument software and Graphpad Prism 5. A stabilization period of minimum 20 min was performed, followed by NaCl 0.9% i.p. Subsequently the animals were injected intraperitoneally every 25th min with increasing concentrations of diazepam (1, 3 and 10 mg/kg i.p.).

In general the cardiovascular effects of benzodiazepines in humans, at clinically relevant doses, are mild [58]. However, during i.v. infusion, or with overdose, benzodiazepines may cause hypotension and respiratory depression [59, 60]. Short acting, fast on-set benzodiazepines such as midazolam are often used as premedication before surgical interventions. When midazolam is injected i.v. it produces a rapid drop in blood pressure and an increase in heart rate [59], comparable to what is seen in conscious animals. A study conducted to evaluate the influence of benzodiazepines on the autonomic neurocardiac regulation in humans found a similar rapid increase in resting heart rate and a concomitant reduction in vagal tone, assessed by changes in heart rate variability. A role for a baroreflex-induced tachycardia was ruled out as no significant fall in blood pressure was recorded [61].

Because the cardiac action potential in guinea pigs resembles more closely the human cardiac action potential as compared to other rodents, especially with respect to the repolarizing currents, guinea pigs are a preferred animal model for initial screening for cardiovascular safety liabilities [62]. In guinea pigs artificially ventilated with isoflurane (1% or 2.5%, $O_2 : N_2O$ 1 : 1) administration of diazepam (1, 3 & 10 mg/kg i.p.) produced no significant changes in heart rate as compared to saline injection (Figure 2). No significant

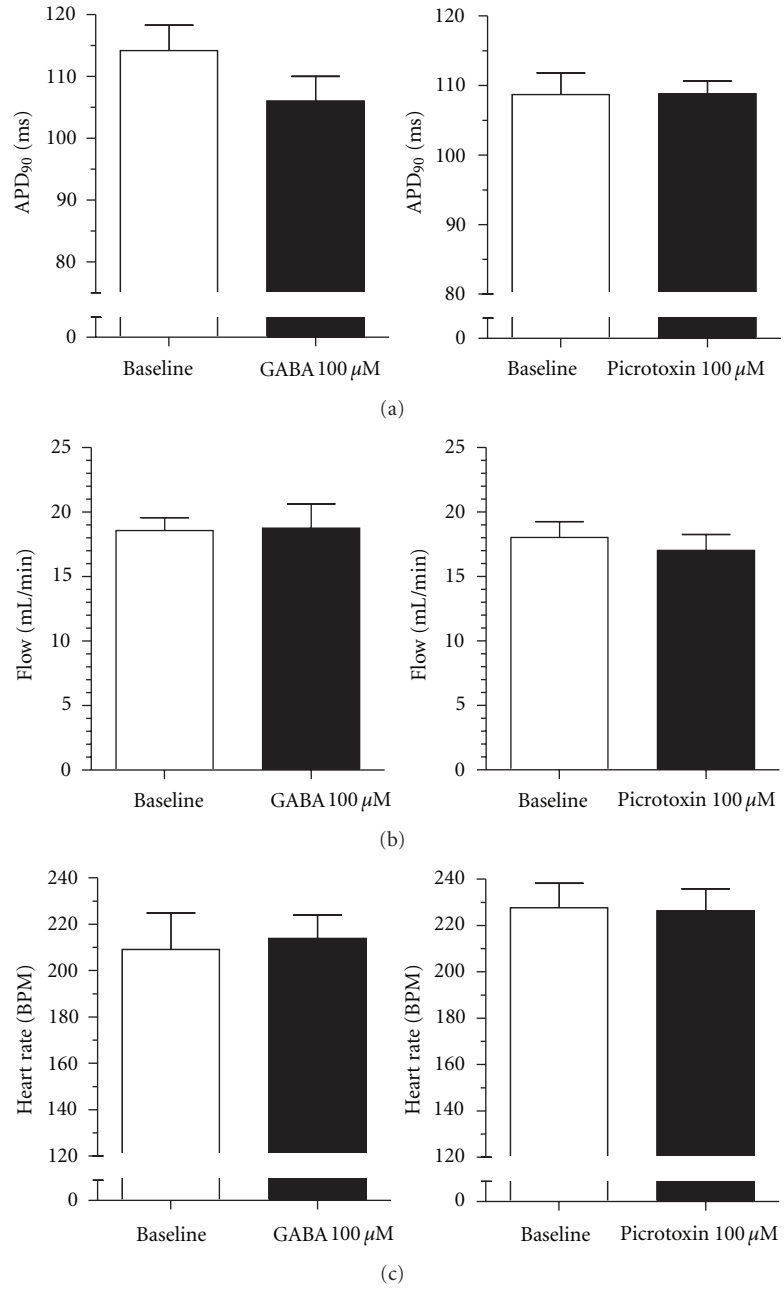

Figure 3: Effect of GABA 100 μM (left column) or picrotoxin 100 μM (right column) on isolated retrograde perfused female guinea pig hearts. Hearts were excised, mounted in a Langendorff apparatus, instrumented, and perfused with krebs-henseleit solution at a constant pressure of 60 mmHg as previously described [29]. Hearts were left to stabilize for a minimum of 30 min. After 20 min baseline recordings, where flow and heart rate was monitored, the hearts were paced from the right atrium for 2 min at 240 BPM. This protocol was repeated in the presence of GABA 100 μM or picrotoxin 100 μM ($n = 5$; GABA: 677 ± 99 g; Picrotoxin: 672 ± 100 g). GABA 100 μM or picrotoxin 100 μM produced no significant effect on action potential duration (a), flow (b), and heart rate (c).

changes in heart rate corrected QT-interval, PR-interval, or temperature were observed (data not shown).

The lack of effect of diazepam in the guinea pig on heart rate as compared to rat could be a species- or dosage-dependent phenomenon, but it could also stem from the use and depth of anaesthesia which might already augment the GABAergic input to the cardiac vagal neurons to such an extent that further potentiation of $GABA_A$ receptors would not cause any vagolytic effect. In order to circumvent this we will establish an *in vivo* setup using radiotelemetry implants in order to investigate the *in vivo* effects of positive allosteric modulators of $GABA_A$ receptors in conscious guinea pigs. This will also allow for simultaneous recordings of ECG, blood pressure, temperature, and locomotor activity. Such

experimental condition will optimize the amount of data that can be extracted from a single experiment and aid in interpretation of drug-induced cardiovascular effects.

5. Effect of GABA on the Isolated Heart

In addition to the central mediated $GABA_A$ receptor effects on the heart, studies have also suggested the presence of $GABA_A$ receptors in the heart. One study found mRNA expression encoding the $GABA_A$ receptor ε subunit in the human cardiac conduction study [63] and in mouse $GABA_A$ receptor protein was detected in the heart [64]. GABA has been found in the guinea pig heart using [^{3}H]-GABA, especially in the area of the SA node and in the intrinsic cardiac ganglion [8, 65]. It appears that there is no direct GABAergic pathway connecting the nervous system to the heart. Yet, GABA might exert its effect on the intrinsic cardiac neurons, where it appears to play indirect modulatory effects [8, 66, 67]. The physiological role of GABAergic currents in the intrinsic cardiac ganglion and their impact on heart rate control need further investigation. However, in rat GABA-evoked currents have been measured from intrinsic cardiac neurons, but the current amplitude declined with age suggesting a role of $GABA_A$ receptors in the development of the rat heart [65]. The intrinsic cardiac neurons, or intrinsic cardiac ganglion, consist of both parasympathetic cholinergic and sympathetic adrenergic postganglionic neurons that receive input from the parasympathetic preganglionic neurons in the brainstem and the preganglionic sympathetic neurons found in the spinal cord. From here these neurons project to the sinoatrial node. This classical view of the autonomic ganglion functioning only as a passive relay station from the central nervous system to the pacemaker cells of the heart is too simple, because both interneurons and afferent neurons are also found in the intracardiac ganglion. This allows sensory information about the chemical and mechanical state of the heart to be signalled to other neurons within the intrinsic cardiac ganglion. This integration of signals from both cardiac and extracardiac afferents and how they interact with the cardiac adrenergic and cholinergic motorneurons are important for regulating cardiac function (for review see [16]).

The isolated perfused heart is often used for cardiovascular safety pharmacology. The importance of $GABA_A$ receptors in this preparation has been investigated. In rats, adding GABA to the perfusion solution resulted in a dose-dependent reduction in heart rate [68]. We did not observe bradycardia in the isolated perfused guinea pig heart at a GABA concentration, which was found to affect the heart rate in rat, nor was there any significant effect of GABA on action potential duration or coronary flow. Likewise blocking $GABA_A$ receptors by administration of picrotoxin did not produce any significant effects (Figure 3).

In order to obtain a more thorough overview of possible GABAergic effects on isolated heart we also tested the effect of positive allosteric modulation. In contrast to agonist application in the form of GABA, we found that diazepam produced a concentration-dependent reduction in heart rate (Figure 4). This effect on heart rate was however not

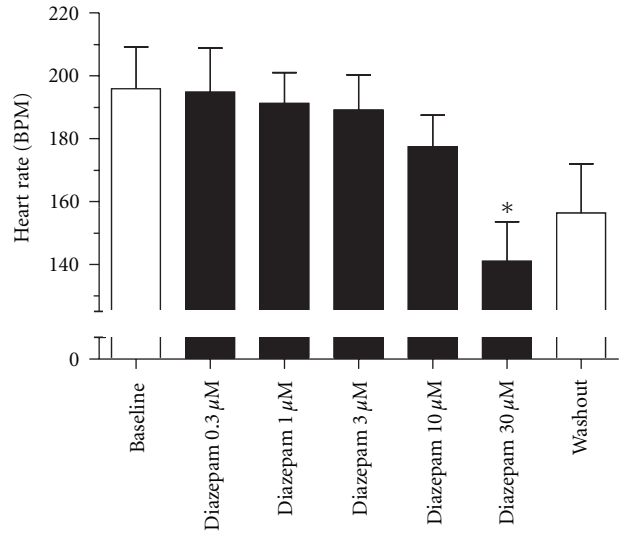

Figure 4: Effect of diazepam on isolated retrograde perfused female guinea pig hearts (868 ± 45 g). After a minimum of 30 min stabilization, the hearts underwent 20 min of baseline recording. Subsequently the hearts were exposed to diazepam at increasing concentrations in 20 min intervals. Diazepam induced a dose-dependent reduction of heart rate and significantly shortened the heart rate at 30 μM ($n = 5$).

prevented by coadministration of picrotoxin (Figure 5(a)), suggesting that $GABA_A$ receptors are not involved in the bradycardiac effect. Another study found that diazepam produced a negative inotropic response in isolated perfused guinea pig hearts that was not prevented by cotreatment with $GABA_A$ or $GABA_B$ receptor antagonists [69]. Further, diazepam application had no effect on action potential duration (APD) addressed by APD_{90} values or on coronary flow (Figures 5(b) and 5(c)). Diazepam has been found to inhibit the cardiac calcium channel recorded from isolated guinea pig cardiomyocytes [69], and to inhibit recombinant L-type voltage-gated calcium channels [70]. The reduced calcium influx can explain the observed $GABA_A$ receptor-independent effects of diazepam on heart rate and contractility. However, it should be noted that the concentrations needed to produce calcium channel block, and the bradycardia and negative inotropy observed in the isolated guinea pig hearts are many folds above the therapeutic free plasma concentration (~0.2 μM) and would so only be encountered during overdose.

6. Conclusion

This paper focused on the effect of GABA on the cardiac vagal neurons and on the isolated heart. It is important to recognize that regulation of heart rate not only involves these parts, but is part of a complex integrated system involving neurons located from the level of the insular cortex to the level of the heart [16]. Because GABA is the main inhibitory neurotransmitter in the central nervous system, the cardiac effects of modulating $GABA_A$ receptor activity, especially with respect to $GABA_A$ receptor subtype selective compounds, are hard to predict and require careful investigation.

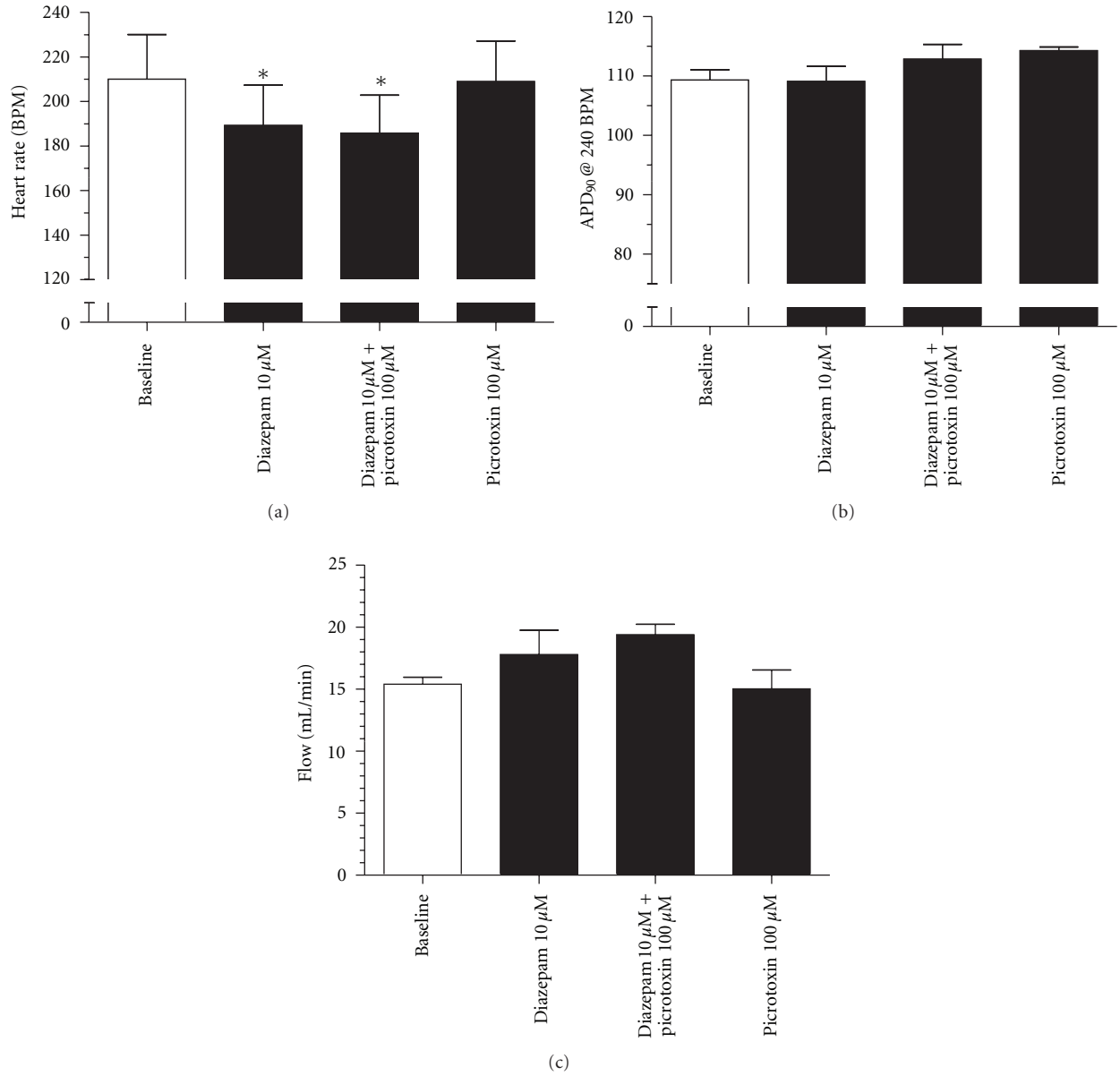

FIGURE 5: The effect of diazepam (10 μM), diazepam (10 μM) + picrotoxin (100 μM) (co-administration), and picrotoxin (100 μM) on heart rate (a), action potential duration (b), and coronary flow (c). Hearts were left to stabilize for a minimum of 30 min. 20 min of baseline recordings were performed, where heart rate and flow were monitored. The hearts were then paced from the right atrium at 240 BPM for 2 min in order to measure the action potential duration at a fixed heart rate. This (20+2) protocol was repeated for the different drugs investigated ($n = 5$; $^*P \leq 0.05$ (1-way ANOVA); (715 ± 129 g)).

In this respect it will be interesting to obtain more knowledge about the $GABA_A$ receptor subunit composition in the neuronal pathways involved in heart rate control. Considering the profound effects of anaesthetics on the GABAergic system, and on neuronal pathways involved in heart rate control, it argues for the use of conscious freely moving animals at early stages during cardiovascular safety pharmacology profiling of novel compounds targeting the $GABA_A$ receptor. Such cardiovascular safety pharmacology investigations could well be combined with behavioural assessment [71]. This will aid in interpretation of drug-induced effects and increase the amount of data generated per animal, thereby reducing the number of animals used.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2011, Article ID 926361, 12 pages
doi:10.1155/2011/926361

Review Article

Neurosteroid Binding Sites on the $GABA_A$ Receptor Complex as Novel Targets for Therapeutics to Reduce Alcohol Abuse and Dependence

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Received 10 May 2011; Accepted 17 July 2011

Academic Editor: Naheed R. Mirza

Despite the prevalence of alcohol abuse and dependence in the US and Europe, there are only five approved pharmacotherapies for alcohol dependence. Moreover, these pharmacotherapeutic options have limited clinical utility. The purpose of this paper is to present pertinent literature suggesting that both alcohol and the neurosteroids interact at the $GABA_A$ receptor complex and that the neurosteroid sites on this receptor complex could serve as new targets for the development of novel therapeutics for alcohol abuse. This paper will also present data collected by our laboratory showing that one neurosteroid in particular, dehydroepiandrosterone (DHEA), decreases ethanol intake in rats under a variety of conditions. In the process, we will also mention relevant studies from the literature suggesting that both particular subtypes and subunits of the $GABA_A$ receptor play an important role in mediating the interaction of neurosteroids and ethanol.

1. Introduction

The suggestion that neuroactive steroids could have potential as new pharmacotherapies for alcohol abuse and dependence followed shortly after the discovery that ethanol administration released specific neurosteroids. These same data also directly implicated the endogenous neurosteroids as potential contributors to the behavioral effects of ethanol [1, 2]. However, elucidating the interaction between the neuroactive steroids and ethanol has been especially difficult because both produce a wide variety of molecular and behavioral effects and both act at multiple receptors [3, 4]. Complicating matters even further, neurosteroids also have both genomic and nongenomic effects [4] that are often only dissociable in terms of their time course. Thus, the goal of this paper is to present pertinent literature regarding the interaction of ethanol and the neurosteroids while also highlighting research from our laboratory suggesting that one neurosteroid in particular, dehydroepiandrosterone (DHEA), may be a key to discovering promising new therapeutics for treating alcohol abuse and dependence. In this process, we also hope to provide compelling evidence for the involvement of the $GABA_A$ receptor complex and the role specific subunits of this complex may play in the effects of DHEA on ethanol intake.

If there is any doubt that new treatments for alcohol abuse and dependence are needed, one need only to review some of the most recent epidemiological data on excessive alcohol use. In 2009, an estimated 18.6 million persons aged 12 or older met criteria for alcohol dependence or abuse, representing 7.4 percent of the US population [5]. Despite the prevalence of this problem, there are only five approved pharmacotherapies for alcohol dependence in the US and Europe [6]. Furthermore, these pharmacotherapeutic options have limited clinical utility. For instance, the opioid antagonist naltrexone has been shown to have limited

success apart from individuals with a family history of alcohol dependence, those with an enhanced opioid response to ingestion of alcohol, those who self-report enhanced alcohol cravings, and individuals with a specific μ-opioid receptor polymorphism [7–9]. Acamprosate, a synthetic homotaurine derivative, has been shown to decrease alcohol intake, purportedly via modulation of glutamate [9] and glycine [10] receptors. However, acamprosate had no direct effect on recombinant glutamate or glycine receptors expressed in *Xenopus* oocytes at low, clinically relevant concentrations [11], and therefore, the mechanism by which acamprosate modulates ethanol consumption is still undefined. Experiments involving acamprosate suggest that it is only fully effective in highly motivated subjects with a "goal of abstinence" [12] and that the combined experience of acamprosate with ethanol is necessary for decreasing ethanol intake [13].

2. Importance of the $GABA_A$ System in the Behavioral Effects of Ethanol

Although a variety of neurotransmitters and signaling pathways have been shown to be involved in the behavioral effects of ethanol (e.g., [14–16]), central GABAergic activity is widely accepted to be one of the most important components of ethanol's effects as a CNS depressant [17, 18]. Behaviorally, this supposition is supported by research showing that benzodiazepines and barbiturates that positively modulate the $GABA_A$ receptor complex can substitute for ethanol in drug-discrimination procedures [19, 20]. Electrophysiological and genetic techniques have also furthered our understanding of the interaction between ethanol and the $GABA_A$ receptor complex by showing that it has both direct and indirect effects on the composition of this heteropentameric chloride ion channel. For example, *in vitro* studies with native and recombinant $GABA_A$ receptors indicate ethanol is able to enhance GABA-mediated currents at receptors containing a δ subunit (which are found almost exclusively extrasynaptically *in vivo*) and at doses of ethanol consistent with those achieved during typical episodes of social drinking in humans [18, 21, 22]. Studies with mice in which the δ subunit has been knocked out have shown the importance of δ subunit-containing $GABA_A$ receptor complexes in mediating many of the effects of ethanol. These knockout mice are less sensitive to the anticonvulsant effects of ethanol, demonstrate a decreased hyperexcitability during ethanol withdrawal, and show a lower preference for ethanol compared to wild-type controls. In contrast, δ subunit knockouts did not differ from controls in ethanol-induced anxiolysis, ataxia, hypnosis, or hypothermia [23].

Because δ subunits are only found in $GABA_A$ receptors that also contain an α4 or α6 subunit, the importance of these α subunit subtypes has been the subject of several investigations and debate. For instance, Hanchar et al. [24] found that cerebellar granule neurons from Sprague-Dawley rats with a naturally occurring mutation in the extrasynaptic α6 subunit (arginine (R) to glutamine (Q) in position 100) had an enhanced response to ethanol. Specifically, they reported an increased tonic current amplitude, tonic current noise, and spontaneous inhibitory postsynaptic current. However, using similar methods, Botta et al. [25] found that this mutation did not increase the sensitivity of $GABA_A$ receptors to ethanol; rather, they reported that ethanol modulated the currents of these channels indirectly via a presynaptic mechanism. The importance of the α4 subunit in mediating the effects of ethanol also remains to be determined, as α4 knockout mice had similar anxiolytic, hypothermic, ataxic, and hypnotic responses to ethanol compared to wild-type littermates [26].

In addition to modulating $GABA_A$ receptors directly, ethanol can also modulate them indirectly by altering the levels of GABA-modulating neurosteroids, such as 3α,5α-THP (allopregnanolone) and 3α,5α-THDOC (allotetrahydrodeoxycorticosterone) [1, 2, 27–29]. These neurosteroids are currently thought to contribute to the various behavioral effects of ethanol, including its sedative-hypnotic [30, 31], anxiolytic [32], and discriminative-stimulus effects [33–35]. For example, a reduction in the levels of 3α,5α-THP and 3α,5α-THDOC by the 5α-reductase inhibitor finasteride blocked the acquisition of ethanol drinking and the development of ethanol preference in male C57BL/6J mice [36]. In healthy, adult social drinkers, finasteride also reportedly decreased the subjective effects of ethanol, leading some investigators to speculate that these neuroactive steroids were integral for producing ethanol's subjective effects [37]. In rats trained to discriminate ethanol from saline, 10 mg/kg of pregnanolone partially substituted (60%–70% drug-lever responding) for the discriminative-stimulus effects of 1 g/kg of ethanol subsequent to chronic administration of either saline or ethanol during adolescence [38]. Similarly, in rats trained to discriminate 5.6 mg/kg of pregnanolone from saline, 1 g/kg of ethanol only partially substituted for this neurosteroid [39]. Together, these symmetrical discrimination data indicate that the neurosteroid pregnanolone has overlapping, but not identical, discriminative-stimulus effects with ethanol.

In contrast to the partial substitution found with pregnanolone, Gurkovskaya and Winsauer [38] demonstrated that the discriminative-stimulus effects of DHEA, which comes from a common precursor pregnenolone, were unlike those of ethanol in rats trained to discriminate 1 g/kg of ethanol from saline. Furthermore, DHEA only modestly shifted the curve for ethanol-lever responding to the right when it was administered shortly before varying doses of ethanol (0.18–1.8 g/kg). Bienkowski and Kostowski [33] also reported a similar finding in that the sulfated derivative of DHEA, DHEAS, was ineffective at blocking the discriminative-stimulus effects of ethanol. Thus, the effects of DHEA on the discriminative-stimulus effects of ethanol are similar to those of RO15-4513, a partial inverse agonist at the benzodiazepine receptor site, which negatively modulates the $GABA_A$ receptor complex and has only been shown to modestly attenuate the subjective effects of ethanol (for review, see [14]). When these data are considered together, there seems to be little evidence to suggest that compounds that negatively modulate the $GABA_A$ receptor alter the discriminative-stimulus effects of ethanol even though these

drugs can attenuate some of the other behavioral effects of ethanol.

Another mechanism by which ethanol enhances GABAergic activity indirectly is by increasing presynaptic GABA release [40–42]. Roberto et al. [42] found that direct infusion of 44 mM ethanol to slices of neurons from the central amygdala of rats reduced paired-pulse facilitation and increased the frequency of spontaneous inhibitory post-synaptic potentials and currents (IPSP/IPSCs), changes that the investigators concluded were indicative of increased presynaptic GABA release. In addition, studies have shown increases in the frequency of miniature ISPCs with 100 mM ethanol in Golgi cells from rat cerebellar slices and 70 mM ethanol in spinal motor neurons [40, 41].

Just as GABAergic activity contributes to ethanol's CNS depressant and discriminative-stimulus effects, it is also thought to be integrally involved in mediating the reinforcing effects of ethanol. This notion has been strongly supported by studies showing that decreases in $GABA_A$ receptor activity can decrease ethanol intake. More specifically, $GABA_A$ receptor antagonists as well as inverse agonists at the benzodiazepine receptor site have been shown to decrease both ethanol preference and operant responding for ethanol [43–47]. For example, injection of 2 ng of the competitive $GABA_A$ receptor antagonist SR 95531 directly into the central nucleus of the amygdala decreased operant responding for ethanol in male rats, demonstrating a direct link between $GABA_A$ receptor modulation and the reinforcing effects of ethanol [48]. These results were similar to those with RO15-4513 [49], which has been shown to decrease ethanol intake and to antagonize the intoxicating effects of ethanol [45, 50–52] when administered under an operant schedule of food- and ethanol-reinforced responding. In addition, RO15-4513 has been shown to reverse the memory-impairing effects of ethanol [53–55], and this reversal was attributed to both compounds' interaction with ethanol at the $GABA_A$ receptor.

Although ethanol's effects on the brain are pervasive, the role of GABA and dopamine in parts of the mesolimbic dopamine system, such as the ventral tegmental area, central nucleus of the amygdala, and nucleus accumbens, are of particular interest with regard to the reinforcing effects of ethanol. Ikemoto et al. [56] found that dopaminergic neurons in the anterior and posterior portions of the ventral tegmental area are differentially regulated by $GABA_A$ receptor modulators, as evidenced by a series of studies in which rats self-infused the $GABA_A$ receptor antagonist picrotoxin into the anterior, but not posterior, ventral tegmental area. Conversely, rats self-infused the $GABA_A$ receptor agonist muscimol into the posterior, but not anterior, ventral tegmental area [56, 57]. This was further clarified in a microdialysis study by Ding et al. [58], in which they found that the anterior ventral tegmental area was predominantly under GABA-mediated tonic inhibitory control, whereas the posterior tegmental area was predominantly under the control of dopamine-mediated inhibition. These data, therefore, suggested that the posterior ventral tegmental area may be of more importance than the anterior ventral tegmental area in the reinforcing effects of ethanol. The ventral tegmental area has direct projections to the nucleus accumbens, an area of the brain classically associated with the translation of "motivation to action," or a link between areas of the brain associated with reward and those associated with drug seeking [59]. Furthermore, ethanol consumption in alcohol-preferring rats has been shown to increase extracellular dopamine content in the nucleus accumbens [60].

3. Effects of DHEA on $GABA_A$ Receptors

The discovery of steroid synthesis in the brain quickly resulted in numerous studies into the physiological roles of these "neurosteroids," with an emphasis on their apparent nongenomic effects [29, 61–64]. In 1990, Majewska et al. [65] demonstrated that the sulfated form of DHEA (DHEAS) bound to the $GABA_A$ receptor on rat neurosynaptosomes. Further, they showed that DHEAS binding decreased GABA-mediated current using a whole-cell voltage-clamp technique. Le Foll et al. [66] confirmed these findings using a whole-cell voltage-clamp technique in frog pituitary cells and also determined that 10 μM of DHEA and DHEAS were equally effective at decreasing GABA-induced currents. The next year, Imamura and Prasad investigated the effects of DHEA and DHEAS on GABA-mediated chloride influx in neurosynaptosomes derived from rat cortex, hippocampus, and cerebellum. These investigators determined the effects of multiple concentrations of DHEA and DHEAS on GABA-mediated chloride influx and concluded that DHEAS altered chloride influx with greater potency than DHEA [67]. Park-Chung et al. [68] also found a difference in potency between DHEA and DHEAS, as 100 μM DHEAS was nearly twice as effective at decreasing GABA-induced current as an equal concentration of DHEA in *Xenopus* embryos expressing $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors.

Because multiple studies have shown that DHEAS is more potent than DHEA, the binding characteristics of DHEAS at the $GABA_A$ receptor have been more widely studied and characterized. Studies that have investigated the putative binding sites for DHEAS suggest that neurosteroids that are negative modulators of the $GABA_A$ receptor complex act at sites distinct from those that are positive modulators. For some neurosteroids, such as pregnanolone, the addition of a negatively charged sulfate group changes the GABA-modulating capacity of the neurosteroid from positive to negative. Substitution of a hemisuccinate group for the sulfate imparts the same effect on modulator activity, indicating that the negative charge of the compound influences its activity [68]. These data support the suggestion that sulfated and unsulfated steroids modulate $GABA_A$ receptor activity through different sites [68, 69]. Unfortunately, similarities between DHEAS and DHEA binding are unknown, and additional research will be necessary to clarify these issues.

The binding sites for neurosteroids that positively modulate the $GABA_A$ receptor complex, such as 3α,5α-THP and THDOC, have been more thoroughly investigated [62, 70, 71]. The results from these studies have indicated that these steroids act at one of two putative neurosteroid binding sites. The first site is thought to reside within the transmembrane domains of the α and β subunit interface, whereas the

second site is thought to reside on the α subunit (for review, see [70]). The failure of DHEA to attenuate the pregnanolone-induced disruptions in behavior maintained under a differential-reinforcement-of-low-rate schedule [72] suggests that DHEA binds to a site on the $GABA_A$ receptor separate from pregnanolone. Therefore, DHEA is suspected to act at a site on the $GABA_A$ receptor distinct from the binding site of sulfated neurosteroids such as DHEAS and from neurosteroids that are positive modulators of the $GABA_A$ receptor such as pregnanolone.

Despite the differences in potency between DHEA and DHEAS, DHEA may have greater clinical utility because of its capacity to cross the blood-brain barrier. The sulfate group of DHEAS imparts greater hydrophilicity to the compound, largely limiting its capacity for diffusing into the central nervous system without first being hydrolyzed to the free steroid [73]. The more lipophilic DHEA crosses the blood-brain barrier in large amounts, as evidenced by recent work in this laboratory. In this experiment, adult male Long-Evans rats were administered 56 mg/kg of DHEA and then sacrificed along with vehicle-treated control rats at time points ranging from 15 minutes to six hours after injection for brain steroid analysis. Steroids were extracted using the solid-phase technique established and validated by Newman et al. [74] and then analyzed using a commercially-available ELISA (DHEA Saliva ELISA kit, IBL International, Hamburg, Germany). As shown in Figure 1, DHEA levels in the hippocampus, hypothalamus, and frontal cortex of the brain were over twentyfold greater than in vehicle-treated controls. In fact, fifteen minutes following intraperitoneal (i.p.) injection, DHEA was present at concentrations shown by Majewska [61] to negatively modulate the $GABA_A$ receptor.

General support for the behavioral effects of DHEA as a negative modulator of the $GABA_A$ receptor complex comes from a study by Amato et al. [72], who demonstrated that the acute effects of DHEA administration on behavior were similar to other negative or neutral $GABA_A$ modulators under a differential-reinforcement-of-low-rates (DRL) schedule in rats. This study compared a variety of positive modulators to DHEA and the negative modulator β-CCM and the neutral modulator flumazenil across several dependent measures. Interestingly, DHEA was similar to β-CCM and flumazenil in producing little or no effect on response rate or the temporal pattern of responding. These findings directly contrast with the effects of the positive modulators ethanol, pregnanolone, lorazepam, and pentobarbital on behavior maintained under the same schedule, as these drugs increased response rate and disrupted the temporal pattern of responding.

The negative modulators of the $GABA_A$ receptor complex also contrast with the positive modulators in terms of their effects on anxiety. For instance, the benzodiazepines and barbiturates that positively modulate the $GABA_A$ receptor complex typically decrease anxiety in animal models as indicated by increases in suppressed behavior [78–80], time spent in open arms of the elevated plus maze [81, 82], and exploration in the open field test [83]. In humans, benzodiazepines are prescribed clinically as anxiolytics. Unlike these drugs, the negative modulators such as the beta carbolines that are inverse agonists at the benzodiazepine binding site are anxiogenic [82]. While one would expect all negative modulators of the $GABA_A$ receptor complex to be anxiogenic, this does not seem to be the case for DHEA as several studies have demonstrated that DHEA is anxiolytic in situations involving chronic stress [84–86]. Presumably, the anxiolytic effects of DHEA can be attributed to its antiglucocorticoid properties, especially considering most subjects under chronic stress have increased levels of cortisol. The DHEA/cortisol ratio is of particular significance, as the antiglucocorticoid effects of DHEA are postulated to be the means by which DHEA was able to reduce depression in humans [87, 88]. Charney [84] has also suggested that DHEA may be valuable for reducing the response to stress, particularly in patients with post-traumatic stress disorder.

In addition to the antiglucocorticoid effects of DHEA, the capacity of DHEA to modulate the release of other GABA-modulating neurosteroids may also be involved in its anxiolytic effects. DHEA administration has been shown to increase peripheral levels of 3α,5α-THP in postmenopausal women that received 25 mg/day for three months [89] and both peripheral and CNS levels of 3α,5α-THP in female

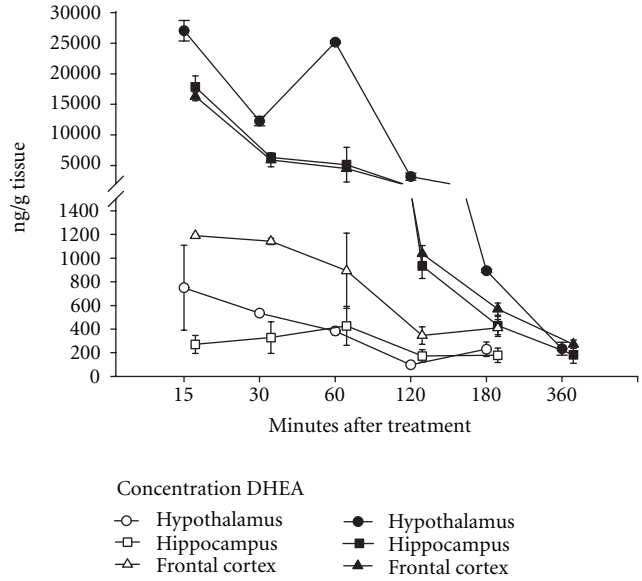


Figure 1: The amount of DHEA (ng/g) present in the hypothalamus, hippocampus, and frontal cortex of rats over a six-hour time period after a single acute intraperitoneal injection. Adult male Long-Evans rats received either 56 mg/kg of DHEA ($n = 6$) or an equal volume of cyclodextrin vehicle ($n = 5$). A DHEA-treated subject was sacrificed with a vehicle-treated control at 15, 30, 60, 120, and 180 minutes after injection, while the final DHEA-treated subject was sacrificed 360 minutes after injection. Brains were collected, flash frozen, and later dissected using the Glowinski technique [75]. Steroids were extracted from the hypothalamus, hippocampus, and frontal cortex of each subject using the solid-phase extraction method described and validated by Newman et al. [74]. Briefly, tissue from each region was prepared in an aqueous matrix and steroids were extracted from each sample using a C18 column primed with ethanol and equilibrated with water. Each sample was eluted, dried, and resuspended in deionized water, and DHEA levels were determined using ELISA.

rats that received 2 mg/kg for 14 consecutive days [90]. Together with the finding that two weeks of DHEA administration decreased central levels of pregnenolone sulfate [91], another neurosteroid that negatively modulates the $GABA_A$ receptor [64, 66], these data suggest that long-term DHEA administration may increase overall GABAergic tone despite its capacity for negatively modulating the $GABA_A$ receptor complex acutely. Whether this is a direct effect of DHEA or a compensatory response to chronic DHEA remains an important question that will require further investigation.

As mentioned previously, the five subunits that comprise the $GABA_A$ receptor complex affect the responsiveness of these receptors to various endogenous and exogenous substances such as the neurosteroids and benzodiazepines [71, 92, 93]. Moreover, the repeated stimulation of $GABA_A$ receptor subtypes can induce changes in the subunits comprising these receptors. The α4 subunit, for example, has been shown to be particularly sensitive to changing levels of neurosteroids [93] and was upregulated following chronic administration of progesterone (a precursor to 3α,5α-THP) and following withdrawal of progesterone treatment. This particular subunit is also of interest because it was upregulated following chronic treatment with benzodiazepines, and its expression decreased the sensitivity of the $GABA_A$ receptor complex to benzodiazepines [94]. For this reason, $GABA_A$ receptors containing an α4 subunit are often referred to as "benzodiazepine insensitive" receptors. The capacity of $GABA_A$ ligands to modify $GABA_A$ receptor subunit expression is, therefore, another putative mechanism by which DHEA treatment might alter ethanol intake and preference.

This notion led us to investigate the effect of DHEA administration on the expression of the α4 subunit of the $GABA_A$ receptor complex. In this study, twenty-four drug-naïve male Long-Evans hooded rats received either 56 mg/kg of DHEA ($n = 12$) or vehicle ($n = 12$) daily for a ten-day period. On the final day of treatment, subjects were sacrificed and the brains collected for analysis. Quantitative analysis of mRNA transcripts indicated that DHEA-treated rats had an approximately threefold increase in expression of α4 subunit mRNA in the hypothalamus compared to vehicle-treated controls, as shown in Figure 2. Interestingly, the expression of the α4 subunit mRNA in the frontal cortex did not differ between treatment groups. Together, these data suggested that the capacity of DHEA to alter α4 subunit expression is brain-region dependent, and this was further supported by Western-blot analysis showing that α4 subunit protein expression was increased in the hypothalamus following DHEA treatment compared to control (see Figure 3). In addition, expression of the δ subunit, which is expressed nearly exclusively in receptor complexes with either the α4 or α6 subunits, was not altered by DHEA treatment. More studies are certainly warranted to determine the implication of these findings.

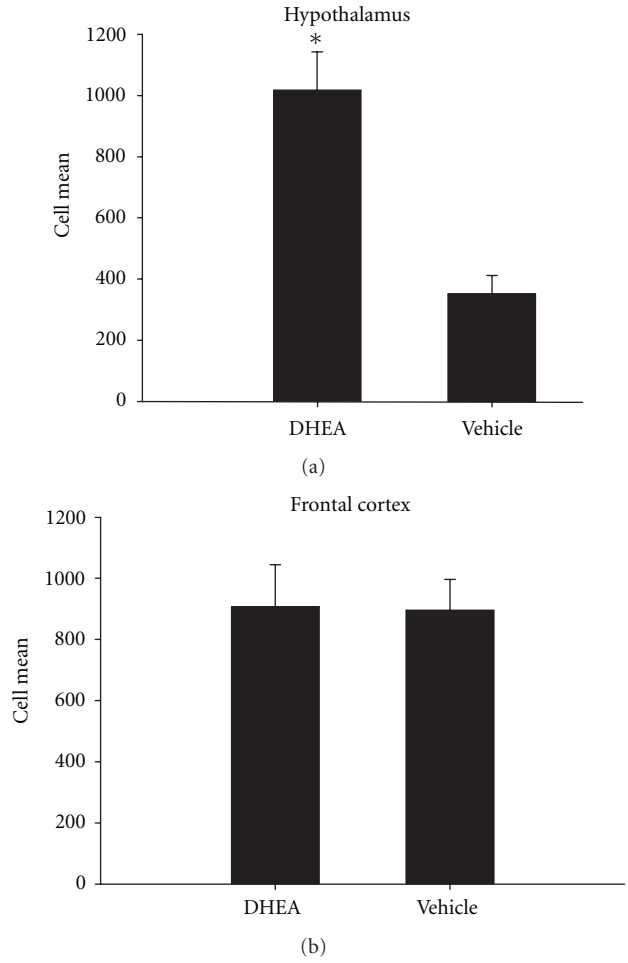
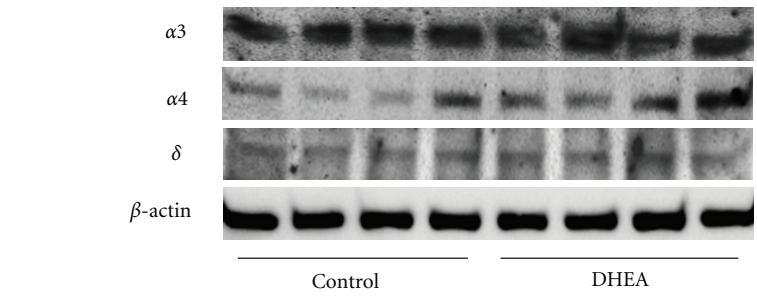


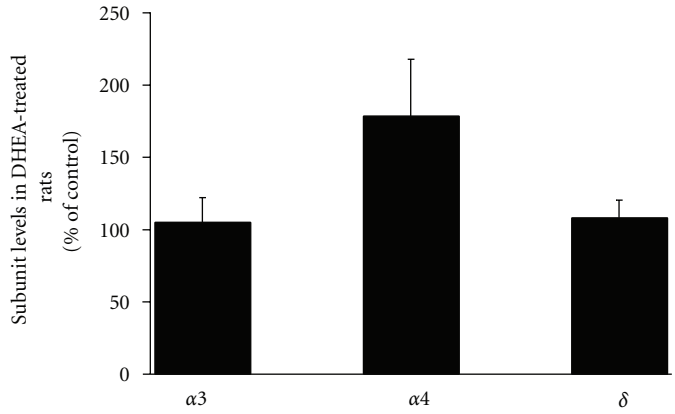
Figure 2: Mean number of $GABA_A$ alpha-4 subunit transcript copies per cell in the hypothalamus (top panel) and frontal cortex (bottom panel) of rats administered 56 mg/kg of DHEA or vehicle. Drug-naïve male rats received either DHEA ($n = 12$) or an equal volume of cyclodextrin vehicle ($n = 12$) for ten consecutive days; on the tenth day, subjects were sacrificed and their brains were collected. Brains were dissected using the Glowinski technique [75], and each brain region was pooled and homogenized. Due to the high lipid content of the samples, a spin column technique was utilized for the RNA extraction. RNA analysis was performed using TaqMan assay kits (Applied Biosystems, Foster City, Calif, USA). Approximately 1 to 2 μL of each sample were used to determine the RNA concentration in each sample using Nanodrop. Values are expressed as a fraction of a normalizing gene, ribosomal 18S RNA.

4. DHEA Decreases Ethanol Intake

Working under the hypothesis that negative modulators of the $GABA_A$ receptor complex generally decrease ethanol intake, we initiated a series of studies to determine if DHEA could produce the same effect. Using a relatively standard ethanol preference procedure, our first study compared the effects of DHEA and pregnanolone on home-cage ethanol intake and found that DHEA was more effective at reducing the intake of an 18% (v/v) ethanol solution than pregnanolone [95], which has been shown to positively modulate the $GABA_A$ receptor complex. These results were important for several reasons. First, they showed that the neurosteroids remain a relatively unexplored class of drugs with enormous therapeutic potential. Second, they showed that neurosteroids with the capacity to negatively modulate the $GABA_A$



(a)



(b)

Figure 3: Expression of the $\alpha 4$, $\alpha 3$, and δ subunits of $GABA_A$ receptors in the hypothalamus of rats administered 56 mg/kg of DHEA for 10 consecutive days as measured by Western blot analysis. 100 μg of tissue from each area was resuspended in lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 0.5 mM sodium orthovanadate, 2 mM okadaic acid, 10% glycerol, 1% Nonidet P40, 2% protease inhibitor) and processed for protein extraction using MicroRoto for Lysis Kit (Bio-Rad, Hercules, Calif, USA). The Bradford Method [76] was used to determine protein concentration, and then samples were diluted, separated by SDS-PAGE, and transferred to nitrocellulose PDVF membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were immunoblotted for two hours at room temperature with two specific antibodies, a rabbit anti-$\alpha 4$ antibody at a 1 : 500 dilution (Santa Cruz Biotechnology, Santa Cruz, Calif, USA), and a mouse anti-β-actin diluted in a proportion of 1 : 2000 (Santa Cruz Biotechnology). A specific secondary antibody (PerkinElmer Life Sciences, Waltham, Mass, USA) followed at a dilution of 1 : 2000. Expression was visualized using ECL Plus (PerkinElmer) and a Fuji Film luminescent image analyzer (LAS-1000 Plus, Fuji Photo Film Co. Ltd., Tokyo, Japan). The images were then quantified by densitometry using the Image Gauge program [77], and the expression value of each subunit was normalized to β-actin values.

receptor complex may be as valuable, or more valuable, as therapeutics for alcohol abuse and dependence than positive modulators, which could putatively serve as substitution therapies for alcohol. An important methodological detail in this study was that neurosteroid injections were administered daily until a criterion for stable ethanol intake was achieved; namely, each dose of neurosteroid was administered until ethanol intake did not vary by more than ±20% for 3 days or for a total of 8 days, in which case the last 3 of those 8 days were used for comparison purposes. This criterion was largely instituted because (1) the intake of low concentrations of ethanol (or low doses of other self-administered drugs) is inherently variable, (2) acute administration of a potential therapeutic may not always be representative of a drug's capacity to reduce self-administration, and (3) therapeutics for drug dependence are generally administered chronically as opposed to acutely. However, using this criterion raised several critical questions regarding DHEA's mechanism of action. First, were multiple injections necessary to achieve the effect on ethanol intake, and second, were the decreases in ethanol intake an effect of DHEA or one of several metabolites including the sex hormones testosterone and estradiol?

To address these questions, Worrel et al. [96] administered 7-keto DHEA, a metabolite of DHEA that is not metabolized to testosterone or estradiol [97], to the subjects from the Gurkovskaya et al. [95] preference study and found that this compound produced effects as large as DHEA. In fact, while 10 mg/kg of 7-keto DHEA produced an effect comparable to DHEA, 56 mg/kg of 7-keto DHEA produced a larger decrease in ethanol intake than DHEA. Another important aspect of this study was that 7-keto DHEA decreased ethanol intake after the initial injection, which occurred 15 minutes prior to the 30-minute preference

session when ethanol and water were presented. Together, these data indicated that a major metabolite of DHEA might be responsible for the effect of DHEA, metabolism of DHEA to the sex hormones was not necessary to produce an effect on ethanol intake, and repeated administration was not necessary to produce an effect with 7-keto DHEA. Given the onset of the effect of 7-keto DHEA on ethanol intake, these data also suggest that a nonsteroidal, rather than steroidal, mechanism of action might be responsible for DHEA's observed effects.

In more recent studies conducted in our laboratory, we have established ethanol self-administration under an operant schedule of reinforcement in order to compare the effects of DHEA (and 7-keto DHEA) on voluntary versus schedule-controlled ethanol intake. To establish ethanol-maintained behavior, rats were trained to respond under a fixed-ratio 10 schedule in which every 10 responses on a lever dispensed 0.1 mL of 18% ethanol to a concave spout located on the front wall of an operant chamber. After ethanol intake stabilized under these contingencies, the substitution of different ethanol concentrations was undertaken to compare the concentration-effect curve for ethanol under the FR-10 schedule with the curve established under the home-cage preference procedures. Interestingly, although intake of the lower concentrations of ethanol was more robust under the home-cage preference procedure than the operant procedure, the intake and dose of ethanol between the two procedures was more similar for the higher ethanol concentrations. In particular, substitution of a 32% ethanol concentration for the 18% ethanol concentration produced similar intake in milliliters and in the dose consumed (see Figure 4). More important, doses of DHEA that decreased ethanol intake under the home-cage preference procedure also decreased ethanol intake under an operant procedure (e.g., 56 mg/kg; Figure 5).

As a means of showing the potential developmental influence of DHEA on ethanol preference, we administered DHEA, lorazepam, or vehicle to three groups of male rats during adolescence and then assessed preference and intake of ethanol during adulthood [98]. Lorazepam was included specifically as a comparison to DHEA, because it is well known as a positive allosteric modulator of the $GABA_A$ receptor complex. Briefly, each group of adolescent rats received a total of 15 injections (12 of one dose and 3 of a higher dose) on postnatal days (PND) 35–64, and then after a period of no treatment received 23-hours access to water, saccharin, or an ethanol/saccharin solution over several days on two separate occasions (PND 88 and again at PND 111). On the last occasion, the concentration of ethanol in the ethanol/saccharin solution was also increased to determine if the adolescent treatments altered the concentration-effect curves for each group. In general, this study demonstrated that lorazepam administration during adolescence increased adult preference for ethanol compared to vehicle or DHEA administration, whereas DHEA decreased adult preference for ethanol and saccharin compared to vehicle administration. These data were remarkable not only because they showed the long-term effect of positive allosteric modulation of $GABA_A$ receptors on later, adult ethanol intake, but they suggest the potential for endogenous levels of DHEA to play an integral role in shaping adult preference and intake either through its putative effects on the $GABA_A$ receptor complex or through other as yet unknown mechanisms [98].

5. Summary and Conclusions

Although the exact mechanism by which DHEA decreases ethanol intake is still under investigation, studies from both the literature and our laboratory strongly indicate that it can interact both directly and indirectly with the $GABA_A$ receptor complex and that its behavioral effects are very similar to those of several other negative $GABA_A$ receptor modulators. Consistent with data generated over the past several years [21, 22], our data also emphasize the potential role of extrasynaptic $GABA_A$ receptors in the interaction of the neurosteroid DHEA and alcohol. For instance, recent electrophysiological and biochemical data have indicated that $GABA_A$ receptors containing a δ subunit are potently affected by both ethanol and neurosteroids and that these "extrasynaptic" receptors likely contribute to tonic IPSP and IPSCs in many brain regions. Furthermore, $GABA_A$ receptors with δ subunits are thought to be associated exclusively with $\alpha 4$ and $\alpha 6$ subunits *in vivo*. If this is the case, the upregulation of the $\alpha 4$ subunit could then affect the responsiveness of δ-containing $GABA_A$ receptors, and ultimately, the behavioral effects of ethanol or the neurosteroids.

Similar to our molecular data pointing to a DHEA-ethanol interaction, our behavioral studies show that DHEA can dose dependently decrease ethanol intake in outbred rats. Interestingly, some of these data were gathered prior to definitively knowing whether peripherally administered DHEA crossed the blood brain barrier and whether DHEA or one of its hormonal metabolites was responsible for the effect. Since then, however, we have conducted studies showing that DHEA readily crosses the blood brain barrier after peripheral administration (data shown above) and that metabolism of DHEA to one of the sex hormones (i.e., either testosterone or estradiol) is not necessary to obtain the decrease in ethanol intake [96]. Moreover, we have shown DHEA can decrease ethanol intake that is voluntary [95] or controlled by an operant schedule of reinforcement.

Unfortunately, the effect of DHEA on ethanol intake cannot be attributed exclusively to its capacity for negatively modulating $GABA_A$ receptor though there is a significant amount of data showing that this capacity may be its most prominent nongenomic effect [65, 67]. Without question, the difficulty identifying a binding site for DHEA on the $GABA_A$ receptor complex has made the investigation into DHEA's mechanism of action more problematic. As indicated in this paper, the binding site for DHEA would seem to be different from the site for sulfated neurosteroids [68, 69] and from the site for positive $GABA_A$ modulators [70]. From a behavioral perspective, however, DHEA produces effects similar to other negative modulators in rats responding under at least one operant schedule of reinforcement (i.e., a DRL schedule). The most notable exception to DHEA's profile as a negative modulator seems to be its capacity for

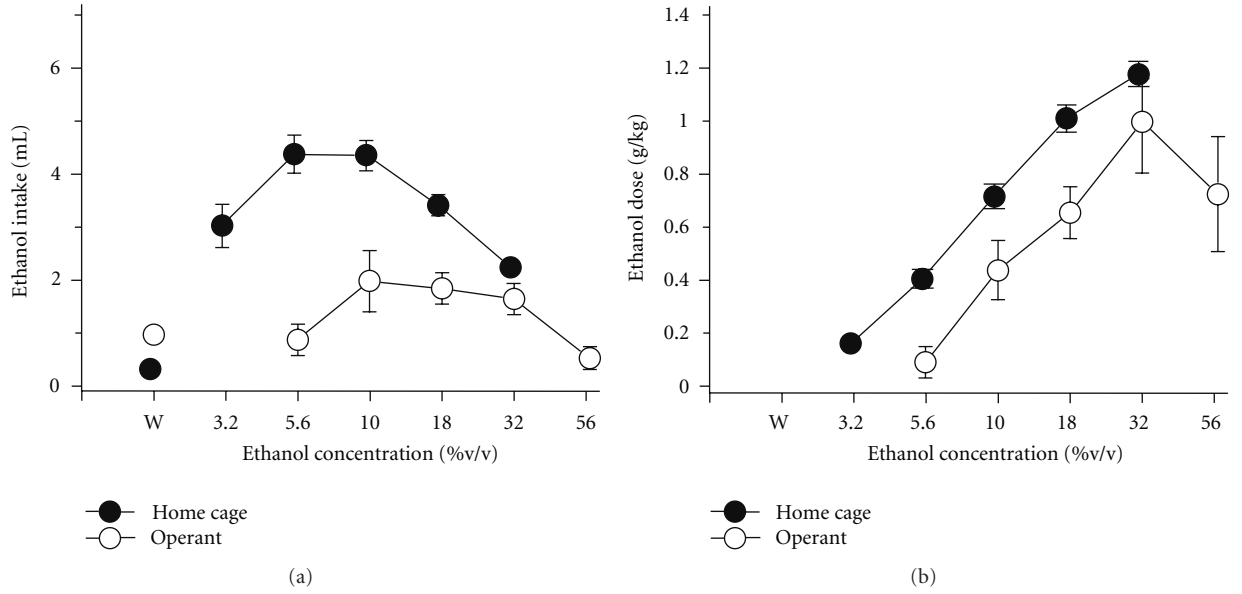


Figure 4: Effects of ethanol concentration on ethanol intake (mL) and the dose of ethanol (g/kg) consumed under home cage ($n = 22$) and operant ($n = 5$) self-administration procedures. Filled circles represent voluntary home cage ethanol intake, whereas unfilled circles represent operant ethanol intake under a fixed ratio (FR) 10 schedule of reinforcement. The points and vertical lines above "W" indicate the means ± standard error of the mean (SEM) for sessions in which water was available (control). The points with vertical lines in the concentration-effect data indicate the mean ± SEM for each ethanol concentration. The points without vertical lines indicate instances in which the SEM is encompassed by the point.

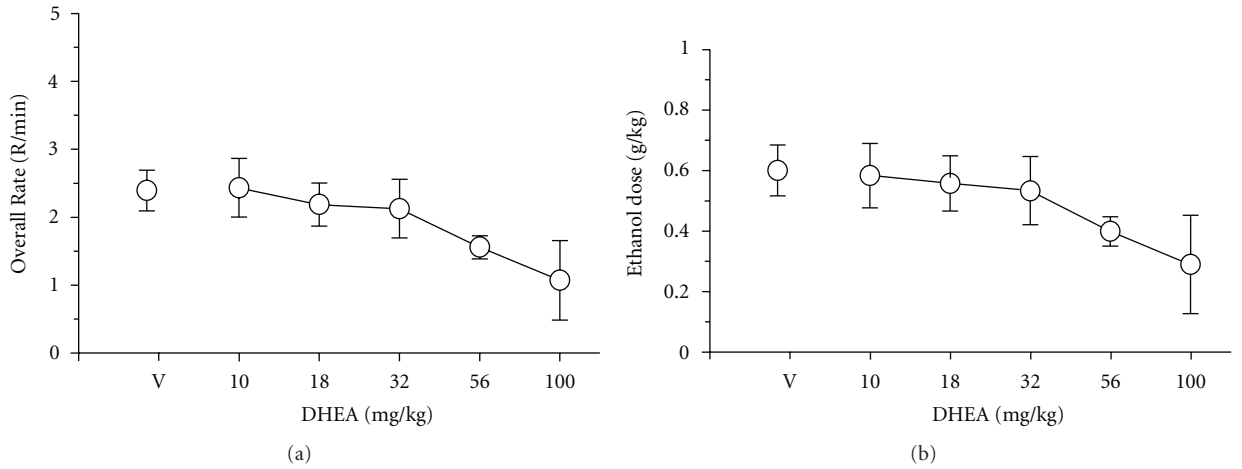


Figure 5: Effects of intraperitoneal administration of DHEA on rats ($n = 5$) responding under an FR-10 schedule for 0.1 mL of 18% (v/v) ethanol. The dependent measures were response rate in responses/min and the dose of ethanol presented in g/kg. The points and vertical lines above "V" indicate the means and standard error of the mean (SEM) for sessions in which vehicle was administered (control). The points with vertical lines in the dose-effect data indicate the mean ± SEM for sessions in which DHEA was administered. The points without vertical lines indicate instances in which the SEM is encompassed by the point.

producing anxiolytic, rather than axiogenic, effects [84–86]. This could be viewed as a therapeutic benefit for a medication that is used to treat alcohol abuse and dependence. Furthermore, unlike negative modulators such as RO15-4513, there is very little evidence that DHEA or 7-keto DHEA have proconvulsant effects [49, 99]. By contrast, numerous small clinical trials with DHEA have shown adverse effects predominantly related to the androgenic effects of DHEA [100, 101]. For instance, common adverse effects in women taking 200 mg of DHEA per day include acne and hirsutism. These effects may be averted, however, by administering 7-keto DHEA, which is not converted to sex hormones [97] and reduces ethanol intake similarly to DHEA [96].

Acknowledgments

This work was supported by USPHS AA09803 (P. J. Winsauer) and AA019848 (M. E. W. Hulin) from the National

Institute on Alcohol Abuse and Alcoholism. Special thanks to Ms. Conni Corll for her expert assistance in quantifying α-4 subunit mRNA.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2011, Article ID 153218, 11 pages
doi:10.1155/2011/153218

Research Article

Chronic Treatment with a Promnesiant GABA-A α5-Selective Inverse Agonist Increases Immediate Early Genes Expression during Memory Processing in Mice and Rectifies Their Expression Levels in a Down Syndrome Mouse Model

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Received 6 April 2011; Revised 27 July 2011; Accepted 31 July 2011

Academic Editor: Naheed R. Mirza

Decrease of GABAergic transmission has been proposed to improve memory functions. Indeed, inverse agonists selective for α5 GABA-A-benzodiazepine receptors (α5IA) have promnesiant activity. Interestingly, we have recently shown that α5IA can rescue cognitive deficits in Ts65Dn mice, a Down syndrome mouse model with altered GABAergic transmission. Here, we studied the impact of chronic treatment with α5IA on gene expression in the hippocampus of Ts65Dn and control euploid mice after being trained in the Morris water maze task. In euploid mice, chronic treatment with α5IA increased IEGs expression, particularly of *c-Fos* and *Arc* genes. In Ts65Dn mice, deficits of IEGs activation were completely rescued after treatment with α5IA. In addition, normalization of *Sod1* overexpression in Ts65Dn mice after α5IA treatment was observed. IEG expression regulation after α5IA treatment following behavioral stimulation could be a contributing factor for both the general promnesiant activity of α5IA and its rescuing effect in Ts65Dn mice alongside signaling cascades that are critical for memory consolidation and cognition.

1. Introduction

Down syndrome (DS) affects 0.45% of human conceptions [1] and is the first cause of mental retardation. This disorder is induced by total or partial trisomy of human chromosome 21 (HSA21) that delays both physical and mental development of affected children. In particular, cognitive skills, including learning and memory functions, are severely impaired in DS subjects.

Although being the focus of intense research activity [2, 3], attempts at developing treatments for counteracting cognitive defects in DS patients have not yet been successful.

Since fifteen years, DS animal models have been engineered to mimic DS physiopathogeny. Ts65Dn mice [4], one of the most studied DS models, have 167 three-copy genes corresponding to more than half of the genes from HSA21. These mice develop gradual learning and memory impairments when compared to euploid animals (for review, see [5]) in conjunction with morphological anomalies. In addition, Ts65Dn mice show abnormal synaptic plasticity as exemplified by long-term potentiation (LTP) deficits [6].

Data from recent years strongly suggest that changes in LTP and associated learning and memory function in DS mice might result from an imbalance between excitatory and

inhibitory neurotransmission. More precisely, it has been demonstrated that increased GABAergic activity in the brain of Ts65Dn mice could be responsible for altered cognitive phenotypes [7, 8], opening new avenues for therapeutic opportunities. Treatments relying on GABA-A antagonists such as picrotoxin and pentylenetetrazole (PTZ) have indeed rescued deficits in DS mice; GABA-A antagonists can restore normal LTP [8] and also normalize cognitive phenotypes in learning tests such as the novel object recognition [9] and Morris water maze [10]. Altogether, these studies suggest the potential use of GABA antagonists for stimulating cognitive performances in DS subjects. However, it is known that such drugs also have convulsant effects which definitively preclude their use as cognitive enhancers in humans.

As an alternative to GABA-A antagonists, GABA-A inverse agonists such as β-carbolines acting at the benzodiazepine recognition site decrease the efficacy of GABAergic transmission and have promnesiant effects [11–14]. Their use in humans is, however, hampered by their convulsant/proconvulsant and anxiogenic side effects [15, 16].

It is nonetheless assumed that various pharmacological profiles can be obtained using ligands with specific affinities for the different α1, α2, α3, or α5 benzodiazepine receptor subtypes [17] that are unevenly distributed in the brain [18–20]. It is known that the α5 subunit-containing receptors are largely expressed in the hippocampus [21, 22], a brain region involved in learning and memory processes that is dysfunctional in DS individuals [23, 24]. In mice, invalidation or mutation of the gene coding for the α5 subunit potentiate synaptic plasticity [25] and concurrently improve cognitive performances [25–27] without inducing anxiogenic or proconvulsant/convulsant side effects [28–32]. Recently, we demonstrated that acute and chronic treatment with an α5-selective inverse agonist, and referred to herein as compound α5IA, initially developed by Merck Sharp and Dohme Research Laboratories [33, 34] can restore cognitive deficits in a DS mouse model [35]. We further showed that following treatment with α5IA, the immediate early gene (IEG) product Fos is selectively increased in brain regions involved in learning and memory in control and DS mice [35].

In order to obtain insights into gene regulation pathways involved in the pharmacological effect of α5IA, we studied gene expression profiles in hippocampi of euploids and Ts65Dn mice behaviorally exposed to the Morris water maze (MWM) task, and treated or not with α5IA. We found that chronic treatment with α5IA globally increases the expression of IEGs and in particular of *c-Fos* and *Arc*. These effects could be related to the memory-enhancing properties classically described for α5IA [28–32].

In Down syndrome mice, we observed an abnormally low level of IEG induction after behavioral stimulation. In addition, some three-copy genes were significantly overexpressed, including *Sod1* gene. Chronic treatment of Ts65Dn mice with α5IA normalized the expression level of *Sod1* and in parallel restored a physiological level of IEGs expression. This double-action mode can explain the rescuing effect observed following α5IA treatment in DS mice.

2. Material and Methods

2.1. Animals. Male mice were produced at the Intragene resource center (TAAM, CNRS UPS44 Orléans, France) and bred on a mixed genetic background B6C3⟨B⟩, derived from C57BL/6J (B6) and a congenic inbred line C3H/HeH for the BALB/c wild-type Pde6b allele [36], thus avoiding retinal degeneration and impaired visual acuity. On this background, Ts65Dn mice showed similar behavioral phenotypes when compared to the original Ts65Dn line (AD and YH, personal communication; see also [37]). Mice were acclimated in our animal facility for at least 2 weeks before initiating behavioral testing. All experiments were conducted in accordance with the ethical standards of French and European regulations (European Communities Council Directive of 24 November 1986). A total number of 24 mice (12 Ts65Dn and 12 euploid mice) were behaviorally trained in the MWM task. Only a subset of these animals (7 and 10-11 mice per genotype for microarray and QPCR analysis, resp.) were processed for the molecular biology analysis described in the present work, (Figure 1).

2.2. α5IA Synthesis and Formulation. The drug used is 3-(5-methylisoxazol-3-yl)-6-(1-methyl-1,2,3-triazol-4-yl)methyloxy-1,2,4-triazolo[3,4-a]phthalazine (α5IA). It was synthesized by Orga-Link SARL (Magny-les-Hameaux, France), according to Sternfeld and collaborators [34]. The hydrochloride salt was prepared by dissolving the base in hot ethanol and adding a solution of 5% hydrochloric acid in ethanol until the solution was slightly acidic. Upon cooling, a precipitate formed which was collected by filtration, washed with cold ethanol, and dried.

The HCl salt of α5IA was solubilized in a mixture of DMSO, Cremophor El (BASF, Ludwigshafen, Germany), and hypotonic water (ProAmp) (10 : 15 : 75). α5IA or vehicle (solubilization solution) was injected intraperitoneally (i.p.) at 5 mg/kg [35].

2.3. Morris Water Maze. Cognitive stimulation was performed during 12 consecutive days in the MWM as previously described [35]. In brief, training consisted in a goal-location task in a pool (2–4 learning trials per daily sessions). Euploid and Ts65Dn mice were injected each day with either vehicle or α5IA (6 mice per group), 30 min before starting the session. Distance travelled to find the platform has been used as learning index. We compared the performances of vehicle-treated Ts65Dn mice to the 3 other groups (α5IA-treated Ts65Dn mice, vehicle-treated euploids, and α5IA-treated euploids) using Student's *t*-tests. Statistical significance was set to a P value <0.05.

2.4. cRNA Probe Preparation and Hybridization. Twenty five min following the last MWM training session, animals were sacrificed, and their brains were extracted and processed for gene expression profiling. Total RNAs were obtained from frozen individual hippocampi and treated with DNAse using NucleoSpin RNA II kit (Macherey Nagel, France) in accordance with the manufacturer's protocol. The quality

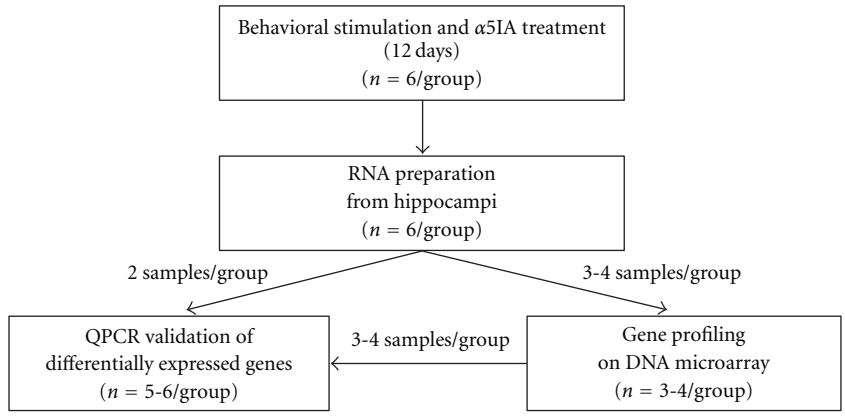

Figure 1: Experimental design used for the genomic studies: microarray and QPCR.

and quantity of each RNA preparation were assessed on the Agilent 2100 Bioanalyzer with RNA 6000 NanoChips (Agilent Technologies).

Hundred ng of each RNA were amplified and labelled with Cy3 using the Low Imput Quick Amp labeling kit (Agilent Technologies) according to the manufacturer's instructions. After purification and quantification on a Nanoview (ThermoFisher Scientific), 2 μg of each Cy3-cRNA were hybridized overnight on Whole Mouse Genome 4 × 44 K v1 Microarrays (Agilent Technologies) according to the manufacturer's instructions.

2.5. Microarray Expression Data Analysis. Microarrays data were acquired on an Innoscan 900 (Innopsys, France) with a resolution of 2 μm and analyzed with Mapix 5.0.0 software (Innopsys, France). For each array, raw data consisted of the median feature intensity and background feature intensity (F-B) at wavelength 532 nm. These raw data were log 2 transformed and quantile normalized under the R freeware (http://www.r-project.org/). Analysis of variance (ANOVA) with two main factors, Genotype (Ts65Dn versus euploid) and Treatment (α5IA versus vehicle), was then performed on the normalized data using the software MeV 4.6.2 (http://www.tm4.org/mev/). Gene ontology (GO) category enrichment analysis was realized using the web-based GOrilla application (http://cbl-gorilla.cs.technion.ac.il/). Statistical significance was set to a P value <0.05.

2.6. Real-Time Quantitative PCR. RNAs from dissected hippocampi (500 ng) were individually reverse transcribed into cDNAs overnight at 37°C using the Verso cDNA kit (ThermoFisher Scientific, Waltham, USA) according to the manufacturer's instructions. qPCR assays were performed in a Lightcycler 480 System (Roche), in the presence of 200 nM of each primer: (*c-Fos* 5′agggagctgacagatacactcc3′_forward and 5′tgcaacgcagacttctcatc3′_reverse; *Homer1* 5′gatggagctgaccagtaccc3′_forward and 5′tggtgtcaaaggagactgaaga3′_reverse; *Ifnar2* 5′ggacagcgttaggaagaagc3′_forward and 5′tggaagtaagtctctaaggacaaatg3′_reverse; *Egr2* 5′ctacccggtggaagacctc3′_forward and 5′aatgttgatcatgccatctcc3′_reverse; *Bdnf* 5′agtctccaggacagcaaagc3′_forward and 5′tgcaaccgaagtatgaaataacc3′_reverse; *Arc* 5′ggtgagctgaagccacaaat3′_forward and 5′ttcactggtatgaatcactgctg3′_reverse; *Sod1* 5′caggacctcattttaatcctcac3′_forward and 5′tgcccaggtctccaacat3′_reverse; *pPib* 5′ttcttcataaccacagtcaagacc3′_forward and 5′accttccgtaccacatccat3′_reverse for normalization), 100 nM of specific hydrolysis probe (designed with Universal Probe Library, Roche Applied Science) and 1X Lightcycler 480 Probes Master mix (Roche, France) and normalized using the Lightcycler 480 SW 1.5 software. Data were analyzed using an analysis of variance (ANOVA) with two main factors: genotype (Ts65Dn versus euploid) and treatment (α5IA versus vehicle), and Fisher's post hoc complementary analysis was carried out when required by the experimental design to assess complementary statistical effects. Pearson correlation coefficients between IEGs expression and behavioral data (mean distance travelled during the first three days trial of the MWM task) were calculated. All analyses were performed using Statistica v6 (StatSoft, Inc., Tulsa, Okla, USA) or GraphPad Prism (GraphPad Software, La Jolla, Calif, USA) softwares. Statistical significance was set to a P value <0.05.

3. Results

3.1. Gene Expression Profiles after Treatment with α5IA in Euploid and Ts65Dn Mice Hippocampi. We showed previously that Ts65Dn mice are impaired in the MWM task and that their learning proficiency can be restored following α5IA treatment [35]. In the present study, mice were trained in the MWM task using a similar protocol and treated daily with α5IA (5 mg/kg). Behavioral data were analyzed although the number of animals was small (5 or 6 per group) and precluded any robust statistical analysis (ANOVA). During the three first training sessions, vehicle-treated Ts65Dn mice travelled a longer distance to find the platform as compared to α5IA-treated Ts65Dn mice or euploid mice treated with vehicle or α5IA ($t_{16} = 7.23$; $P = .016$; Figure 2). Thus, as previously described in [35], we showed that behavioral deficit of the Ts54Dn mice in the MWM task was recovered following α5IA treatment. Twenty five minutes after completion of this long-term behavioral stimulation (12 days),

Table 1: Analysis of variance (ANOVA) of microarray data: genotype (Euploids versus Ts65Dn mice) and treatment (Vehicle versus α5IA-treated) were the two main factors. Three-copy genes and IEGs were analyzed separately.

		Analysis of variance (ANOVA) of microarray data		
	Total	Genotype-modulated genes	Treatment-modulated genes	Interaction-modulated genes
All genes	13024	848	781	1260
3N genes	56	6	3	5
	Genes	*GART*, *Ifnar-2*, *Kcnj6*, *Itsn1*, *Hlcs*, and *Sod1*	*App*, *Kcnj6*, and *Sod1*	*Cbr*1, *Gabpa*, 4931408A02Rik, *Hmngn1*, and *Pcp4*
IEGs and *BDNF*	16	5(***)	3(*)	1
	Genes	*BDNF*, *Cox2*, *Homer1*, *GS2*, and *Arc*	*Fos*, *Egr2*, and *BDNF*	*BDNF*

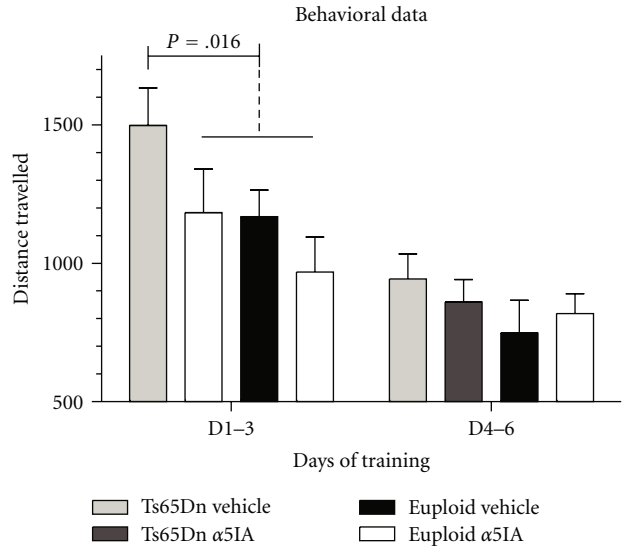

Figure 2: Effects of α5IA treatment in Ts65Dn mice trained in the MWM task. TS65Dn mice had impaired performance in the MWM task (comparison with other groups: $P < .025$). This deficit was rescued by treatment with α5IA.

mice were sacrificed and their hippocampi dissected. RNAs were extracted and amplified, labeled using *in vitro* transcription, and then hybridized on microarrays.

Among 41,000 genes present on the microarray, 13,024 were found to be expressed. Data were normalized and analyzed using ANOVA with two factors: genotype and treatment (Table 1). We found an effect of genotype (euploid versus Ts65Dn) on 848 genes representing 4.52% of whole genes expressed, an effect of treatment (vehicle versus α5IA 5 mg/kg) on 781 genes (4.17%) and a genotype-treatment interaction effect on 1,260 genes (6.73%). Principal component analysis (PCA) using all the genes expressed did not reveal any segregation of animal groups (data not shown), indicating that genotype and treatment did not globally affect expression profiles. Among the differentially expressed genes, we searched for enrichment of genes belonging to particular gene ontology (GO) categories. As shown in Table 2, 17 and 19 GO categories were significantly enriched among genes differentially expressed according to genotype and treatment, respectively. Eighteen of these GO categories were directly related to neurogenesis (Table 2 in bold).

We then analyzed the expression data focusing on genes of interest such as the expressed genes that are in three copies in Ts65Dn mice (3N genes, $n = 56$) and immediate early genes (IEGs, $n = 16$) which are involved in active memorization processes, and the product of which was shown to be increased by α5IA treatment in a previous study [35].

3.2. Gene Expression Changes of 3N Genes after α5IA Treatment. PCA on the 56 3N genes expressed in hippocampus showed a partial segregation between euploid and Ts65Dn mice (Figure 3). Expression levels of 3N genes were significantly different in Ts65Dn mice as compared to euploid mice (t-test $P < .05$) with a mean ratio of 1.13 and 1.10 between ts65Dn and euploid mice under vehicle or α5IA treatment, respectively, suggesting a global increase of expression of three-copy genes. ANOVA with two factors (genotype and treatment) specifically on 3N genes revealed an effect of genotype on 6 genes that represented 10.71% of the total number of expressed triplicate genes (56): *Gart, Ifnar-2, Kcnj6, Itsn1, Hlcs*, and *Sod1* (Table 1). The mean expression ratio Ts65Dn/Euploid for these 6 three-copy genes was found to be 1.22 ($t_{12} = 3.49$; $P = .0045$). In addition, we found that α5IA treatment impacted on the expression levels of 3 three-copy genes (*App, Kcnj6,* and *Sod1*). Interactions between genotype and treatment were significant for 5 genes (*Cbr1, Gabpa*, 4931408A02Rik, *Hmngn1,* and *Pcp4*). Among differentially expressed genes, there was no significant enrichment of 3N genes. However, a strong tendency to overrepresentation of three-copy genes modulated by the genotype factor in comparison to the overall distribution was observed (chi-square: $P = .057$; Table 1).

Among these genes modulated by the genotype factor, our attention was particularly drawn to the *Sod1* gene whose role in DS was largely demonstrated [38, 39]. In order to confirm the microarray results, QPCR analysis was performed on RNAs from 13 mice (3-4 per group) used for microarray and on RNAs from onther 8 mice (2 per group). Two-way ANOVA analysis on *Sod1* gene showed a treatment by genotype interaction effect ($F_{1,17} = 16.77$; $P < .00005$; Figure 4). *Sod1* expression level of vehicle-treated Ts65Dn mice increased in comparison to vehicle-treated mice

Table 2: Analysis of GO categories among the genes differentially expressed in the hippocampus of Ts65Dn mice after α5IA and behavioral stimulation. In bold GO categories related to neurogenesis processes.

GO term	Description	P value
Enrichment analysis of GO biological processes associated with genotype		
GO:0051272	Positive regulation of cellular component movement	$2.93E-4$
GO:0007216	**Metabotropic glutamate receptor signaling pathway**	$\mathbf{2.96E-4}$
GO:0032879	Regulation of localization	$3.3E-4$
GO:0016265	**Death**	$\mathbf{3.55E-4}$
GO:0009798	Axis specification	$3.58E-4$
GO:0006414	Translational elongation	$3.77E-4$
GO:0030335	**Positive regulation of cell migration**	$\mathbf{4.21E-4}$
GO:2000147	**Positive regulation of cell motility**	$\mathbf{4.21E-4}$
GO:0008219	**Cell death**	$\mathbf{4.49E-4}$
GO:0065008	Regulation of biological quality	$4.66E-4$
GO:0012501	**Programmed cell death**	$\mathbf{5.3E-4}$
GO:0008624	**Induction of apoptosis by extracellular signals**	$\mathbf{5.44E-4}$
GO:0040017	Positive regulation of locomotion	$7.32E-4$
GO:0008283	**Cell proliferation**	$\mathbf{7.77E-4}$
GO:0000578	Embryonic axis specification	$7.96E-4$
GO:0006916	**Antiapoptosis**	$\mathbf{8.14E-4}$
GO:0007049	**Cell cycle**	$\mathbf{9.3E-4}$
Enrichment analysis of GO biological processes associated with α5IA treatment		
GO:0046883	Regulation of hormone secretion	$4.35E-5$
GO:0030335	**Positive regulation of cell migration**	$\mathbf{4.89E-5}$
GO:2000147	**Positive regulation of cell motility**	$\mathbf{4.89E-5}$
GO:0040017	Positive regulation of locomotion	$9.19E-5$
GO:0051272	Positive regulation of cellular component movement	$1.04E-4$
GO:0048869	**Cellular developmental process**	$\mathbf{1.49E-4}$
GO:0007176	**Regulation of epidermal growth factor receptor activity**	$\mathbf{3.38E-4}$
GO:0040008	**Regulation of growth**	$\mathbf{5.16E-4}$
GO:0051270	Regulation of cellular component movement	$5.9E-4$
GO:0000302	**Response to reactive oxygen species**	$\mathbf{5.98E-4}$
GO:0030334	**Regulation of cell migration**	$\mathbf{6.81E-4}$
GO:0048519	Negative regulation of biological process	$7.57E-4$
GO:0009888	**Tissue development**	$\mathbf{8.46E-4}$
GO:0006012	Galactose metabolic process	$9.12E-4$
GO:0009896	Positive regulation of catabolic process	$9.15E-4$
GO:0030154	**Cell differentiation**	$\mathbf{9.24E-4}$
GO:2000145	**Regulation of cell motility**	$\mathbf{9.33E-4}$
GO:0035413	Positive regulation of catenin import into nucleus	$9.67E-4$
GO:0031331	Positive regulation of cellular catabolic process	$9.98E-4$

($F_{1,17} = 7.01$; $P < .05$) with a mean ratio of 1.27 (Figure 4). Treatment reduced the level of *Sod1* expression in α5IA-treated Ts65Dn mice ($F_{1,17} = 14.43$; $P < .01$). Thus, *Sod1* expression in Ts65Dn mice under α5IA treatment was similar to vehicle-treated euploid mice, suggesting that chronic treatment with α5IA allowed *Sod1* to return to a physiological level of expression in the hippocampus of Ts65Dn mice.

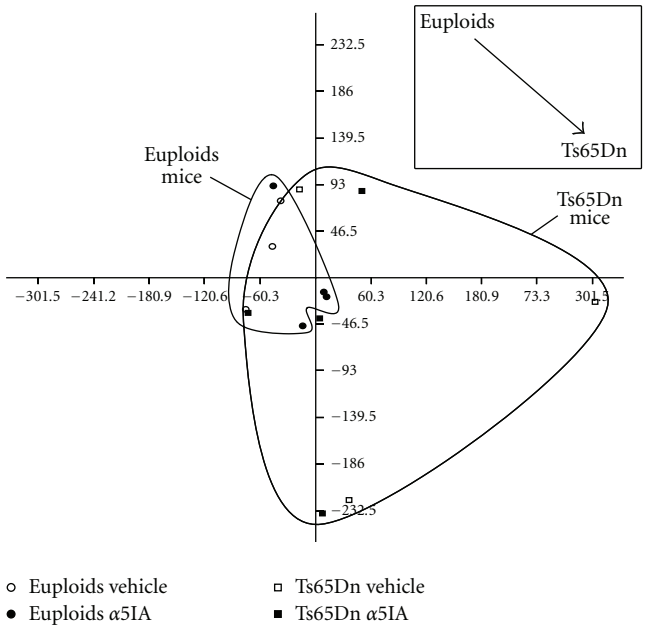

Figure 3: PCA on three-copy genes from Ts65Dn mice measured on microarrays. The first two principal components discriminated between euploid and Ts65Dn mice.

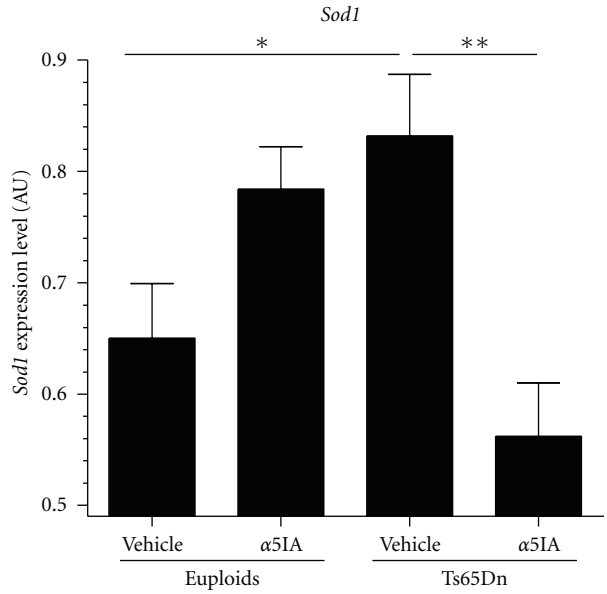

Figure 4: QPCR expression level of *Sod1* gene in euploid and Ts65Dn mice after chronic treatment with vehicle or α5IA. $^{*}P < .05$, $^{**}P < .01$, and two-way ANOVA with Fisher's post hoc comparisons.

3.3. Gene Expression Changes of IEGs Genes after α5IA Treatment. PCA on the 16 IEGs expressed in the hippocampus showed a total separation between euploid and Ts65Dn mice but also a partial segregation as a function of treatment (Figure 5). Two-way ANOVA on these 16 IEGs revealed an effect of genotype (euploid versus Ts65Dn) on 5 genes (*Bdnf, Cox2, Homer1, RGS2,* and *Arc*), an effect of treatment (vehicle versus α5IA 5 mg/kg) on 3 genes (*c-Fos*, *Egr2,* and *Bdnf*), and a genotype by treatment interaction effect on one gene (*Bdnf*). Among the differentially expressed genes, there was a significant enrichment of IEGs modulated by genotype and treatment (chi-square: genotype $P < .001$, chi-square: treatment $P < .05$) supporting an effect of genotype and treatment on IEGs expression levels (Table 1).

We selected 4 IEGs classically described as expressed during behavioral stimulation: *Arc*, *Homer1*, *c-Fos,* and *EGR2* for validation using QPCR analysis on 21 samples (5-6 per group), 8 of which were not used in the microarray experiments (Figure 6). Two-way ANOVA analysis with within-subjects design on IEGs expression showed genotype ($F_{1,16} = 7.90$; $P < .05$), treatment ($F_{1,16} = 11.72$; $P < .01$), and gene ($F_{4,64} = 91.66$; $P < .001$) effects. The expression level of IEGs was increased in euploid mice (treated or not with α5IA) as compared to Ts65Dn mice (Fisher's post hoc test: $P = .012$). The mean expression ratio Ts65Dn/euploid for these 4 genes was found to be 0.82. In contrast, α5IA-treated mice (euploid or Ts65Dn) showed higher levels of expression of IEGs relative to vehicle-treated mice (Fisher's post hoc test: $P = .0034$). The mean expression ratio α5IA/vehicle for these genes was 1.29.

Individual IEGs expression levels were normalized against the vehicle-treated euploid mice value that corresponds to the physiological level of expression. In euploid mice, all IEGs increased after α5IA treatment. This increase was statistically significant for *c-Fos* and *Arc* genes (one sample t-test: $t_4 = 6.44$, $P = .003$, and $t_4 = 2.89$; $P = .04$, resp.). In Ts65Dn mice, the basal level of expression of IEGs was lower as compared to euploids. *C-Fos* and *Egr2* expression was drastically reduced (one sample t-test: $t_3 = 3.62$, $P = .036$, and $t_3 = 20.38$; $P = .0003$, resp.). Interestingly, IEGs expression profiles were normalized to euploid mice levels after α5IA treatment. In addition, we found inverse correlations between the expression levels of *Fos, Egr2, Homer1,* and *Arc* deduced from QPCR and the mean distance travelled during the first three training sessions of the MWM ($-0.645 < r < -0.494$; Figure 7).

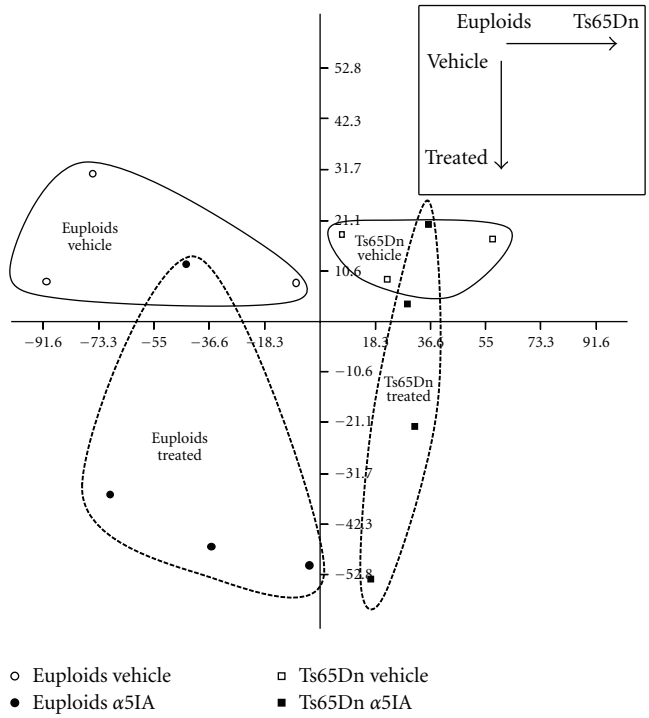

Figure 5: PCA on IEGs expression measured on microarrays. The first principal component fully discriminated euploid and Ts65Dn mice. The second principal component partially discriminated vehicle- and α5IA-treated mice.

4. Discussion

We have previously shown that treatment with α5IA alleviates learning and memory deficits of Ts65Dn mice [35] We also demonstrated that α5IA increased the expression of the IEG product Fos in specific brain regions involved in learning and memory following cognitive stimulation. Importantly, following α5IA administration, both genotypes were observed to display significant and comparable Fos induction. This potentiation of brain activity might therefore be the substratum of the general promnesiant effects of α5IA independently of the disease status. In order to gain more insight into mechanisms of the general promnesiant effects as well as the rescuing effects in Ts65Dn mice, we studied gene expression regulation networks in mice trained in the MWM task. During this continuous training episode, mice received daily injections of α5IA (5 mg/kg) for a total of 12 days. Gene expression was then measured using DNA microarrays from hippocampal RNA extracts obtained 30 min after the last training session.

4.1. Hippocampal Gene Expression Networks Regulated by α5IA in Control Euploid Mice

4.1.1. Effects on HSA21 Genes. Microarray and QPCR analysis on the expression of genes from the region of mouse chromosome 16 which is triplicated in Ts65Dn mice and is orthologous to human chromosome 21 (HSA21) genes did not reveal any effect of chronic treatment with α5IA. These results suggest that genes from this triplicated region are not interfering with the activity of α5IA and hence do not modify the α5 subunit-containing GABA-A-benzodiazepine receptors or their signaling pathways.

4.1.2. Effects on IEGs Expression during Memory Processes. Following memory stimulation, chronic treatment with α5IA enhanced IEG activation in euploid mice. It is likely that higher IEG transcripts following α5IA treatment will result in an increase of IEG protein products (e.g., Fos protein)

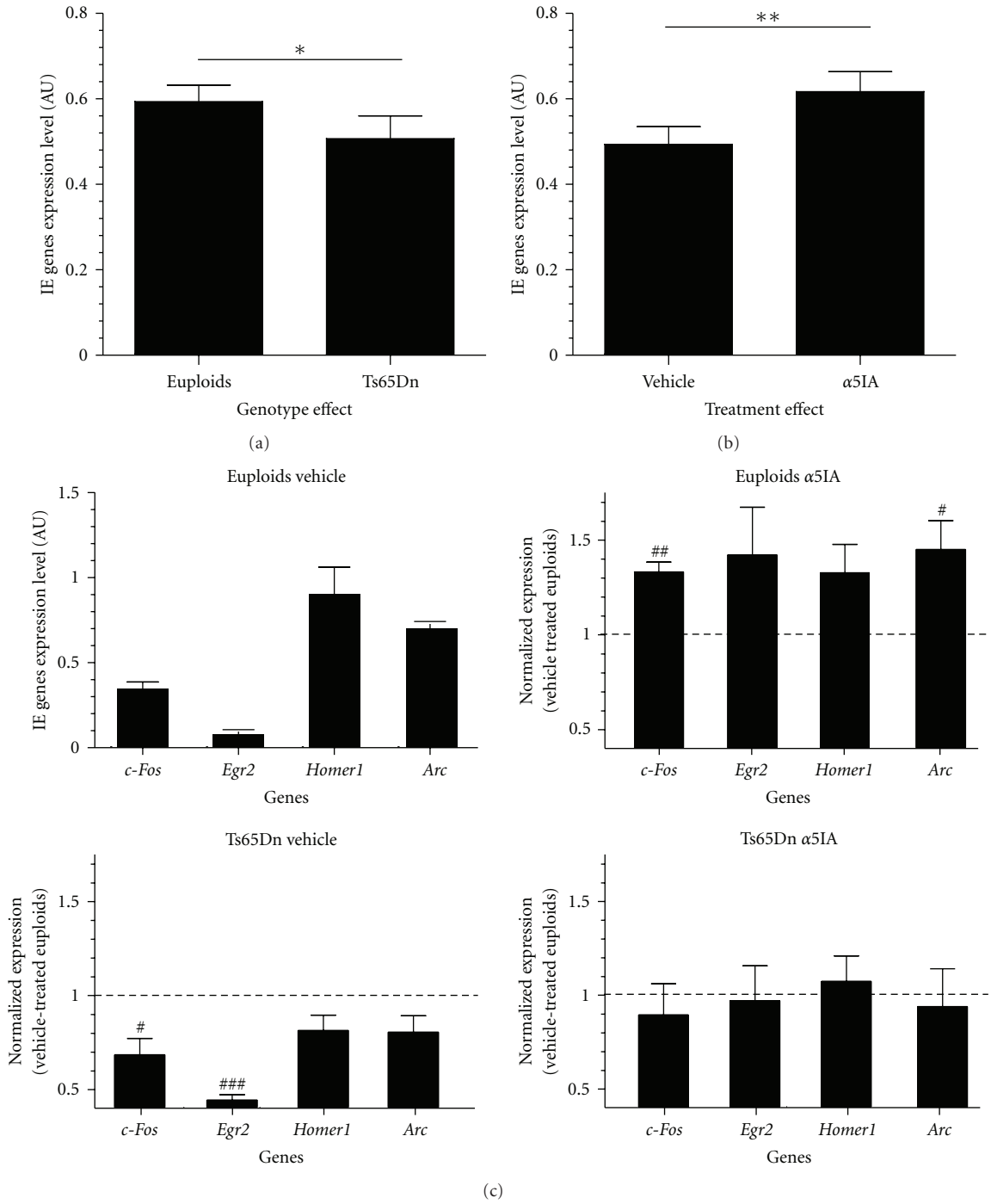

Figure 6: QPCR expression level of selected IEGs. (a) Mean cumulated expression levels of five selected IEGs in euploid and Ts65Dn mice; (b) effect of α5IA treatment on the mean level of expression of five selected IEGs; (c) expression of *c-Fos, Egr2, Bdnf, Homer-1,* and *Arc* in the four genotype and treatment experimental groups. $^{*}P < .05$, two-way ANOVA with Fisher's post hoc comparisons; $^{\#}P < .05$, $^{\#\#\#}P < .001$, and one-sample t-test.

as confirmed in our previous study [35], while without behavioral stimulation, α5IA did not increase the IEG product Fos (data not shown). These results suggest a state dependency (cognitive stimulation) of the effects of α5IA on IEG expression. IEG expression regulation after α5IA treatment following behavioral stimulation could be a contributing factor for both the general promnesiant activity of α5IA and its rescuing effect in Ts65Dn mice alongside signaling cascades that are critical for memory consolidation and cognition.

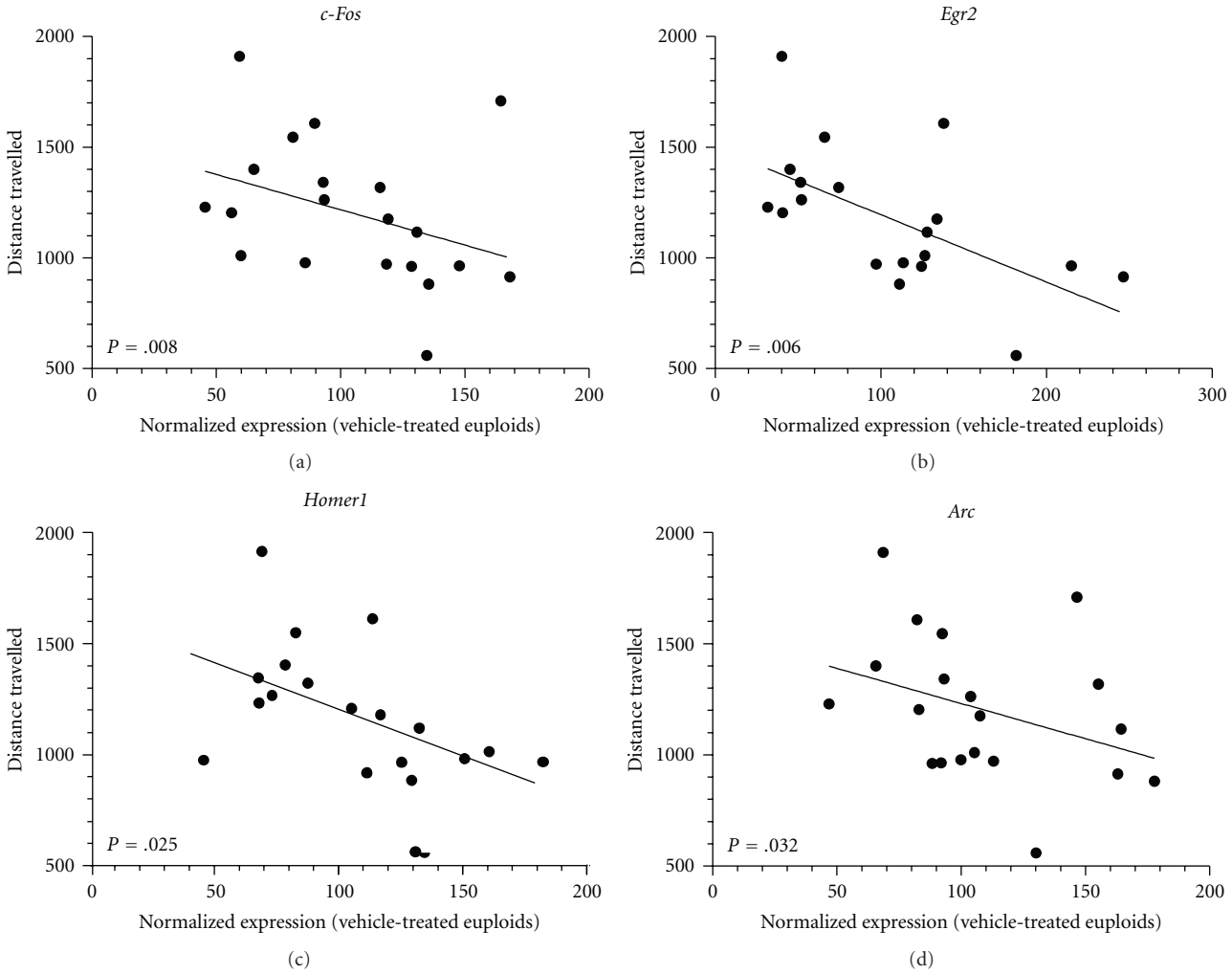

Figure 7: Correlation between IEGs expression levels and behavioral performances. Expression levels of IEGs were negatively correlated to the mean distance travelled during MWM testing ($-0.645 < r < -0.494$, Pearson correlation) underlining a tight relationship between learning proficiency and IEGs activation.

4.2. Hippocampal Gene Expression Networks Regulated by α5IA in Ts65Dn Mice

4.2.1. Effects on HSA21 Genes

Gene Expression Differences between Ts65Dn and Euploid Hippocampi. Of the 108 HSA21 genes present on the microarray and expressed in the hippocampus, only 6 were differentially expressed between Ts65Dn and euploid mice: *Gart, Ifnar-2, Kcnj6, Itsn1, Hlcs* and, *Sod1*. To our knowledge, this is the first time that gene expression profiles have been established in the hippocampus of adult Ts65Dn mice. We focused our attention on the *Sod1* gene which has been extensively studied in DS and confirmed our results using QPCR. LTP deficits observed in Ts65Dn [8] could be due to overexpression and thus increased activity of *Sod1* in the hippocampus since overexpression of *Sod1* gene in transgenic mice is sufficient to impair LTP [40]. Increased level of *Sod1* has also been shown to enhance the sensitivity to degeneration and apoptosis leading to a reduction of hippocampal neuronal progenitors [41]. This could explain enrichment for numerous GO categories related to neurogenesis among the genes differentially expressed between Ts65Dn and euploids. Such effects on neurogenesis-related genes may contribute to the memory deficits observed in Ts65Dn mice [42] and also in humans with memory dysfunction [43]. Since *Sod1* and IEGs expressions are inversely modulated in Ts65Dn mice as compared to euploids before and after treatment, it could also be speculated that *Sod1* and IEGs are functionally regulated, IEGs inhibiting *Sod1* expression and conversely increased *Sod1* levels decreasing IEGs after behavioral stimulation.

Chronic Treatment with α5IA Restores the Expression of Sod1in Ts65Dn Mice to Normal Physiological Levels. Chronic

treatment of Ts65Dn mice with α5IA normalized the level of expression of *Sod1* in the hippocampus. Although the exact mechanisms responsible for this effect are unclear, we can postulate that chronic treatment with α5IA could alleviate cognitive deficits at least partly through the normalization of *Sod1* expression in the hippocampus. Since *Sod1* overexpression impairs hippocampal neurogenesis and long-term synaptic plasticity, α5IA could reverse these deleterious effects by decreasing the expression levels of *Sod1*. Stimulating or restoring neurogenesis might thus participate in the recovery of cognitive functions of Ts65Dn mice, as suggested also by enrichment of GO categories associated with proliferation and cell death among differentially expressed genes.

4.2.2. Effects on IEGs Expression

Reduction of IEG Activation Pattern in Ts65Dn Mice. It is known that IEGs play a key role in learning and memory mechanisms which are deficient in Ts65Dn mice. Indeed, long-term memory requires activation of specific IEGs [44]. Neuronal IEGs mostly code for transcription factors, growth factors, cytoskeletal proteins, metabolic enzymes, or proteins involved in signal transduction [45]. Memorization of new information requires the establishment of a pattern of IEGs expression. It has been shown that age-related memory deficits in rats result in an overall decrease in the expression of IEG in the hippocampus, particularly *Homer-1*, *Arc*, and different members of EGR family [46]. After behavioral stimulation in the MWM task, we found, using microarray and QPCR, a significant reduction in the overall level of IEGs in Ts65Dn mice as compared to euploids. This reduction was observed in particular for *c-Fos* and *Egr2*. For *Homer-1*, and *Arc,* decreased expression in Ts65Dn mice was just below the level of significance. This could be due to differences in the kinetics of waves of expression of these particular IEGs [44].

Chronic Treatment of Ts65Dn Mice with α5IA Restores Normal IEG Activation Pattern. Ts65Dn mice treated chronically with α5IA showed normalized levels of activation of IEG following memory stimulation, particularly *c-Fos, Egr2, Homer-1* and *Arc* that could be related to the recovery of MWM performance deficits observed in the present study and demonstrated previously [35]. Normalization of the activation profile of IEG following behavioral stimulation could thus be responsible for the rescuing effects of α5IA observed in Ts65Dn mice. The close relationship between IEGs expression levels and cognitive performances was indeed suggested by the significant inverse correlations found between the expression levels of *Fos, Egr2, Homer1* and *Arc* and performances in the MWM task. In addition, α5IA treatment was shown to restore Ts65Dn mice performances in the MWM task and to normalize the expression levels of *c-Fos, Egr2, Homer-1*, and *Arc*.

4.3. General Promnesiant and Rescuing Effects of α5IA. The general α5IA promnesiant effect observed in euploid and Ts65Dn mice could be explained by the acute pharmacological action of the drug directly on α5 GABA-A-benzodiazepine receptors. Indeed, α5 inverse agonists decrease GABAergic transmission and promote the excitability of postsynaptic neurons in rodents [28–32]. The previously described increase of Fos protein immunoreactivity after short-term memory stimulation combined with α5IA acute treatment could thus be the consequence of immediate release of GABA inhibition [35]. Following repetitive cognitive stimulations as in the MWM task that involves hippocampus-dependent memory, we evidenced IEGs activation deficits in the hippocampus of Ts65Dn mice that were rescued after chronic α5IA treatment. However, we cannot exclude that a single injection of α5IA would have a similar effect on IEGs expression levels. It thus appears that the rescuing effects of α5IA on long-term memories are more likely the consequence of hippocampal IEGs expression normalization than the long term effect of repetitive GABAergic modulations. In addition, as mentioned above, it is likely that the normalization of *Sod1* overexpression by α5IA is also important to promote cognitive rescuing.

5. Conclusions

We have identified genomic changes related to chronic treatment with α5IA, an α5-selective GABA-A receptor inverse agonist. Under physiological conditions in which α5IA has been shown to be promnesiant, increase of IEGs activation has been observed and in particular of *c-Fos* and *Arc* genes. This increase of activation could allow a more efficient storage of information during memory process.

Under pathological conditions such as DS in which deficits in learning and memory have been described, we were able to demonstrate an effect of chronic treatment with α5IA at the level of expression of different genes in Ts65Dn mice. Indeed, chronic administration of α5IA restored a normal level of *Sod1* expression which is involved in hippocampal neurogenesis and LTP. In addition, chronic treatment with α5IA normalized the pattern of IEGs activation that is deficient in Ts65Dn mice. These genomic changes observed after chronic treatment with α5IA could be responsible for the restoration of learning and memory functions in Ts65Dn mice.

Acknowledgments

The authors wish to thank the Fondation AMIPI-Bernard Vendre, the AnEUploidy program (LSHG-CT-2006-037627), and the Fondation Jérôme Lejeune for their financial support. They are grateful to Dr. I Rivals for helpful discussions on statistics and to zoo technicians from UPS 44(Orléans) for providing animal care.

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Hindawi Publishing Corporation
Advances in Pharmacological Sciences
Volume 2011, Article ID 790590, 12 pages
doi:10.1155/2011/790590

Review Article

Augmentation of Tonic $GABA_A$ Inhibition in Absence Epilepsy: Therapeutic Value of Inverse Agonists at Extrasynaptic $GABA_A$ Receptors

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Received 17 March 2011; Accepted 16 May 2011

Academic Editor: Keith Wafford

It is well established that impaired GABAergic inhibition within neuronal networks can lead to hypersynchronous firing patterns that are the typical cellular hallmark of convulsive epileptic seizures. However, recent findings have highlighted that a pathological enhancement of GABAergic signalling within thalamocortical circuits is a necessary and sufficient condition for nonconvulsive typical absence seizure genesis. In particular, increased activation of extrasynaptic $GABA_A$ receptors ($eGABA_AR$) and augmented "tonic" $GABA_A$ inhibition in thalamocortical neurons have been demonstrated across a range of genetic and pharmacological models of absence epilepsy. Moreover, evidence from monogenic mouse models (stargazer/lethargic) and the polygenic Genetic Absence Epilepsy Rats from Strasbourg (GAERS) indicate that the mechanism underlying $eGABA_AR$ gain of function is nonneuronal in nature and results from a deficiency in astrocytic GABA uptake through the GAT-1 transporter. These results challenge the existing theory that typical absence seizures are underpinned by a widespread loss of GABAergic function in thalamocortical circuits and illustrate a vital role for astrocytes in the pathology of typical absence epilepsy. Moreover, they explain why pharmacological agents that enhance GABA receptor function can initiate or exacerbate absence seizures and suggest a potential therapeutic role for inverse agonists at $eGABA_ARs$ in absence epilepsy.

1. Introduction

Typical absence epilepsy is characterised by the regular occurrence of nonconvulsive seizures that result in periods of sudden and brief (average ≈10 seconds, range ≈4–40 seconds) loss of consciousness. In the electroencephalogram (EEG), human absence seizures are typified by the appearance of generalized, synchronous, and bilateral "spike (or polyspike) and slow wave discharges" (SWD) occurring at frequencies between 2.5–4 Hz [1, 2]. Although typical absence seizures are significant clinical features of many generalized idiopathic epilepsies (IGEs), as defined by the classification of the International League Against Epilepsy (ILAE) [3], they are the only neurological symptom presented in childhood absence epilepsy (CAE). CAE has an annual incidence of approximately 2–8 per 100,000 children under 16 years of age, with seizure onset typically occurring between 3 and 8 years of age and seizure frequency often as high as several hundred events per day [2]. Absence seizures associated with CAE are not triggered by visual or other sensory stimuli and are not usually associated with neurometabolic or neurophysiological deficits, a factor which is thought to contribute to ~70% spontaneous remission rates in adolescence [2, 4]. Nonetheless, in this pure absence epilepsy phenotype, there is a consensus, based upon older invasive studies and more recent imaging investigations, that seizure genesis and propagation occur as a result of aberrant electrical activity in reciprocally connected thalamic and cortical regions (i.e., thalamocortical circuits) without significant involvement of other brain areas including hippocampus and limbic regions which are often associated with convulsive seizures [5–9]. In fact, recent observations in humans suggest that seizure genesis occurs due to paroxysmal activation of discrete frontal and parietal cortical territories prior to spread into

other cortical and thalamic regions [5–8]. This review will, therefore, focus on the key cellular elements of thalamocortical circuits and in particular upon thalamocortical neurons.

γ-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the brain, and its actions are mediated largely by a family of ubiquitously expressed ligand-gated ion channels known as $GABA_A$ receptors [10]. $GABA_A$ receptors are pentameric assemblies comprising several distinct subunits which open upon GABA binding leading to an increase in membrane permeability to both chloride and bicarbonate ions [11]. Typically this occurs when GABA is released from presynaptic terminals causing a transient rise in GABA concentration within the synaptic cleft and activation of postsynaptic receptors. The resulting brief change in membrane conductance underlies "phasic" $GABA_A$ergic inhibition and generation of the "classical" inhibitory postsynaptic potential (IPSP). However, it has come to light relatively recently that $GABA_A$ receptor activation can occur in a much more spatially and temporally diffuse manner [10]. It has been demonstrated in several brain regions including the cerebellum [12], hippocampus [13], and thalamus [14–16] that very low (nM) concentrations of GABA, which are found in the extracellular space, can persistently activate a population of nonsynaptic $GABA_A$ receptors resulting in a "tonic" increase in membrane conductance. These peri- or extrasynaptic $GABA_A$ receptors ($eGABA_ARs$) differ from their synaptic counterparts in having a significantly higher affinity for GABA as well as markedly slower rates of desensitization [10, 17–19] although it has been recently demonstrated in the visual thalamus that significant desensitization of $eGABA_ARs$ can occur at ambient GABA concentrations [20]. The divergence in the properties of synaptic $GABA_ARs$ versus $eGABA_ARs$ is conferred by receptor subunit composition, in particular, the inclusion of the δ subunit in the case of dentate gyrus granule cells (DGCs), cerebellar granule cells (CGCs), thalamocortical neurons and some cortical neurons [13–16, 21, 22], and α_5 subunits in CA1 and CA3 hippocampal pyramidal cells [23–25]. Nineteen $GABA_A$ receptor subunits have been cloned from the mammalian CNS ($\alpha_{(1-6)}$, $\beta_{(1-3)}$, $\gamma_{(1-3)}$, δ, ε, θ, π, $\rho_{(1-3)}$) offering the potential for an enormous heterogeneity in $GABA_A$ receptor assembly. In reality however, only about twenty to thirty of the potential combinations have been shown to exist in the brain. The most commonly expressed subunit combination is α_1, β_2, γ_2 (with stoichiometry of 2α and 2β subunits and a single γ subunit [26, 27]) whilst other common arrangements include $\alpha_2\beta_3\gamma_2$ and $\alpha_3\beta_3\gamma_2$. Significantly, light microscopic immunofluorescence and EM immunogold methods have established that the postsynaptic densities of GABAergic synapses are highly enriched with receptors including $\alpha_{(1-3)}$, α_6, $\beta_{(2-3)}$, and γ_2 subunits [28, 29] suggesting that these subunits form the $GABA_A$ receptors responsible for classical "phasic" inhibition. However, in contrast to the aforementioned subunits which are enriched in the postsynaptic density but also abundant at extrasynaptic locations [30, 31], some $GABA_A$ receptor subunits, especially δ, are not found in the synapse and are exclusively peri- or extrasynaptically located [21]. Extrasynaptic receptors containing the δ subunit are commonly found to coassemble with α_4 or α_6 subunits ($\alpha_4/\alpha_6\beta_X\delta$) whilst α_5-containing receptors are also mostly extrasynaptic despite usually containing the typically synaptically located γ_2 subunit ($\alpha_5\beta_X\gamma_2$). A recent study by Kasugai et al. [32] has demonstrated the presence of α_1 and α_2 subunits as well as β_3 subunits at extrasynaptic locations on the soma of CA1 pyramidal neurons suggesting these subunits may also contribute to $eGABA_AR$ signalling and perhaps confer specific pharmacological properties.

Thalamocortical (TC) neurons of the dorsal lateral geniculate nucleus (dLGN, visual thalamus) [14], the ventrobasal nuclei (VB, somatosensory thalamus) [14–16], and the medial geniculate body (MGB, auditory thalamus) [33] of rodents have been demonstrated *in vitro* to have robust GABAergic tonic currents. In voltage-clamped TC neurons, application of the $GABA_A$ receptor antagonist SR-95531 not only completely blocks the phasic inhibitory postsynaptic currents (IPSCs) but also produces a reduction in input conductance accompanied by a decrease in current variance that is indicative of block of tonically active $eGABA_ARs$. In the thalamus, it has been estimated that approximately 80–90% of total $GABA_A$ receptor-mediated inhibition occurs through tonic currents resulting from activation of extrasynaptic $GABA_ARs$ [14, 16]. In fact, it has been suggested that tonic conductance in TC neurons (when normalized to whole cell capacitance) may be larger than in other regions expressing $eGABA_ARs$ including the cerebellum and dentate gyrus [16]. In all of the previously described thalamic nuclei, there is a high expression of the $GABA_A$ receptor δ-subunit [22, 33–35], and several studies have shown, using selective pharmacological agents [14–16], δ-subunit knockout ($\delta^{-/-}$) [36] and α_4-subunit knock-out ($\alpha_4^{-/-}$) [37] mice that the thalamic tonic current is mediated largely by $\alpha_4\beta_2\delta$ subunit-containing receptors. In particular, $eGABA_ARs$ in the thalamic nuclei are highly sensitive to the potent and selective activator of α_4-δ-containing [38] receptors 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridin-3-ol (THIP, Gaboxadol) [14–16, 37] as well as ethanol [39], taurine [40], and the anaesthetic isoflurane [41], all of which act to enhance tonic inhibition. Conversely, the α_1-selective agent zolpidem and the nonselective benzodiazepine midazolam increase the decay time of sIPSCs in VB neurons without effects on tonic currents and the inverse agonist Ro 15-4513, a potent activator of α_4-γ_2 subunit-containing receptors [38], also had no effect of tonic current in VB [15]. Functionally, $eGABA_ARs$ in the thalamus have been suggested to play a role in switching the behavioural state-dependent TC neuron firing modes [14] and modulating the temporal precision of rebound low-threshold Ca^{2+} spikes (LTS) [34]. Furthermore, tonic inhibition in TC neurons is likely to play a significant role in the modulation of slow wave sleep (SWS) activity given the integral role of TC neurons in generating low-frequency (<4 Hz) oscillations in corticothalamic circuits [14, 16]. However, the potential importance of $eGABA_ARs$ in pathological seizure activity associated with typical absence epilepsy has only recently been elucidated.

2. Enhanced Tonic $GABA_A$ Inhibition in Thalamocortical Neurons of Genetic Absence Epilepsy Models

It has been demonstrated *in vitro* using several different genetic animal models of absence seizures that the tonic $GABA_A$ current in TC neurons of the VB thalamus is enhanced in animals displaying an epileptic phenotype compared to their respective nonepileptic control animals (Figure 1) [36]. This was first shown in the polygenic GAERS model but has subsequently also been demonstrated for various mice models with known, but divergent, spontaneous monogenic mutations, including stargazer and lethargic mice. In GAERS animals, there is a clear developmental profile for this increased GABAergic function (Figure 1(a)). Up to postnatal day sixteen, the tonic current in VB of GAERS is similar to that of the nonepileptic control (NEC) strain. However, in the 24 hour period between the postnatal day 16-17, there is a significant (almost doubling) increase in the amplitude of the tonic current in VB TC neurons of the epileptic animals [36] that remains elevated well past the time of seizure onset (around the postnatal day 30 in this strain). These data suggest that, rather than occurring as a consequence of seizure onset, the pathological enhancement of tonic GABA inhibition during development in GAERS may be proepileptogenic. Moreover, despite the full developmental profile for the monogenic lethargic and stargazer mice being unknown, it is clear in these models that a significant enhancement of tonic current in TC neurons is present after seizure onset, (Figure 1(b)) [36]. In contrast, no tonic $GABA_A$ current is detected in the GABAergic NRT neurons of GAERS or NEC animals (unpublished observation) as is indeed the case in normal Wistar rats [14].

The pathological augmentation of tonic $GABA_A$ currents in TC neurons of genetic absence models is, however, not due to increased vesicular GABA release, overexpression of δ-subunit containing $eGABA_ARs$, or misexpression of synaptic $GABA_ARs$ but results from a dysfunction of GABA re-uptake by the transporter GAT-1 [36]. In fact, despite being far less abundant in the thalamus than GAT-3 [42], GAT-1 appears to play a major role in the regulation of extrasynaptic GABA concentration and activation of $eGABA_ARs$ [36]. In acute brain slices prepared from both GAERS and stargazer animals, block of GAT-1 using the specific antagonist NO711 produced no effect upon the magnitude of tonic current observed in VB TC neurons, (Figures 1(c), 1(d), and 1(e)). In marked contrast, the block of GAT-1 in neurons of nonepileptic mice and rats facilitated a significant enhancement of tonic current that reached levels similar to those found in neurons from epileptic animals, (Figures 1(c), 1(d), and 1(e)). Furthermore, in nonepileptic animals, blockade of GAT-3 using SNAP5114 resulted in an increase in tonic current that was significantly less than that observed in GAERS or stargazer suggesting that the ability of GAT-1 to compensate for the loss of GAT-3 is erased in the epileptic strains, (Figures 1(c), 1(d), and 1(e)). These findings are made all the more significant by the fact that expression of both GAT-1 and GAT-3 in the thalamus appears to occur exclusively in nonneuronal cells, specifically astrocytes [42, 43]. A malfunction in GAT-1 also underlies the increased tonic $GABA_A$ current in TC neurons of lethargic mice [36]. In contrast to GAERS and stargazer mice; however, the action of this transporter is not inhibited in lethargic mice but appears to be reversed. These data expand upon previous findings that demonstrated a reduction in GABA uptake by GAT-1 [44] and increased levels of extracellular GABA [45] in the VB thalamus of GAERS compared to NEC. Moreover, NO711 increases tonic $GABA_A$ current by a similar amount in dentate gyrus granule cells of GAERS and NEC [36], indicating that GAT-1 activity is not compromised in a brain area that does not participate in the generation of typical absence seizures and where the distribution of this transporter is primarily neuronal. Indeed, the basal tonic current of dentate gyrus granule cells is not different between GAERS and NEC [36], and in stargazer mice, tonic current in both DGCs and CGCs is actually reduced compared to WT littermates [46]. Interestingly, it has been demonstrated previously in CGCs of $GABA_AR$ α_1 subunit knock-out ($\alpha_1^{-/-}$) mice that tonic currents in these neurons are also enhanced via a reduction of GAT activity that is not due to reduction in GAT-1 or GAT-3 expression or increased expression of either α_6 or δ subunit-containing receptors [47].

In summary; therefore, genetic models of typical absence seizures (i.e., GAERS, stargazer, and lethargic mice) show a brain region-specific enhancement of tonic $GABA_A$ current, which in TC neurons is due to increased extracellular GABA level that in turn results from a malfunction in GABA uptake by astrocytic GAT-1.

3. Pharmacological Models of Typical Absence Epilepsy and the Role of $GABA_B$ Receptors

As well as resulting from genetic modifications, SWDs can be generated in genetically "normal" animals through administration of various pharmacological agents. The best-established pharmacological model of typical absence seizures is achieved by the systemic administration of γ-hydroxybutyric acid (GHB) [48–50]. However, it has been known for some time that systemic administration of THIP, a selective agonist at δ subunit-containing extrasynaptic $GABA_ARs$, also elicits SWDs in normal animals, (Figure 2(a)) [51]. In the context of the involvement of enhanced thalamic tonic $GABA_A$ inhibition in several genetic models of absence epilepsy, the pharmacological induction of seizures by THIP becomes more readily explainable. This is because, as previously disclosed, THIP can potently enhance tonic $GABA_A$ currents of TC neurons in nonepileptic rats, (Figure 2(b)), [36] and mice [15, 16], thus mimicking the enhanced thalamic tonic inhibition observed in genetic models. On the other hand, the effects of GHB, which does not bind to $GABA_ARs$ and is believed to elicit absence seizures by activation of $GABA_BRs$ [50], become more difficult to interpret in light of the apparent necessity for enhanced $eGABA_AR$ signalling during SWDs. However, it has now been demonstrated in brain slices of Wistar rats that GHB enhances tonic $GABA_A$ currents in TC neurons, (Figure 2(c))

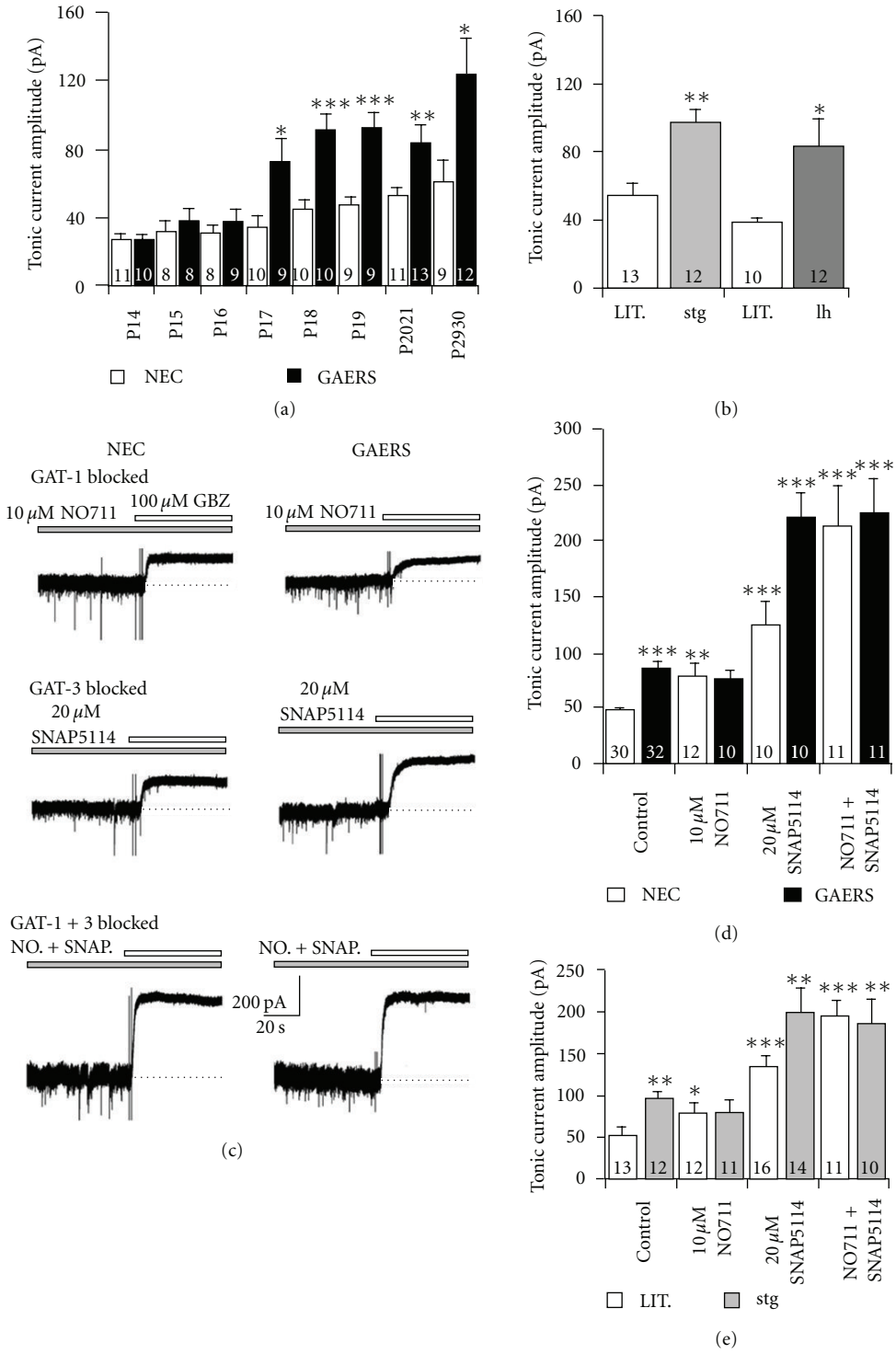

Figure 1: GAT-1 transporter dysfunction produces enhanced tonic $GABA_A$ currents in VB TC neurons in animal models of absence epilepsy. (a) The developmental profile of enhanced thalamic tonic current observed in GAERS animals compared to NEC. At P17 (prior to seizure onset), a significant increase in current amplitude is observed in the epileptic animal that remains elevated up to seizure onset (P30). (b) Tonic $GABA_A$ currents in VB TC neurons of monogenic stargazer (stg) and lethargic (lh hour) mice are significantly greater than nonepileptic littermates after seizure onset. (c) Block of GAT-1 using NO711 in NEC animals elevates tonic current amplitude to levels similar to those observed in GAERS animals. No further enhancement of tonic current in GAERS is observed when GAT-1 is blocked. Block of GAT-3 produces significant increases in tonic current in both NEC and GAERS animals although the increase is smaller in NEC where GAT-1 remains functional. Simultaneous block of GAT-1 and GAT-3 results in very large tonic currents in both GAERS and NEC animals, which are not significantly different from each other. (d) Graph summarising the experiments depicted in (c). (e) Graph depicting the same series of experiments performed in stargazer mice illustrating the similar effects in both models. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Number of recorded neurons for each condition is indicated in bars. (a–e) reproduced from [34].

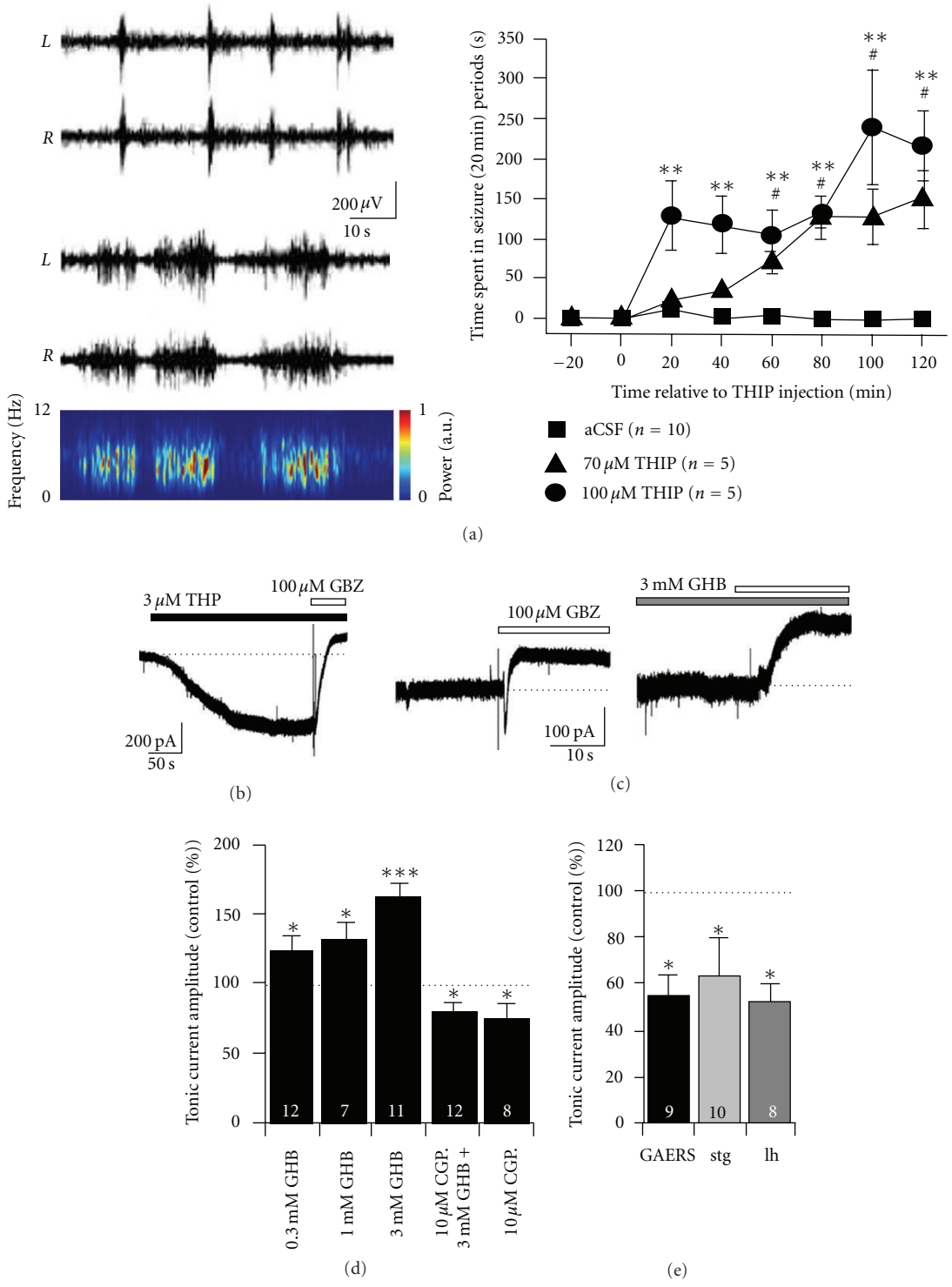

Figure 2: GHB and THIP enhance tonic $GABA_A$ currents in VB thalamus *in vitro* and induce SWDs *in vivo*. (a) Examples of SWDs in bilateral EEG traces recorded from Wistar rats following selective activation of $eGABA_ARs$ by intrathalamic application of THIP via microdialysis (100 μM). The top trace shows seizures occurring in the first hour after THIP administration and the bottom the second hour. The spectrogram (corresponding to the right hemisphere of the lower traces) clearly shows an increase in oscillatory power in the 5–7 Hz range typical of SWDs in rats. The graph (right) summarises the concentration-dependent emergence of SWDs after THIP application as the total time spent in seizure during 20 minutes bins. (b) THIP produces robust enhancement of tonic $GABA_A$ currents in VB TC-neurons in acute brain slices *in vitro*. (c) GHB produces increased tonic current in VB TC neurons. (d) Graph summarising the concentration-dependent enhancement of thalamic tonic currents by GHB and the blocking effect of the $GABA_BR$ antagonist CGP55845. (e) Block of $GABA_BRs$ by CGP55845 produces a reduction of tonic $GABA_A$ currents in VB TC neurons of epileptic GAERS, stargazer, and lethargic mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Number of recorded neurons for each condition is indicated inset into bars. (a–e) reproduced from reference [34].

[36]. The effects on tonic GABAergic inhibition *in vitro* are dose dependent with concentrations used reflecting those that are required to elicit absence seizures *in vivo*, (Figure 2(d)) [52]. Moreover, the effects of GHB are not due to nonspecific binding interactions since the GHB-mediated enhancement of tonic current is negated by the $GABA_BR$ antagonist CGP55845, (Figure 2(d)). In fact, application of CGP55845 alone significantly reduces the tonic $GABA_A$ current amplitude in TC neurons of Wistar rats to 74% of the control values, indicating that facilitation of extrasynaptic $GABA_ARs$ by $GABA_BRs$ contributes approximately one quarter of the tonic $GABA_A$ current in normal rats. Importantly, CGP55845 also reduces the tonic current in GAERS, stargazer, and lethargic mice to about 55, 65, and 57% of control, (Figure 2(e)), respectively, [36] suggesting that facilitation of extrasynaptic $GABA_AR$ function by $GABA_BR$ activation contributes up to half of the pathologically enhanced tonic current in these genetic models.

This GHB-mediated enhancement of thalamic tonic currents is fascinating in the context of another genetic disorder related to GABAergic system function. Succinic semialdehyde dehydrogenase (SSADH) deficiency is an autosomal recessively inherited disorder that results in loss of activity in SSADH (an enzyme responsible for metabolism of GABA), reduced GABA breakdown, and excessive accumulation of both GABA and GHB in the cerebrospinal fluid [53, 54]. Clinical symptoms are varied but include delayed intellectual, speech and language development, ataxia and, significantly, generalised absence seizures [55–57]. Using a recently developed SSADH knock-out ($SSADH^{-/-}$) mouse [53, 58, 59], we have been able to demonstrate that in these animals, which replicate the epileptic phenotype displayed in humans with SSADH deficiency, there is a significant enhancement of tonic $GABA_A$ currents in TC neurons compared to their WT counterparts, Figures 3(a) and 3(c) [60]. Moreover, as previously described for other genetic models of absence seizures, a large proportion of the enhanced tonic current is sensitive to block by $GABA_BR$ antagonists further supporting the role of these metabotropic receptors in the pathology of absence seizures, (Figures 3(b) and 3(d)) [60].

In summary; therefore, a GAT-1 malfunction in thalamic astrocytes of mouse and rat genetic models leads to an increase in ambient GABA in the sensory thalamus, which in turn elicits an enhancement in tonic $GABA_A$ inhibition through direct activation of extrasynaptic $GABA_ARs$ and indirect facilitation of extrasynaptic $GABA_ARs$ via activation of $GABA_BRs$.

4. Enhanced Tonic $GABA_A$ Inhibition of TC Neurons Is Necessary and Sufficient for Typical Absence Seizure Generation

As previously described, SWDs of typical absence epilepsy appear to be initiated in deep layers (V/VI) of the cortex where intracellular recordings show rhythmic paroxysmal depolarisations occurring in phase with the EEG spike [61–63]. The action potentials associated with these synchronous depolarisations in turn providestrong rhythmic input to thalamic nuclei. In NRT neurons *in vivo*, the strong converging corticothalamic input that result from cortical volleys during SWDs produces bursts of excitatory postsynaptic potentials (EPSPs) that trigger T-type Ca^{2+}-channel-mediated LTS and bursts of action potentials. In contrast, TC neurons receive both monosynaptic excitation directly from corticothalamic inputs and disynaptic inhibition via the NRT. *In vivo* intracellular recordings made in GAERS have shown that during ictal activity TC neurons typically receive sequences of one EPSP plus four to six IPSPs arriving in phase with each EEG spike and that action potential firing is rare [62, 64]. This is likely due to the much stronger corticothalamic excitatory inputs into NRT neurons compared to TC neurons [65] and the robust nature of the LTS-driven action potential bursts of NRT neurons [62, 64]. Thus, it is highly probable although it remains to be directly demonstrated that strong GABAergic input into TC neurons during SWDs produces activation of $eGABA_ARs$ and that the corresponding increase in tonic current contributes to the observed downregulation of TC neuron output during ictal activity.

To assess the impact that the enhanced tonic $GABA_A$ current of TC neurons might have in the expression of absence seizures, experiments in freely moving animals are required. Under these conditions, both the behavioural and EEG components of the seizures can be assessed, and data are not confounded by the concomitant use of anaesthetics and/or analgesics. Thus, unrestrained GAT-1 KO mice ($GAT\text{-}1^{-/-}$), which have not undergone any pharmacological treatment and whose TC neurons display enhanced tonic $GABA_A$ currents *in vitro* express ethosuximide-sensitive typical absence seizures (Figures 4(a), 4(b) and 4(c)) [36]. Furthermore, the direct injection of the selective GAT-1 blocker NO-711 into the VB by reverse microdialysis initiates ethosuximide-sensitive typical absence seizures in previously nonepileptic Wistar rats (Figures 4(d) and 4(e)) [36]. On the other hand, in $\delta^{-/-}$ mice, which exhibit a nearly ablated tonic $GABA_A$ inhibition in TC neurons (Figure 5(a)), systemic administration of GHB fails to induce absence seizures (Figures 5(b) and 5(c)) [36]. Intrathalamic injection of a δ subunit-specific antisense oligodeoxynucleotide in GAERS strongly decreases both the tonic $GABA_A$ current and spontaneous seizures 1-2 days after injection, whereas a missense oligodeoxynucleotide has no effect (Figures 5(d), 5(e), and 5(f)) [36]. Finally, intrathalamic administration of THIP in normal Wistar rats elicits absence seizures in a concentration-dependent manner, which as expected are blocked by systemic administration of ethosuximide [36]. Taken together, these data show that enhanced tonic $GABA_A$ inhibition in TC neurons is both necessary and sufficient for the generation of typical absence seizures.

5. Conclusions and Future Perspectives

Augmented tonic $GABA_A$ inhibition in TC neurons represents the first potential molecular mechanism that is common to both well-established pharmacological and genetic models of typical absence seizures. Despite having a range of divergent genetic mutations, GAERS (polygenic),

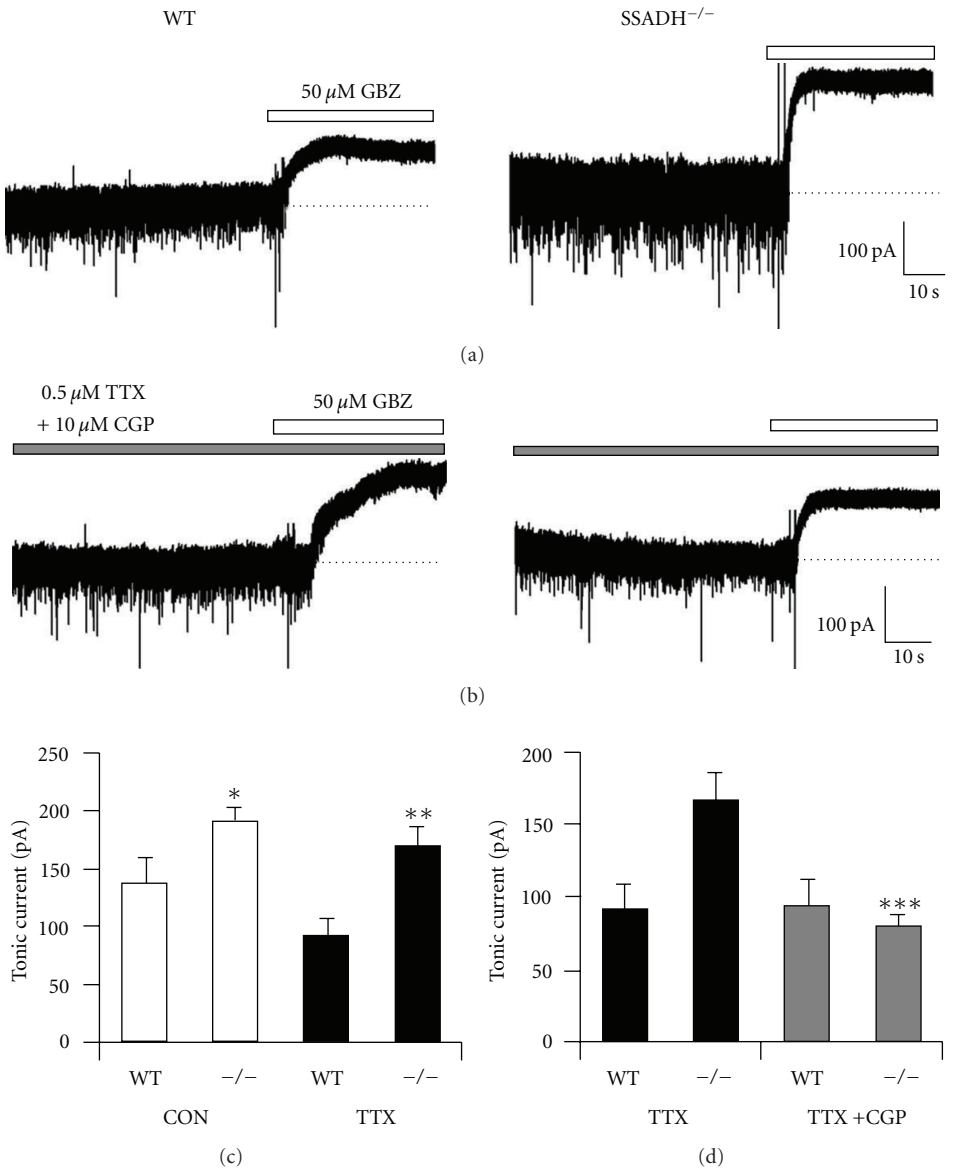

Figure 3: SSADH-deficient mice have enhanced tonic $GABA_A$ currents in VB TC neurons. (a) and (c) VB TC neurons from $SSADH^{-/-}$ mice display enhanced tonic $GABA_A$ currents compared with their WT control littermates. (b) and (d) In TTX, tonic current amplitudes in both $SSADH^{-/-}$ and WT mice are reduced compared to control conditions. CGP55845 reduces the amplitude of the tonic $GABA_A$ current observed in $SSADH^{-/-}$ to a similar level found in WT mice. (a–d) reproduced from reference [58].

stargazer (Ca^{2+} channel γ_2 subunit, TARP-γ_2), lethargic (Ca^{2+} channel β_4 subunit), $SSADH^{-/-}$ and GAT-$1^{-/-}$ mice all display SWDs characteristic of typical absence epilepsy, whereas in $\delta^{-/-}$ mice drugs that commonly produce SWDs are ineffective. Importantly, because powerful $GABA_A$ IPSPs can be recorded in the vast majority of TC neurons during absence seizures *in vivo* [64, 66], these findings also indicate that model systems that aim to reproduce typical absence seizures by blocking $GABA_A$Rs of TC neurons are inherently flawed.

The discovery of a malfunction in GAT-1 as the underlying abnormality that produces increased tonic $GABA_A$ inhibition in TC neurons of genetic absence models shifts the emphasis from a neuronal to an astrocytic aetiology for this type of nonconvulsive epilepsy. Impaired GAT-1 activity in GAERS is not caused by decreased thalamic or cortical expression of GAT-1 mRNA or protein levels. Also, no genetic variants are present in GAT-1 cDNA from GAERS, stargazer, or lethargic mice nor are the mutations responsible for absence seizures in stargazer and lethargic mice present in GAERS. Future studies, therefore, may investigate whether GAT-1 is unable to reach the outer astrocytic membrane and/or whether there are abnormalities in its phosphorylation processes.

Experimental typical absence seizures can be elicited or aggravated by selective $GABA_B$R agonists and can be

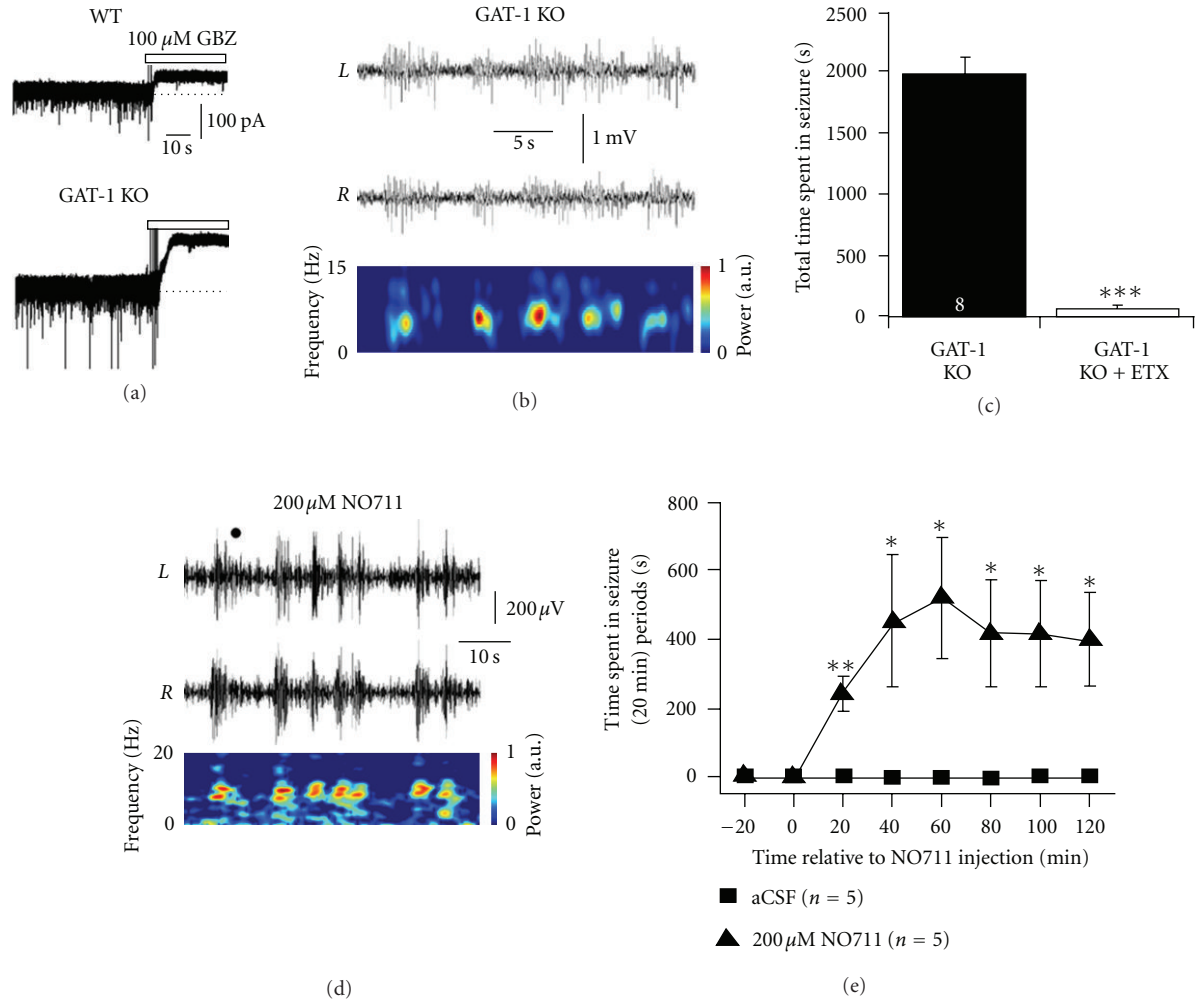

FIGURE 4: Loss of functional GAT-1 in TC neurons leads to SWDs. (a) In adult (P68-74) GAT-1$^{-/-}$ mice, tonic $GABA_A$ currents in VB TC neurons are significantly larger than in WT littermates. (b) Simultaneous bilateral EEG traces reveal that adult GAT-1$^{-/-}$ mice also display SWDs (WT animals did not display SWDs—data not shown). The spectrogram at the bottom corresponds to the EEG signal from the right (R) hemisphere. (c) Treatment of GAT-1$^{-/-}$ mice with the anti-absence drug ethosuximide (200 mg per kg body weight i.p.) significantly reduces the total time spent in seizures. (d) Bilateral EEG traces from a normal Wistar rat following intrathalamic administration by reverse microdialysis of 200 μM of the selective GAT-1 blocker NO711 (spectrogram of the L trace is illustrated below). (e) Time course of the induction of SWDs by intrathalamic administration of NO711. (a–e) reproduced from [34].

blocked by selective $GABA_BR$ antagonists, applied either systemically or intrathalamically. Because about 50% of the tonic $GABA_A$ current observed in TC neurons of GAERS, stargazer, and lethargic and SSADH$^{-/-}$ mice is abolished by a $GABA_B$ antagonist [36, 60], the behavioural and EEG effects of selective $GABA_B$ drugs on typical absence seizures can no longer be simply explained by the ability of these drugs to affect $GABA_B$ IPSPs and/or presynaptic $GABA_BRs$ but should also take into account the positive modulation by $GABA_BRs$ of the tonic $GABA_A$ inhibition in TC neurons.

From a clinical perspective, it is important to stress that all the results reviewed above provide a mechanistic explanation for the aggravation of absence seizures that is observed in humans and experimental animals following either systemic or intrathalamic administration of drugs that increase GABA levels, including tiagabine, a GABA uptake blocker, and vigabatrin, a GABA transaminase blocker [67–70]. Thus, the classical approach of treating seizures by increasing inhibition through positive modulation of GABAergic neurotransmission is particularly ineffective in absence epilepsy. In this circumstance, a selective *reduction* of tonic $GABA_A$ inhibition in thalamic neurons presents perhaps the best possible therapeutic intervention. Intriguingly, a recent study demonstrated that excessive tonic $GABA_A$ergic inhibition is also a feature of cortical neurons surrounding the infarct site (peri-infarct) after induction of stroke in experimental models (in this model, a reduction in GAT-3/4 expression in neurons was observed) [71]. In the motor cortex, where the stroke was induced, $eGABA_ARs$ largely contain α_5 and δ subunits. The α_5 selective benzodiazepine inverse agonist L655,708 produced a significant reduction

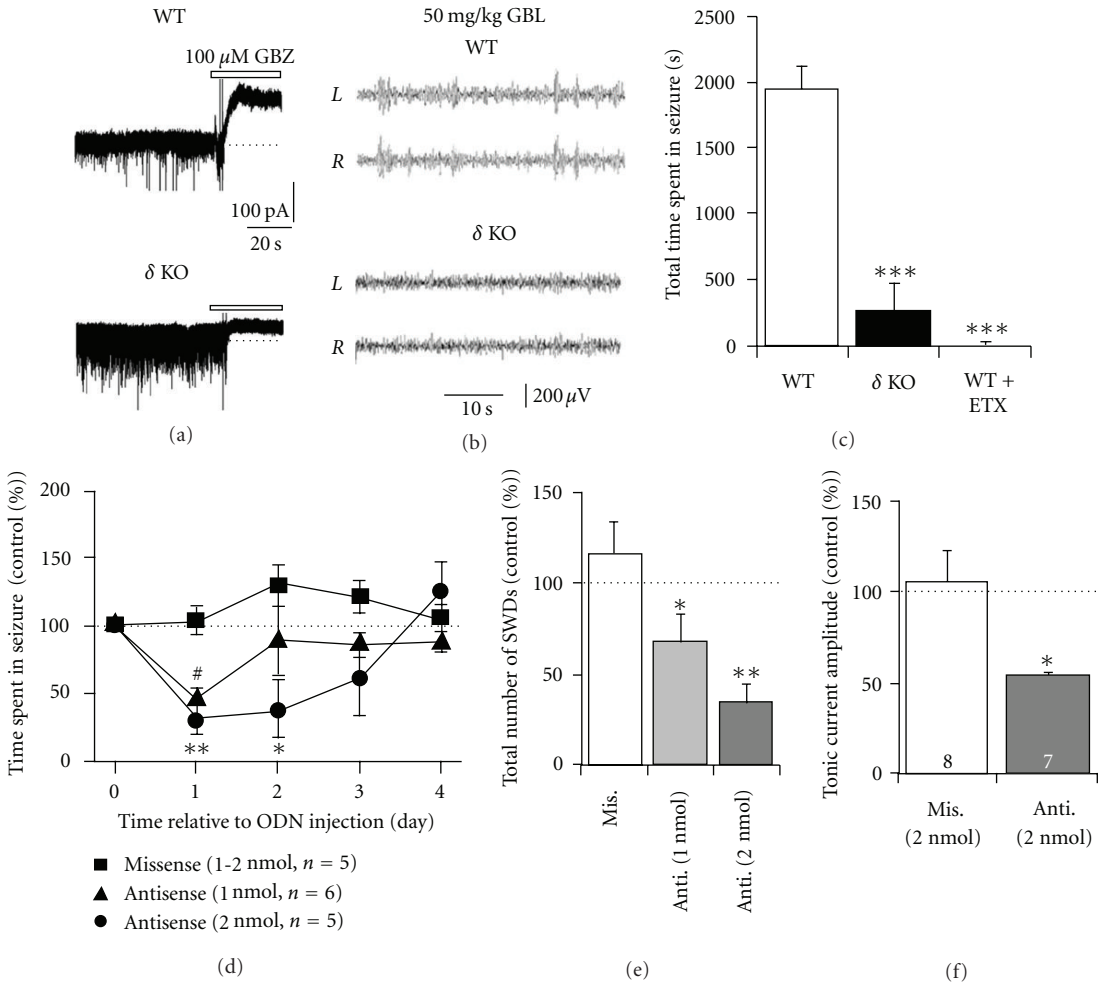

Figure 5: δ subunit containing eGABA$_A$RS in thalamocortical neurons are crucial for expression of SWDs. (a) Tonic GABA$_A$ currents are nearly completely ablated in VB TC neurons from $\delta^{-/-}$ mice. In comparison, WT mice display robust tonic currents as revealed by focal application of GBZ to the recording chamber. (b) Bilateral EEG traces demonstrating that GBL (the GHB prodrug) induces SWDs in WT littermates but not in $\delta^{-/-}$ mice. (c) Ethosuximide-sensitive SWDs that are observed in GBL-injected WT mice are significantly reduced in $\delta^{-/-}$ mice. Graph summarises the total time spent in seizure. (d) Intrathalamic injection of δ subunit-specific antisense oligodeoxynucleotides (ODN) produced a significant reduction in time spent in seizure in GAERS for two days post injection. In contrast sham injection of a missense ODN had no significant effect on the occurrence of SWDs. (e) Graph summarising the effect of ODN injection into VB thalamus upon seizure number normalized to preinjection control values. (f) Graph summarising the effect of anti- and missense ODN injection into VB thalamus of GAERS animals upon tonic current amplitude measured in vitro. Acute brain slices were prepared 1 day after intrathalamic injections were administered. (a–f) reproduced from [34].

in the tonic current amplitude in peri-infarct neurons of slices from poststroke animals as well as improving the performance of animals in an *in vivo* motor task [71]. In a similar manner, the gain of function of eGABA$_A$Rs in typical absence seizures provides compelling preclinical data for the development of inverse agonists selective for α_4-δ subunit containing GABA$_A$Rs which may have potential therapeutic value in this type of nonconvulsive epilepsy.

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