Title page

Anxiolytic-Like Activity of Pregabalin in the Vogel Conflict Test in $\alpha_2\delta$-1 (R217A) and $\alpha_2\delta$-2 (R279A) Mouse Mutants

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ABSTRACT

The $\alpha_2\delta$ auxiliary subunits ($\alpha_2\delta$-1 and $\alpha_2\delta$-2) of voltage-sensitive calcium channels are thought to be the site of action of pregabalin (Lyrica®), a drug that has been shown to be anxiolytic in clinical trials for generalized anxiety disorder. Pregabalin and the chemically-related drug, gabapentin have similar binding and pharmacology profiles, demonstrating high-affinity in vitro binding to both $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits. Two independent point mutant mouse strains were generated in which either the $\alpha_2\delta$-1 subunit (arginine-to-alanine mutation at amino acid 217; R217A) or the $\alpha_2\delta$-2 subunit (arginine-to-alanine mutation at amino acid 279; R279A) were rendered insensitive to gabapentin or pregabalin binding. These strains were used to characterize the activity of pregabalin in the Vogel conflict test, a measure of anxiolytic-like activity. Pregabalin showed robust anti-conflict activity in wild-type littermates from each strain at a dose of 10 mg/kg but was inactive in the $\alpha_2\delta$-1 (R217A) mutants up to a dose of 320 mg/kg. In contrast, pregabalin was active in the $\alpha_2\delta$-2 (R279A) point mutants at 10 and 32 mg/kg. The positive control, phenobarbital, was active in mice carrying either mutation. These data suggest that the anxiolytic-like effects of pregabalin are mediated by binding of the drug to the $\alpha_2\delta$-1 subunit.
**Introduction**

Pregabalin (S-(+)-3-isobutylGABA or Lyrica®) has shown efficacy in randomized, placebo-controlled clinical trials for the treatment of generalized anxiety disorder (Feltner et al., 2003; Rickels et al., 2005; Montgomery et al., 2006), and has also shown efficacy in clinical trials for fibromyalgia, and neuropathic pain associated with diabetic neuropathy and postherpetic neuralgia (Arnold et al., 2010; Dworkin et al., 2010). Pregabalin and gabapentin (Neurontin®) are both alkyl derivatives of GABA at the 3-carbon position and share the property of potent and selective binding to a site in brain membranes identified as the calcium channel \(\alpha_2\delta\) subunit (Gee et al., 1996). Despite its structural resemblance to GABA, pregabalin has not been found to bind with high affinity to any drug target associated uniquely with GABA synapses, and therapeutic effects of drugs in this class are thought to be mediated instead by binding to the voltage-gated calcium channel \(\alpha_2\delta\) subunit (\(\text{Ca}_{\alpha_\delta}\) protein), specifically \(\alpha_2\delta-1\) and \(\alpha_2\delta-2\) (\(K_D = 6.0 \text{nM and 7.2 nM, respectively}\); Li, Taylor, Weber, Piechan, Prior, Bian, Cui, Hoffman and Donevan, Pregabalin, an anticonvulsant, analgesic, and anxiolytic drug, is a potent and selective ligand for \(\alpha_2\delta-1\) and \(\alpha_2\delta-2\) calcium channel subunits, submitted for publication). Neither pregabalin nor gabapentin bind to \(\alpha_2\delta-3\) or \(\alpha_2\delta-4\) (Taylor et al., 2007). Subsequent to binding at \(\alpha_2\delta-1\) and \(\alpha_2\delta-2\), these drugs modify neurotransmitter release at a variety of synapses in brain and spinal cord, which is thought to lead to the observed therapeutic effects (Dooley et al., 2007). Furthermore, it has recently been shown that by blocking thrombospondin binding to \(\alpha_2\delta-1\), gabapentin potently inhibits the formation of excitatory synapses (Eroglu et al., 2009), a mechanism that may also contribute to the therapeutic effects of \(\alpha_2\delta\) ligands.

The pharmacologic actions of pregabalin have been studied extensively in rodents. Results with a series of compounds that are structurally-related to pregabalin and gabapentin suggest that high-affinity binding to \(\alpha_2\delta\) accurately predicts anxiolytic-like activity in the rat Vogel conflict model, one of the most predictive assays for identifying compounds with anxiolytic-like
properties (Vogel et al., 1971; Millan and Brocco, 2003; Belliotti et al., 2005). Gabapentin and pregabalin have also been reported to produce anxiolytic-like activity in the elevated plus maze, Geller conflict test, and an animal model for posttraumatic stress disorder (Field et al., 2001; Zohar et al., 2008).

Bian and colleagues (Bian et al., 2006) reported a significant reduction in ex vivo $[^{3}H]$pregabalin and $[^{3}H]$gabapentin binding in autoradiography studies with brain and spinal cord from $\alpha_{2}\delta$-1 (arginine-to-alanine mutation at amino acid 217; R217A) mutant mice. The results suggest that the $\alpha_{2}\delta$-1 protein is the major high affinity binding site for pregabalin in the central nervous system. Further, Field and colleagues (Field et al., 2006) reported that while wild-type (WT) and $\alpha_{2}\delta$-1 (R217A) mice had similar pain thresholds following formalin footpad injection and chronic constriction injury, the analgesic effects of pregabalin were absent in mice expressing the $\alpha_{2}\delta$-1 (R217A) mutation. These results suggest that drug binding to the $\alpha_{2}\delta$-1 subunit is required for the analgesic effects of pregabalin in these models.

To determine the importance of $\alpha_{2}\delta$ binding to the anxiolytic effects of pregabalin, we examined the activity of pregabalin in the Vogel conflict test in $\alpha_{2}\delta$-1 (R217A) mutant mice. In addition, we established a congenic strain of mutant $\alpha_{2}\delta$-2 (arginine-to-alanine mutation at amino acid 279; R279A) mice, a mutation that dramatically reduces pregabalin and gabapentin binding to the $\alpha_{2}\delta$-2 protein (Bian et al., 2006; Bian et al., 2008). We characterized the activity of pregabalin in the Vogel conflict model to determine the importance of binding to the $\alpha_{2}\delta$-2 protein to the anxiolytic effects of pregabalin. The results presented here suggest that the anxiolytic-like properties of pregabalin in the Vogel conflict model are mediated by drug binding to the $\alpha_{2}\delta$-1 subunit of voltage-gated calcium channels, with no apparent contribution from drug binding to the $\alpha_{2}\delta$-2 protein.
Materials and Methods

Animals

Point mutant (arginine-to-alanine) $\alpha_2\delta$-1 (R217A) mice, as described by Bian and colleagues (Bian et al., 2006), were backcrossed to the C57BL/6N strain at Charles River Laboratories (Wilmington, MA, USA) using marker-assisted accelerated backcrossing technology (MAX-BAX$^{SM}$) as a congenic breeding strategy (Markel et al., 1997). To generate the $\alpha_2\delta$-2 mutants, standard molecular biology techniques were used. Briefly, a genomic clone containing exon 8 of $\alpha_2\delta$-2 was isolated from a mouse 129SVJ genomic DNA library. The arginine at position 279 was mutated to an alanine in vitro. This mutated fragment was used as a targeting vector to generate the mutant mouse (details in supplemental data). The 129 mice carrying the R279A mutation in $\alpha_2\delta$-2 were backcrossed to the C57BL/6N strain at Charles River Laboratories (Wilmington, MA, USA) using marker assisted backcrossing to generate a mouse strain congenic for the $\alpha_2\delta$-2 mutation (Markel et al, 1997).

The breeding strategy yielded congenic mice that carried the mutation and were otherwise >99.5% genetically identical to inbred C57BL/6N mice. Mice for the experiments were generated using heterozygous [(+/−) x (+/−)] breeding so that WT and homozygous littermates were compared. The same environmental conditions were maintained for each breeding colony. Male mice, received in shipments of 60 WT and 60 mutants at 6 to 8 weeks old, were acclimated to the facility for 2 weeks prior to testing. The background strain C57BL/6N mice from Charles River Laboratories (Wilmington, MA, USA) were received at 8 weeks and tested 1 week later. All mice were housed in isolators in a temperature/humidity-controlled environment on a 12 h light/dark cycle (lights on 6 AM) with food and water available ad libitum. WT and homozygous mutant mice were run concurrently and separate shipments of animals were used for each experiment.
The \(\alpha_2\delta-1\) (R217A) studies were conducted at the Pfizer facility in Ann Arbor, MI. The \(\alpha_2\delta-2\) (R279A) studies were conducted by the same scientist using the same equipment at the Pfizer facility in Groton, CT. Parental background strain studies were successfully replicated at the Groton facility to validate the equipment relocation and we report one set of those experiments here.

All testing was carried out in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals under an approved protocol in accordance with the Institutional Animal Care and Use Committee guidelines.

**Vogel Conflict Protocol**

Shock intensities used for drug testing were experimentally determined for each strain (genotype) as the lowest intensity to reliably suppress drinking behavior (see supplemental data). Reliable and repeatable baselines were reached when both parameters of the criteria were met as follows: 1) drinking behavior was suppressed \(\geq 80\%\) (compared with no-shock control group); and 2) <40\% of the animals responded with >20 shocks. Together these parameters provided baseline levels of shock-suppressed drinking with consistency across experiments.

After 24 h of water deprivation, mice were individually placed into Coulbourn modular operant mouse chambers (Allentown, PA, USA) for a 10-min unpunished drinking session. A 1:1 mixture of evaporated milk and water, with a higher caloric value than water alone, was used for the restricted drinking session. Total volume was restricted similarly across subjects to 100-150 licks, approximately 25\% of their total daily water intake. Mice that consumed this volume were returned to their home cages and water deprivation continued for another 24 h. On the test day (day 2), the same reinforcer (water/evaporated milk) was used. Mice were subjected to a 10-min session with shock delivered on a fixed ratio FR10 schedule (1 shock for every 10 licks).
test subject completed an electrical circuit between the drink tube and metal grid floor, and shock duration was programmed for 1 s but terminated immediately when contact between the animal and drink tube was broken. Licks were detected by an optical lickometer, which consisted of two glass rods (photosensor LED emitter and detector) spanning the width of the drink tube with a light beam piped across the two rods. Each lick was automatically recorded using Graphic State Notation software (Coulbourn Instruments, Allentown, PA, USA). Shock-suppressed drinking was reported as the number of mean shocks for each group during the 10-min test session.

Data generated in the parental background strain were subjected to a one-way ANOVA; the non-parametric, Kruskal-Wallis one-way ANOVA was used if normality was not met. Data generated in the genetically modified mice were subjected to a two-way ANOVA (drug x genotype), but the non-parametric, Kruskal-Wallis one-way ANOVA was applied to each genotype when normality was not met.

Drugs

Pregabalin (Pfizer Inc, Ann Arbor, MI, USA), morphine, and phenobarbital (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in sterile saline and delivered by intraperitoneal injection in a volume of 10 ml/kg of body weight. All drug dosages are expressed as milligrams of drug active moiety (free base) per kilogram of animal body weight and tested at the times previously reported to produce peak behavioral effects: 30 min after dosing for morphine and phenobarbital and 120 min after dosing for pregabalin. Each animal was dosed only once on the test day. All other chemicals, reagents, and solvents were of the highest purity available.

Brain and Plasma Sample Drug Extraction Procedure

Whole mouse brain samples and blanks were homogenized in four volumes of water. A standard pregabalin calibration curve (4.88–10,000 ng/ml) was prepared by serial dilutions in
blank mouse plasma. A mixed matrix method was used to quantitate the drug concentrations in brain samples. To generate the standard curve in brain (19.5–40,000 ng eq/g), 50 µl of blank mouse brain homogenate was added to 50 µl of each plasma standard. Likewise, 50 µl of blank plasma was added to 50 µl of each brain sample. All plasma and brain homogenate samples were diluted to the linear range of the standard curves. A 200-µl aliquot of acetonitrile containing a structurally similar internal standard was added to each 50-µl plasma sample, standard and blank, and to each 100-µl brain sample, standard and blank. Samples were vortexed for 30 s and centrifuged at 4000 rpm for 10 min. Following centrifugation, 50 µl of the supernatant was transferred to a clean 96-well plate to which 50 µl of water was added. Samples were analyzed by liquid chromatography tandem mass spectrometry as described in the next paragraph.

Liquid chromatography tandem mass spectrometry was used to quantitate pregabalin. Samples were injected (5 µl; HTC PAL Leap autosampler, LEAP Technologies, Carrboro, NC, USA) onto an Atlantis C18 50 x 2.1 mm, 5-µM column. Pregabalin was eluted with a linear gradient consisting of 0.1% acetic acid in 10-mM ammonium acetate (“A”) and acetonitrile (“B”) at a flow rate of 250 µl/min produced by two Shimadzu LC-10 ADVP pumps (Columbia, MD, USA). An initial concentration of “A” (95%) was ramped to a final concentration of “A” (0%) over 4 min. The system was returned to the initial conditions in a single step and held there for 1 min. An AB SCIEX 4000 triple quadrupole mass spectrometer (Concord, Ontario, Canada) was used with positive electrospray ionization in multiple-reaction monitoring mode. Quantitation was achieved using AB Analyst 1.4 software (AB SCIEX, Concord, Ontario, Canada).
Results

Brain and Plasma Exposures

To ensure that pregabalin reached appropriate concentrations in the mice that corresponded to anxiolytic exposure ranges in patients, brain and plasma samples were harvested from mice of each genotype (n = 3) 2 h after dosing. Figure 1 shows the results of these analyses. For comparison, human plasma drug concentrations of pregabalin in clinical trials for generalized anxiety disorder with effective dosages of 300 to 600 mg/day given every 8 h ranged from approximately 2.0 μg/ml (trough) to 8.5 μg/ml (peak) (Bockbrader et al., 2010a; Bockbrader et al., 2010b). In mice, doses in the range of 3.2 to 320 mg/kg yielded concentrations of pregabalin that rose in a dose-dependent manner, and there were no significant differences in exposure between any of the genotypes (Fig. 1).

Effect of Strain on Suppressed Drinking Behavior

Suppression curves for each genotype and the C57BL/6N parental background strain were conducted to identify equivalent baseline levels of shock-suppressed drinking behavior for the pharmacology experiments. Equivalent baseline levels of shock-suppressed drinking were achieved when 0.4 mA was used for the C57BL/6N parental strain, both WT lines and the α2δ-2 (R279A) mice, while the α2δ-1 (R217A) point mutants required 0.7 mA to produce the same level of suppressed drinking behavior. The results can be found in Table S1 in the supplemental data. Subsequent experiments were conducted at the experimentally determined intensity appropriate for the genotype being tested. Body weights were tracked during the suppression studies and found to be similar for all strains/genotypes prior to and following water deprivation.

Effects of Drug on C57BL/6N Parent Strain
To show that increases in shock-suppressed drinking behavior in the Vogel conflict assay were not due to an analgesic effect, morphine was tested in C57BL/6N mice (Fig. 2). The mean numbers of shocks were similar in vehicle- and morphine-treated groups (0.32 and 1 mg/kg), and inactivity and sedation observed at the higher doses of morphine (3.2 and 5.6 mg/kg) resulted in a reduction in punished drinking, as expected when sedation begins to interfere with responding. An overall Kruskal-Wallis one-way ANOVA yielded an $H_{3, 58} = 32.6$, $p < 0.001$ with statistical significance ($p < 0.05$; Dunn’s post hoc) at doses of 3.2 and 5.6 mg/kg.

Prior to testing in the mutants, we set out to show that pregabalin and a suitable positive control drug were indeed able to produce robust anxiolytic-like effects in the C57BL/6N parental background strain to demonstrate the rationale for their use in the mutant mice. In light of our previous findings where diazepam activity was blunted in the C57BL/6N strain (but not C57BL/6J strain) in the Vogel conflict test (Lotarski and Kinsora, 2004), we chose phenobarbital, another clinically validated anxiolytic where activity is not mediated through effects on $\alpha_2\delta$, to serve as the positive control. Phenobarbital produced robust anxiolytic-like effects in the C57BL/6N parent strain animals, reflected by an increase in mean shocks relative to the concurrent vehicle-treated group (Fig. 3A). Phenobarbital effects were dose-dependent and an overall Kruskal-Wallis one-way ANOVA yielded an $H_{5, 72} = 32.0$, $p < 0.001$, with statistical significance ($p < 0.05$; Dunn’s post hoc) at doses of 32 and 56 mg/kg compared with the concurrent vehicle control group. Pregabalin was also tested for anxiolytic-like activity in the C57BL/6N strain. Doses ranged from 3.2 to 100 mg/kg and increases in mean shocks were robust and dose-dependent. An overall Kruskal-Wallis one-way ANOVA yielded an $H_{4, 60} = 19.9$, $p < 0.001$, with statistical significance ($p < 0.05$; Dunn’s post hoc analysis) at doses of 10 mg/kg and greater (Fig. 3B).

Effects of Phenobarbital on WT and Mutant Mice
Phenobarbital was used to demonstrate anxiolytic-like activity in the Vogel conflict model in WT and mutant mice from each colony. Using the experimentally determined shock intensity for each genotype, phenobarbital produced similar anxiolytic-like effects in all four genotypes (Fig. 4 and 5). Vehicle-treated control groups exhibited similar shock-suppressed drinking behavior across genotypes and phenobarbital produced dose-dependent and significant increases in mean shocks relative to the concurrent vehicle control group ($p < 0.05$). A two-way ANOVA on WT and $\alpha_2\delta^{-1}$ (R217A) mice yielded a significant effect of dose ($F_{3, 111} = 28.8$, $p <0.001$) but not genotype x dose. Doses of 32 and 56 mg/kg achieved statistical significance ($p < 0.05$) in WT and $\alpha_2\delta^{-1}$ (R217A) mice using the Bonferroni multiple comparisons post hoc analysis. Similarly, a two-way ANOVA on WT and $\alpha_2\delta^{-2}$ (R279A) also yielded a significant effect of dose ($F_{3, 92} = 48.8$, $p <0.001$), but not genotype x dose, and a Bonferroni multiple comparisons post hoc analysis yielded statistical significance ($p<0.05$) in both genotypes at doses of 32 and 56 mg/kg.

Effects of Pregabalin on WT and Mutant Mice

Pregabalin was tested to determine if a mutation that reduces binding of pregabalin to the $\alpha_2\delta^{-1}$ or $\alpha_2\delta^{-2}$ subunit selectively would produce a concomitant change in anxiolytic-like activity in vivo. A wide dose range was explored in littermate WT and $\alpha_2\delta^{-1}$ (R217A) mice in two separate experiments with results presented in Figure 6. In the first experiment, pregabalin (3.2–32 mg/kg) increased shock-suppressed drinking and produced robust anxiolytic-like activity in WT mice (Kruskal-Wallis one-way ANOVA: $H_{5, 57} = 29.0$, $p <0.001$) but had no effect in $\alpha_2\delta^{-1}$ (R217A) mice (Kruskal-Wallis one-way ANOVA: $H_{5, 51} = 2.1$, $p =0.555$). Doses of 10 and 32 mg/kg in the WT mice were statistically significant when compared with the concurrent vehicle control group ($p < 0.05$; Dunn’s post hoc analysis; Fig. 6A). In the second study, pregabalin was tested at much higher doses of 100 and 320 mg/kg, but again, the $\alpha_2\delta^{-1}$ (R217A) mice treated
with pregabalin were not statistically different compared with the vehicle-treated control group (Kruskal-Wallis one-way ANOVA: \(H_{2, 40} = 5.7, p = 0.075\)), whereas robust and significant (Kruskal-Wallis one-way ANOVA: \(H_{2, 45} = 26.2, p < 0.001\)) increases in mean shocks were again observed in the littermate WT control mice (Fig. 6B). This confirms our hypothesis that binding to the \(\alpha_2\delta-1\) subunit is responsible for the anxiolytic-like actions of pregabalin.

In contrast to the \(\alpha_2\delta-1\) (R217A) mutants, pregabalin significantly (Kruskal-Wallis one-way ANOVA: \(H_{3, 54} = 22.2, p < 0.001\)) increased mean shocks in the \(\alpha_2\delta-2\) (R279A) mutant mice in a dose-dependent manner (Fig. 7). In fact, the anxiolytic-like actions of pregabalin in the \(\alpha_2\delta-2\) (R279A) mice were similar to those of their littermate WT control mice (Kruskal-Wallis one-way ANOVA: \(H_{3, 53} = 35.6, p < 0.001\)), with significance observed at doses of 10 and 32 mg/kg for each genotype (\(p < 0.05\); Dunn’s post hoc analysis). These data show that the anxiolytic-like activity of pregabalin in the Vogel conflict model is mediated through binding to the \(\alpha_2\delta-1\) subunit, with no apparent contribution from the \(\alpha_2\delta-2\) subunit.
Discussion

The results presented in this report extend our understanding of the therapeutic activity of pregabalin by showing that the $\alpha_2\delta$-1 (R217A) mice were insensitive to the anxiolytic-like action of pregabalin in the Vogel conflict test. Furthermore, by using the corresponding mutation and generating $\alpha_2\delta$-2 (R279A) point mutant mice, which remain sensitive to the anti-conflict properties of pregabalin in the same test, we have shown that the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels appears to be solely responsible for the anxiolytic activity of pregabalin.

Gabapentin and pregabalin show a wide range of activities in various preclinical models (de-Paris et al., 2000; Field et al., 2001; Lotarski and Kinsora, 2004; Vartanian et al., 2006; Taylor et al., 2007; Zohar et al., 2008) and demonstrate clinical efficacy in several indications (Rickels et al., 2005; Montgomery et al., 2006; Taylor et al., 2007; Arnold et al., 2010; Dworkin et al., 2010). They show selective and potent binding to $\alpha_2\delta$-1 and $\alpha_2\delta$-2, but not to $\alpha_2\delta$-3 or $\alpha_2\delta$-4 voltage-gated calcium channel subunits (Thorpe and Offord, 2010), and the particular importance of the $\alpha_2\delta$-1 subunit in mediating the therapeutic effects of these $\alpha_2\delta$ ligands is gaining support. A study similar to the one reported herein demonstrated that binding of pregabalin or gabapentin to the $\alpha_2\delta$-1 subunit was necessary for their antihyperalgesic effects in a preclinical pain model (Field et al., 2006).

In the current studies, phenobarbital was selected as the positive control. Our Vogel conflict method was originally validated with benzodiazepines in C57BL/6J mice, and we observed robust anxiolytic-like effects similar to those reported by Mathiasen and Mirza (Mathiasen and Mirza, 2005); however, in the C57BL/6N mouse strain (parental background for the $\alpha_2\delta$ mutants), benzodiazepine activity was blunted (Hain et al., 2001; Lotarski and Kinsora, 2004; Mathiasen and Mirza, 2005). It is well known that behavior and pharmacology in mice can be very sensitive to the strain used for testing (Crawley et al., 1997; Silva et al., 1997). Strain differences to the anxiolytic-like effects of benzodiazepines in mice have been reported in the
literature, including lack of activity in some C57 substrains—this further emphasizes the need for pharmacologic testing of parent strains prior to evaluating the genetically modified animals (Crawley et al., 1997; Belzung, 2001; Rodgers et al., 2002). In the current studies, we validated our Vogel conflict method using phenobarbital and pregabalin, each of which produced robust anxiolytic-like effects in the C57BL/6N parental background strain and provided confidence in the rationale for pharmacologic evaluations in the genetically modified mice. Confidence in the specificity of the assay was also tested, and morphine confirmed that analgesics do not increase shock-suppressed drinking in the Vogel conflict test, allowing one to conclude that the outcome is specific to the anxiolytic actions of the drug tested.

The effect of pregabalin on unpunished drinking behavior or thirst was not tested in the present study, and therefore, we cannot exclude that such an effect might contribute to pregabalin’s anti-conflict actions. However, drugs that increase thirst (e.g., isoproterenol) do not necessarily increase punished responding in the mouse Vogel conflict test (Mathiasen and Mirza, 2005).

It is worth noting that shock delivery in our assay is considered mild, in part, because it occurs through the floor and drink tube and terminates immediately when the animal pulls its snout away. This is uniquely different when compared with other tests that employ inescapable shock via only the grid floor, where animals may vocalize, jump, and/or run the perimeter of the chamber in response—those behaviors do not occur in our procedure. The 80% suppression of drinking behavior is utilized because it identifies the lowest intensity that produces reliable and repeatable baselines in our experience and controls for phenotypic differences in shock sensitivity between various strains/genotypes, as observed here where the $\alpha_2\delta$-1 (R217) mice needed a slightly higher intensity to meet the suppression criteria compared with the three genotypes tested (and the parental backcross strain). While this difference is not clearly understood, it may be related to the embryonic stem cell donor strain and a flanking-allele effect (Crawley, 1996). Each mutant mouse was originally created using different 129 embryonic stem
cells, and our limited work in the Vogel conflict test with 129 substrains also showed differences in shock sensitivity in the Vogel conflict test (Lotarski and Kinsora, 2004). While we do not believe shock sensitivity in our assay is a pain-related response, it is worth noting that there was no difference in pain thresholds between WT and α_2δ-1 (R217A) mice (Field et al., 2006) in previous neuropathic pain work. Despite this phenotypic difference, our results with phenobarbital clearly demonstrate that by starting with equivalent baselines, we can reliably measure pharmacologic activity and compare genotypes.

When phenobarbital and pregabalin were tested for anxiolytic-like activity in the α_2δ-1 (R217A) mice, phenobarbital retained activity, but pregabalin did not, despite both compounds showing robust anxiolytic-like effects in the WT littermate mice. While these data suggest that binding of pregabalin to the α_2δ-1 subunit is necessary and sufficient for anxiolytic-like activity in the Vogel conflict assay, the means by which activity at the α_2δ-1 subunit translates to anxiolysis is unclear. Autoradiographic and immunohistochemical analyses of the expression of α_2δ-1 subunits show that α_2δ-1 subunits are expressed in areas of the brain that would be expected to be involved in anxiolytic activity. α_2δ-1 levels are high in hippocampus, entorhinal cortex, and amygdala (Bian et al., 2006; Taylor and Garrido, 2008). However, levels of α_2δ-2, as shown by autoradiography and immunohistochemistry, are expressed primarily in the cerebellum and septum (Gong et al., 2001; Bian et al., 2008). These results are supported by measurement of RNA levels for α_2δ-1 and α_2δ-2 in WT mice and the ducky mutant, a mouse with a spontaneous mutation in α_2δ-2 that yields a much shorter, inactive protein (Thorpe and Offord, 2010).

It remains unclear whether the activity of α_2δ ligands similar to pregabalin is mediated through activity at calcium channels. Recent work has shown that α_2δ-1 interacts with the protein thrombospondin to increase the number of excitatory synapses, that this interaction is independent of activity at calcium channels, and that the interaction and the development of excitatory synapses is blocked by application of gabapentin (Eroglu et al., 2009). In a similar
way, α2δ-3 is involved in the development of motor neuron terminals in drosophila and this activity is independent of calcium channel activity (Kurshan et al., 2009).

Further work is needed to determine if all of the known activities of the α2δ ligands are mediated through their interactions with α2δ-1, and not through interactions with α2δ-2. Our present data suggest that if a novel α2δ-1 selective ligand is discovered, it will be anxiolytic.

**Authorship contributions**

Participated in research design: Donevan, Lotarski, Offord  
Conducted experiments: Lotarski, Osgood, Poe  
Performed data analysis: El-Kattan, Lotarski, Offord, Poe  
Wrote or contributed to the writing of the manuscript: Donevan, Lotarski, Offord, Taylor
References


Footnotes

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**Figure Legends**

**Fig. 1.** Pregabalin concentrations (–2 h, IP; n = 3/dose) in plasma (A) and brain (B) were similar across all four genotypes.

**Fig. 2.** The analgesic morphine (–30 min, IP) did not increase shock-suppressed drinking behavior in the Vogel conflict test in the C57BL/6N parent strain mice (n = 11–12/group). At higher doses (3.2 and 5.6 mg/kg), drinking behavior was significantly decreased and sedation observed. Significant differences (*p < 0.05) compared with vehicle-treated mice.

**Fig. 3.** Phenobarbital (–30 min, IP) (A) and pregabalin (–2 h, IP) (B) produced dose-dependent anxiolytic-like effects in the Vogel conflict test in the C57BL/6N parental background strain mice (n = 12/group). Significant differences (*p < 0.05) compared with vehicle-treated mice.

**Fig. 4.** Phenobarbital (–30 min, IP) produced dose-dependent anxiolytic-like effects in the Vogel conflict test in wild-type (n = 14–15) and α2δ-1 (R217A) (n = 13–14) mice. Significant differences compared with vehicle treated wild-type (*p < 0.05) or α2δ-1 (R217A) (†P < 0.05) mice.

**Fig. 5.** Phenobarbital (–30 min, IP) produced dose-dependent anxiolytic-like effects in the Vogel conflict test in wild-type (n = 11–12) and α2δ-2 (R279A) (n = 11–12) mice. Significant differences compared with vehicle-treated wild-type (*p < 0.05) or α2δ-2 (R279A) (†p < 0.05) mice.

**Fig. 6.** Pregabalin (–2 h, IP) produced dose-dependent anxiolytic-like effects in the Vogel conflict test in wild-type (A, n = 14–15; B, n = 15) but not α2δ-1 (R217A) (A, n = 12–13; B, n =
12–14) mice in two separate experiments across a wide dose range. Significant differences (*p < 0.05) compared with vehicle-treated wild-type mice.

**Fig. 7.** Pregabalin (−2 h, IP) produced dose-dependent anxiolytic-like effects in the Vogel conflict test in wild-type (n = 12–14) and α<sub>2</sub>δ-2 (R279A) (n = 13–14) mice. Significant differences compared with vehicle-treated wild-type (*p < 0.05) or α<sub>2</sub>δ-2 (R279A) (†p < 0.05) mice.
Mean number of shocks

Morphine, mg/kg

Veh  0.32  1  3.2  5.6

*
Figure 3

A

![Graph A showing mean number of shocks vs. Phenobarbital concentration. Veh, 5.6, 10, 18, 32, 56 mg/kg.](image)

B

![Graph B showing mean number of shocks vs. Pregabalin concentration. Veh, 3.2, 10, 32, 100 mg/kg.](image)
Figure 4

![Bar graph showing the mean number of shocks for different phenobarbital doses in wild-type and α₂δ-1 (R217A) mice.](image)

- Wild-type
- α₂δ-1 (R217A)

**Mean number of shocks**

**Phenobarbital, mg/kg**

- Veh
- 10
- 32
- 56

* Indicates significance compared to wild-type, † indicates significance compared to 32 mg/kg.
Figure 5

![Graph showing the mean number of shocks in wild-type and α₂δ-2 (R279A) mice with different doses of phenobarbital. The y-axis represents the mean number of shocks, and the x-axis represents the doses of phenobarbital (Veh, 10, 32, 56 mg/kg). The graph includes error bars indicating variability. Significant differences are indicated by * for wild-type and † for α₂δ-2 (R279A) groups.]
Figure 6

A

Mean number of shocks

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>α₂δ-1 (R217A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td></td>
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</table>

Pregabalin, mg/kg

B

Mean number of shocks

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>α₂δ-1 (R217A)</th>
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</thead>
<tbody>
<tr>
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<td></td>
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</tbody>
</table>

Pregabalin, mg/kg
Figure 7

Comparison of mean number of shocks between Wild-type and \( \alpha_2\delta-2 \) (R279A) genotypes across different doses of pregabalin (mg/kg).