Anxiolytic profile of pregabalin on elicited hippocampal theta oscillation

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ABSTRACT

Previous published work with the novel anticonvulsant, analgesic and anti-anxiety medication, pregabalin (Lyrica<sup>®</sup>), has shown that it has anxiolytic-like actions in several animal behavioral models. However, pregabalin is structurally and pharmacologically different from other classes of known anxiolytic drugs, and the mechanisms that alter brain activity to produce anxiolytic-like actions are not well understood. In an effort to determine more about the cellular mechanisms of pregabalin, we studied its effects on hippocampal theta activity of urethane-anesthetized rats that was elicited by electrical stimulation of the nucleus pontis oralis (nPO) in the brainstem. We found that systemic administration of pregabalin significantly reduced the frequency of stimulation-induced hippocampal theta activity similarly to the effects of diazepam. In addition, pregabalin (but not diazepam) significantly altered the stimulus intensity/frequency relationship, and increased slow delta oscillation (<3.0 Hz) in spontaneous hippocampal EEG in a dose-dependent manner. Our findings suggest that pregabalin may alter evoked theta frequency activity in the hippocampus by reducing neurotransmitter-mediated activation of either the septal nucleus or the hippocampus, and that its actions are unlikely to be mediated by direct activation of GABA neurotransmitter systems. These observations provide further insight to the action of pregabalin, and support the utilization of stimulation-induced theta model in discovery of novel anxiolytic drugs.

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1. Introduction

Pregabalin (Lyrica<sup>®</sup>) is a novel drug first developed as an anticonvulsant, but has since found significant clinical utility in the treatment of various neuropathic pain syndromes, fibromyalgia and in reducing the symptoms of generalized anxiety disorders in humans. Pregabalin is structurally related to gabapentin (Neurotonin<sup>®</sup>), and both compounds show similar pharmacological profiles. Because of the close chemical structural similarity to the neurotransmitter gamma-aminobutyric acid (GABA) much of the early work regarding gabapentin’s and pregabalin’s mechanism of action focused on GABA-related pharmacology. Subsequent work, however, has shown that the cellular and molecular actions of both gabapentin and pregabalin are not related to intrinsic activity at GABA receptors, GABA-related enzymes or other GABAergic related drug targets (Dooley et al., 2007).

Early studies of radioligand binding with tritiated gabapentin and pregabalin identified a saturable, high-affinity binding site in homogenates of brain membranes and in autoradiographs of rat brain (Hill et al., 1993; Suman-Chauhan et al., 1993). Soon afterwards, the protein responsible for high-affinity binding of these compounds was identified as the auxiliary α<sub>2</sub>δ subunit of voltage-gated calcium channels (Gee et al., 1996; Brown and Gee, 1998; Bian et al., 2006). Subsequent to drug binding to the α<sub>2</sub>δ subunit, it is thought that pregabalin alters the action of presynaptic calcium channels and/or additional closely related presynaptic proteins to subtly reduce the release of various neurotransmitters, including glutamate (reviewed in Dooley et al., 2007; Taylor et al., 2007). These changes in neurotransmission within the brain and spinal cord are thought to underlie the pharmacology responsible for analgesic, anticonvulsant and anxiolytic-like actions. Neuronal network activity of the hippocampus, including theta band oscillation is known to be regulated by a number of afferent inputs, originating both from brainstem structures as well as forebrain and cortical areas (Buzsaki, 2002; Vertes and Kocsis, 1997). A significant proportion of these afferent pathways are glutamatergic, and their activity impacts hippocampal theta oscillation via various glutamate receptors known to be present in the hippocampus abundantly (Vertes, 2005).

In the present experiments the effects of pregabalin on stimulation-induced hippocampal theta activity have been studied in anesthetized rats. High frequency (250 Hz) stimulation of the nucleus pontis oralis (nPO), a nucleus of the brainstem reticular formation, induces current-dependent theta oscillations in the hippocampus (McNaughton et al., 2007; Vertes, 1982). Since ascending nPO neurons involved in stimulation-induced...
hippocampal theta are glutamate-containing neurons (Vertes, 2005), it has been presumed that pregabalin, via attenuating stimulation-induced glutamate release will attenuate elicited hippocampal theta oscillation. In addition, this electrophysiological model has a particular relevance for pregabalin pharmacology, since previous work has shown that at least five different types of clinically useful anxiolytic agents each reduce the frequency of stimulus-induced theta rhythm in the hippocampus of anesthetized and non-anesthetized rats at anxiolytic dosages (for review see McNaughton et al., 2007). Therefore, the clinically proven anxiolytic diazepam was used as a positive control in the present study.

2. Materials and methods

2.1. Animals and surgical procedures

Experiments were performed on male, Sprague-Dawley rats (weighing 275–325 g) anesthetized with 15–1.6 g/kg urethane intraperitoneally, under an approved animal use protocol and in compliance with the Animal Welfare Act Regulations (9 CFR parts 1, 2 and 3) and with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health guidelines). A catheter was surgically implanted in the left femoral vein for administration of chlороral hydrate. After cannulation, the animals were placed in a Kopf stereotaxic frame on a temperature regulated heating pad (Harvard Apparatus) set to maintain body temperature at 37–38 °C. A stainless steel screw was placed in the right frontal bone to act as ground, and additional holes were burred into the left parietal bone to accept the recording (hippocampal CA1 region) and stimulating (nPO) electrodes according to the following coordinates (relative to bregma in mm; Paxinos and Watson, 1986): CA1, AP = −3.5, lateral = 2.0 and DV = −2.8 from the surface of the brain; nPO, AP = −7.5, lateral = 1.8 and DV = −6.0 from the surface of the brain. The animals were kept in the stereotaxic frame for the duration of the experiment and were allowed to stabilize for 2 h following placement of the last electrode. At the conclusion of each experiment animals were euthanized and the brains blocked and frozen for histological verification of electrode placement.

2.2. Electrophysiological recordings

Field potentials (electroencephalogram, EEG) were recorded using bipolar, concentric recording electrodes (NE-100X, Rhodes Medical Instruments, Woodland Hills, CA) and a Grass P55 AC differential preamplifier with filters set between 0.3 and 300 Hz. Each signal was digitized (CED Micro1401, Cambridge Electronic Design Ltd., Cambridge CB4 4EF, UK) at a rate of 1000 Hz and stored for off-line analysis using a Fast Fourier Transform analysis using the Spike2 (version 4) software. The brainstem was stimulated using the same type of bipolar concentric electrode used for recording the EEG. The stimulus paradigm consisted of a train of 0.3 ms square pulses delivered over a period of 6 s at a rate of 250 Hz. The 6 s stimulation periods were repeated with an interval of 100 s. The anodal stimulating current began at 0.06 mA, and increased in 0.02 mA increments with successive stimulations, until a maximum of 0.16 mA was reached. In this way, a stimulus–response was obtained ranging from 0.06 to 0.16 mA over a total period of 10 min. This pattern was repeated without interruption for the duration of each experiment. The EEG was continuously recorded for the entire 2 h length of each experiment and thereby included both periods of stimulated EEG as well as the spontaneous EEG between stimulations.

2.3. Data analysis

Signals derived from the last 5 s of each stimulation period, or from the 1 min period of spontaneous EEG preceding each 0.16 mA stimulation, were tapered by applying a standard Hanning window cosine function, then quantified by performing a Fast Fourier Transform analysis using the Spike2 software at a frequency resolution of approximately 0.24 Hz. The first 1 s of stimulation was omitted to avoid stimulus artifact. Total power was determined by summing the total power computed for each frequency band of interest. Peak theta frequency was determined directly for each animal by determining where the peak power occurred in the 4–8 Hz frequency band of the power spectrum. For the individual frequency data presented in Fig. 2, the mean peak frequency for each animal was determined over all the stimulus intensities during the 60–90 min period following drug administration then subtracted from the mean frequency computed from the 30-min period prior to injection to yield the change in frequency due to vehicle/drug administration. For the time-course comparisons (Fig. 3), the average frequency over each 30 min series of stimulations was computed, then normalized to the mean of the three responses prior to drug injection. For the stimulus–response data, frequencies were averaged at discreet stimulation intensities during the first 30 min prior to drug administration, and compared to the average frequencies computed over the 60–90 min period following injection.

2.4. Drugs

All drugs and vehicle control were administered by subcutaneous injection (s.c.). Diazepam was purchased from Sequoia Research Products Ltd, Pangbourne, UK. Pregabalin was synthesized in-house at Pfizer Central Research, Ann Arbor, MI. Both compounds were dissolved in 23% 2-OH-propyl-β-cyclodextrin (Sigma, St. Louis, MO) and the concentrations adjusted so that injection volumes equaled 1 ml/kg body weight. Vehicle control animals received equivalent volumes of 23% 2-OH-propyl-β-cyclodextrin.

2.5. Statistics

Data are expressed as mean ± standard error of the mean (SEM). Comparisons of the change in stimulated-theta frequency between vehicle and drugs was made by a 2-way ANOVA followed by post hoc analysis between groups using a standard Bonferroni t-test. For slope analysis of the stimulus–response measurements, straight lines were fitted to the pre- and post-drug data from individual animals using a simple linear regression program (Microcal Origin, version 7, Northampton, MA, USA). All straight lines fit in this way had correlation coefficients which exceeded 0.9. Changes in slope were then analyzed by performing the Kruskal-Wallis statistic for overall significance followed by comparison between groups using a standard Dunnett's test. Comparisons of power measured in the spontaneous EEG were carried out for the pre- and post-drug periods using the paired t-test.

3. Results

3.1. Effect of nPO stimulation on hippocampal theta

Electrical stimulation of the brainstem nPO in urethane-anesthetized rats consistently elicited highly regular hippocampal theta oscillations, showing current-dependent theta frequency and power as previously reported (Siok et al., 2006; Vertes, 1982). Fig. 1 shows the data from a representative animal demonstrating a shift in theta to higher frequencies, and an increase in theta power in the 4–8 Hz frequency band in response to increasing stimulating current (0.06–0.16 mA). The response in theta frequency to increasing stimulation current was highly consistent from animal-to-animal ranging from 5.4 ± 0.1 Hz at the lowest stimulating current (0.06 mA) to 7.3 ± 0.2 Hz at the highest stimulating current (0.16 mA). Changes in absolute theta power were more variable from animal-to-animal, presumably due to slight differences in electrode placement, but in each case increased with increasing stimulation intensity.

3.2. Both pregabalin and diazepam reduce stimulation-induced theta frequency

For initial comparison, theta frequency was averaged over all the stimulation intensities during the 30-min pre-drug period, and for the 30–90 min period following drug or vehicle administration. Fig. 2a shows the change in average frequency for each individual animal as a consequence of treatment. Diazepam caused a significant, dose-dependent decrease at both 0.32 mg/kg (Δ0.85 ± 0.39 Hz; mean ± SEM; p < 0.01) and 1.0 mg/kg (Δ1.02 ± 0.19 Hz; p < 0.01) compared the vehicle treated animals (Δ0.25 ± 0.1 Hz). No difference from vehicle was seen at the lowest dose of 0.1 mg/kg (Δ0.32 ± 0.23 Hz). Pregabalin also caused a dose-dependent decrease in average theta frequency, showing a significant reduction at the dose of 32 mg/kg, s.c. (Δ−0.60 ± 0.12 Hz; p < 0.01).

Furthermore, the time-course effects of diazepam, pregabalin and vehicle on theta frequency were also analyzed (Fig. 2b). The change in frequency over time with 32 mg/kg pregabalin was clearly distinguishable from both the lower doses of pregabalin as well as from vehicle. Differentiation of the frequency effects between pregabalin and diazepam could also be seen in the time of onset of action for each compound: the effects of diazepam were present 15 min following administration, the shortest testing time, whereas the effect of pregabalin developed more gradually, not reaching significance (p < 0.01) until 30 min after administration.
Due to the slow decline in average frequency over time in rats treated with vehicle alone, steady-state responses to diazepam or pregabalin were not determined.

3.3. Changes in the stimulation–response relationship in hippocampal theta following pregabalin and diazepam treatment

The effects of drug treatment on stimulation-induced hippocampal oscillation elicited by increasing stimulating current have been analyzed by measuring changes both in frequency and power of theta activity. Both pregabalin (32 mg/kg, s.c.) and diazepam (0.32 mg/kg, s.c.), but not vehicle, significantly reduced the frequency of the stimulation driven theta at all stimulation intensities tested (Fig. 3). However, slope analysis of the response curves for the 30 min period prior to drug administration, and for the 60–90 min period post-injection revealed differences in the responses of diazepam and pregabalin. Although diazepam significantly decreased theta frequency over the entire range of stimulating currents, it did not change the slope of the stimulus–response curve indicating a lack of preference for theta frequency as tested within the range of this study. Pregabalin (32 mg/kg) also significantly lowered the frequency of stimulated theta, but in contrast to diazepam, significantly decreased the slope of the frequency stimulus–response (p < 0.01) in all animals, indicating that theta oscillation at the highest frequencies was the most sensitive to pregabalin (Fig. 3). Pregabalin also lowered theta power at stimulation intensities greater than 0.1 mA (i.e. at theta frequencies ≥7.0 Hz), while diazepam or vehicle had no effects on power of stimulation-induced theta (data not shown).
3.4. Effect of pregabalin and diazepam on spontaneous hippocampal EEG activity

Prior to drug administration, and between stimulations, the spontaneous EEG activity in urethane-anaesthetized rats was usually dominated by a regular, low frequency (4.5–5.0 Hz) theta rhythm, with occasional, short periods of non-synchronous, large amplitude, irregular non-theta stages (Fig. 1a and b), as has been described previously (Varga et al., 2002). In order to quantify this activity and test for the impact of each drug, 1-min of continuous, spontaneously occurring EEG was analyzed at 10-min intervals immediately preceding each 0.16 mA stimulation period, for the 30-min period prior to drug administration, and for the 90-min period following injection. Spectrograms for each experimental group were then combined by averaging across animals and cascaded spectrograms were created (Fig. 4). In these analyses, theta was defined as 3–8 Hz since in several animals receiving diazepam, the theta peak slowed to 3.8 Hz (data not shown) but was still clearly distinguishable from 0 to 3 Hz frequency range.

The pre-injection, spontaneous hippocampal EEG activity in all three groups of animals was clearly dominated by theta oscillation. In both vehicle and diazepam (0.32 mg/kg) treated animals, theta persisted over the entire 90-min post-injection, with neither treatment significantly altering the frequency of the peak theta oscillation (vehicle, 5.03 ± 1.15 Hz before injection, 4.70 ± 1.73 Hz 90 min post-injection; diazepam, 4.46 ± 1.15 Hz before injection, 4.33 ± 1.15 Hz post-injection) or power of the 3–8 Hz frequency band (Fig. 5).

By contrast, pregabalin (32 mg/kg) had a profound impact on both 0–3 and 3–8 Hz frequency bands. Over the final 30 min of the 90-min post-pregabalin injection period, power in the theta frequency range decreased significantly (70 ± 9%; p < 0.01; Fig. 5) when compared to the pre-injection period. Peak frequency also exhibited a small, but non-significant, decrease from 4.97 ± 1.15 Hz...
significant increased slow delta oscillation (<3.0 Hz) in spontaneous hippocampal EEG in a dose-dependent manner. These observations provide further insight to the action of pregabalin, and support the utilization of stimulation-induced theta model in discovery of novel anxiolytic drugs.

The hippocampus and its anatomically and functionally connected temporal lobe structures are well known for their involvement in learning and the acquisition and recall of memories (Buzsaki, 2002). The hippocampus, however, has also been linked with the control of emotion (Gray and McNaughton, 2000; Papez, 1937), and hippocampal theta oscillation seems to be strongly associated with affective behavior in particular. Thus, power of theta oscillation shows correlation with the anxiety level in various experimental conditions, and was demonstrated in different experimental animals (Fontani and Carli, 1997; Gray and McNaughton, 2000; Green and Arduini, 1954). For example, it has been reported that mice lacking the serotonin 1A receptors (5-HT1AR) show increased levels of anxiety-related behavior and increased magnitude of hippocampal theta oscillations, specifically in the anxiety-provoking elevated plus maze and not in a familiar environment (Gordon et al., 2005). In contrast, anxiolytic drugs acting on either GABAA or 5-HT1A receptors inhibit oscillatory theta activity in the hippocampal-limbic circuitry, which is thought to contribute to their anxiolytic or sedative effects (Caudarella et al., 1987; McNaughton and Gray, 2000; van Lier et al., 2004). We have recently shown that systemic administration of positive allosteric modulators of GABA<sub>A</sub> receptors, including the alpha-subunit non-selective diazepam, as well as the preferential GABA<sub>A</sub> alpha1 modulator zolpidem and the preferential GABA<sub>A</sub> alpha2/3 modulator L-838417, inhibits theta oscillation of medial septum/diagonal band of Broca (MS/DB) neurons and theta wave activity in the hippocampus simultaneously in chloral hydrate anesthetized rats (Hajos et al., 2004; Ujfalussy et al., 2007).

The present findings demonstrate that pregabalin, just as several other classes of clinically useful anxiolytic drugs, reduces the frequency of stimulation-induced theta oscillation in anesthetized rats. This expands the list of drugs that are active in this model (McNaughton et al., 2007) to include anxiolytics of the calcium channel α2-δ binding class such as pregabalin, in addition to the previously known active drug classes of barbiturates, ethanol, benzodiazepines, serotonin 5-HT<sub>1A</sub> agonists, selective serotonin reuptake inhibitors and tricyclic antidepressants. Our results also supplement previous positive preclinical findings with pregabalin in rodent models of anxiety, including anxiolytic-like actions in the rat Geller conflict test (Singh et al., 1996), the rat Vogel conflict test (Belliotti et al., 2005), the rat elevated plus maze (Singh et al., 1996), and the mouse Vogel conflict test (Lotarski and Kinsora, 2006).

In the present study, pregabalin and diazepam showed similar efficacy, since both reduced frequency of stimulation-induced hippocampal theta to the same extent. In contrast, the minimal effective dose of diazepam was 0.32 mg/kg, whereas the minimal effective dose of pregabalin was 32 mg/kg, indicating a 100-fold difference in their efficacy, correlating well with their clinical dosing (pregabalin 150–600 mg/day, diazepam 5–15 mg/day; Hidalgo et al., 2005). The relatively slow onset of action of pregabalin observed in the present experiments (maximum response approximately 2 h after dosing) is in agreement with the onset of action of pregabalin in animal models of anxiety, including anxiolytic-like actions in the rat (Vartanian et al., 2005). It is likely that the peak effect of pregabalin is delayed by the gradual equilibration of drug in the brain extracellular space following the relatively rapid systemic absorption of pregabalin into the bloodstream (Vartanian et al., 2005).

The mechanism of action of pregabalin responsible for its anxiolytic effects appears to be reduced neurotransmitter release caused by specific drug binding to the presynaptic calcium channel α<sub>2-δ</sub> protein (see Section 1). Our present results are consistent with
this hypothesis. It is known that nPO-stimulation-induced hippocampal theta involves glutamate transmission along the neuroaxis involved in theta generation. In fact, the electrically stimulated ascending nPO neurons are glutamate-containing neurons, such as the neurons projecting from the supramammillary body to the medial septum/diagonal band of Broca (Vertes, 2005). Therefore, it is suggested that pregabalin could reduce stimulus-induced glutamate release in synapses between the brainstem and hippocampus, and this could at least in part explain its anxiolytic effects.

Since it has been demonstrated that ascending neurons of nPO contribute to rapid eye movement (REM) sleep regulation (Xi et al., 2004), a drug-related decrease in frequency of hippocampal theta induced by nPO stimulation as well as reduction in REM sleep could indicate shared mechanisms. Although, both diazepam and pregabalin have been shown to reduce REM sleep (Gottesmann et al., 1998; Kubota et al., 2001), further studies are required to establish the precise relationship between changes in nPO-stimulation-induced hippocampal theta and changes in REM sleep parameters.

While GABA<sub>A</sub> positive allosteric modulators are excellent anxiolytics, it seems that the pharmacology of pregabalin is unrelated to GABA neurotransmission (see Section 1). For example, previous sleep–wake cycle studies combined with quantitative EEG analysis revealed characteristic effects of pregabalin on cortical activity, which were clearly distinct from those induced by benzodiazepine GABA<sub>A</sub> positive allosteric modulators. For example, pregabalin has been shown to increase slow-wave sleep activity, and enhances EEG delta band power of EEG (Kubota et al., 2001). In contrast, GABA<sub>A</sub> positive allosteric modulator anxiolytics reduce slow-wave sleep, as well as reducing the power of EEG delta activity during slow-wave sleep (Jugovac et al., 2006; van Lier et al., 2004). Interestingly, our present results are in accord with the previous published findings of pregabalin effects on EEG of non-anaesthetized rats, namely an increase in power at delta frequency band (less than 3 Hz) (Kubota et al., 2001). However, our present findings suggest that increases in delta power are not restricted to or simply associated with increases in slow-wave sleep, but instead represent a more general facilitation of low frequency EEG oscillations in the delta frequency range.

Other studies have shown that an increase in delta power is observed in the waking EEG of humans who are sleep deprived (De Gennaro et al., 2007), and that a selective serotonin 5-HT<sub>2A</sub> antagonist, MDL 100907, increased the delta power in rat EEG during sleep (Moirarty et al., 2008). Both of these treatments result in changes of EEG delta power similar to the change we observed with pregabalin treatment. Mechanisms underlying the increase in delta power in response to pregabalin treatment are unknown at the present, but it would be of interest to determine whether a reduction in excitatory or inhibitory neurotransmitter inputs (such as glutamatergic inputs from nPO to the medial septal nucleus and diagonal band of Broca or both cholinergic and GABAergic inputs from the septal nucleus to the hippocampus) are responsible for the pregabalin-induced effect.
Importantly, significant anxiolytic action in randomized, blind-
dined clinical trials has been shown for both the \( \alpha_2 \rightarrow \delta \) binding drugs pregabalin (Feltner et al., 2003; Montgomery et al., 2006; Pande et al., 2003; Pohl et al., 2005; Rickels et al., 2005) and gabapentin (Pande et al., 1999). In addition, pregabalin has recently been compared in a meta-analysis of 27 similar placebo-controlled studies with five other different anxiolytic drug classes (each using HAM-A anxiety scores to measure effects in generalized anxiety disorder) and pregabalin had the largest effect size of any of the different anxiolytic treatments (Hidalgo et al., 2007). The present findings demonstrate that pregabalin reduces the frequency of stimulated-theta activity in the hippocampal–limbic circuit in a fashion that has overall profile to other anxiolytic drugs.

Since the overall profile of pregabalin differed from that of diazepam (particularly in right-shifting the stimulus intensity–frequency response relationship, but without altering the slope of the relationship), our findings also are consistent with pregabalin acting at a site that is independent of GABA \( \alpha \) receptors, possibly at the calcium channel \( \alpha_2 \rightarrow \delta \) binding site at synapses within the reticular formation, the medial septum, or the hippocampus. Additional electrophysiological studies using whole-animal models are now needed to determine the cellular basis of the effects that we have reported here. For example, it would be of interest to test whether pregabalin reduces hippocampal theta activity induced by septal stimulation or by hippocampal infusion of cholinergic agonists.

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