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Identification of the α2-δ-1 subunit of voltage-dependent calcium channels as a molecular target for pain mediating the analgesic actions of pregabalin

Mark J. Field*, Peter J. Cox*, Emma Stott*, Heather Melrose*, James Offord†, Ti-Zhi Su‡, Steve Bramwell*, Laura Corradini*, Steven England*, Joanna Wink*, Ross A. Kinloch*, Jan Hendrich§, Annette C. Dolphin§, Tony Webb†, and Dic Williams*

*Department of Pain Therapeutics and Animal Welfare Group, Discovery Biology, Pfizer Global Research and Development, Sandwich Laboratories, 8500 IPC 351, Sandwich, Kent CT13 9NJ, United Kingdom; †Department of Molecular Sciences, Pfizer Global Research and Development, Ann Arbor, MI 48105; and §Laboratory of Cellular and Molecular Neuroscience, Department of Pharmacology, University College London, Andrew Huxley Building, Gower Street, London WC1E 6BT, United Kingdom

Neuropathic pain is a debilitating condition affecting millions of people around the world and is defined as pain that follows a lesion or dysfunction of the nervous system. This type of pain is difficult to treat, but the novel compounds pregabalin (Lyrica) and gabapentin (Neurontin) have proven clinical efficacy. Unlike traditional analgesics such as nonsteroidal antiinflammatory drugs or narcotics, these agents have no frank antiinflammatory actions and no effect on physiological pain. Although extensive preclinical studies have led to a number of suggestions, until recently their mechanism of action has not been clearly defined. Here, we describe studies on the analgesic effects of pregabalin in a mutant mouse containing a single-point mutation within the gene encoding a specific auxiliary subunit protein (α2-δ-1) of voltage-dependent calcium channels. The mice demonstrate normal pain phenotypes and typical responses to other analgesic drugs. We show that the mutation leads to a significant reduction in the binding affinity of pregabalin in the brain and spinal cord and the loss of its analgesic efficacy. These studies show conclusively that the analgesic actions of pregabalin are mediated through the α2-δ-1 subunit of voltage-gated calcium channels and establish this subunit as a therapeutic target for pain control.

Neuropathic pain is a consequence of disease, trauma, or dysfunction of the central or peripheral nervous systems. It can result from a wide range of conditions including diabetes, nerve root compression, herpes zoster infection, cancer, and stroke thus affecting millions of people worldwide. Many patients respond poorly to conventional analgesics, such as nonsteroidal antiinflammatory drugs, and despite the use of alternative therapeutic approaches including tricyclic antidepressants and anticonvulsants, effective management of neuropathic pain remains a significant challenge. The novel compounds pregabalin (Lyrica) and gabapentin (Neurontin) have proven clinical efficacy in neuropathic pain (1, 2) and are effective in other disorders of the nervous system including epilepsy (3) and anxiety (4–6).

Despite over a decade of extensive research, the exact mechanism of action of pregabalin and gabapentin has yet to be elucidated, although a number of putative mechanisms have been postulated [see Taylor et al. for review (7)]. Despite their structural similarity to the inhibitory transmitter gamma amino butyric acid (GABA), neither pregabalin nor gabapentin bind to GABA_A or GABA_B receptors, nor do they interact with GABA uptake transporters (8), and studies suggesting that they may act via a specific presynaptic GABA_A heterodimeric complex (9) have been disproved (8, 10). In 1996, Gee, Brown, and coworkers (11, 12) isolated and sequenced a gabapentin binding protein from porcine brain. This was identified as the α2-δ subunit of voltage-gated calcium channels. The discovery of pregabalin and its low-affinity enantiomer (R)-isobutyryl-gaba suggested a relationship between α2-δ binding and analgesic activity (13). Thus, pregabalin (but not its enantiomer) demonstrated activity in a number of preclinical models of pain (13). However, the importance of the interaction of gabapentin and pregabalin with this protein has not been conclusively demonstrated.

Calcium channels constitute a diverse class of proteins that have been traditionally classified via their pharmacological and electrophysiological properties (14). These channels play a fundamental role in the regulation of many biological processes with transient changes in calcium ion concentration essential for neurotransmitter release and modulation of cell-membrane excitability. α2-δ is one of three regulatory subunits along with β and γ that configure around the pore-forming α1 subunit (15, 16) to form the complete channel complex. The α2-δ protein is encoded by at least four genes. α2-δ-1 was the first to be identified and is the most widely studied, but this has been followed by discovery of three other novel genes, α2-δ-2, α2-δ-3, and α2-δ-4, contributing to the heterogeneity of voltage-dependent calcium channels (VDCCs) (17). Each of these subtypes has been shown to display tissue-specific distribution (17). Recent studies have identified regions critical for gabapentin binding on the α2-δ subunit (11, 18). These molecular studies have shown that a mutation leading to the substitution of a single amino acid, arginine at position 217 with alanine (R217A) on the α2-δ-1 subunit (15, 16) to form the complete channel complex. The α2-δ-1 protein is encoded by at least four genes. α2-δ-1 was the first to be identified and is the most widely studied, but this has been followed by discovery of three other novel genes, α2-δ-2, α2-δ-3, and α2-δ-4, contributing to the heterogeneity of voltage-dependent calcium channels (VDCCs) (17). Each of these subtypes has been shown to display tissue-specific distribution (17). Recent studies have identified regions critical for gabapentin binding on the α2-δ subunit (11, 18). These molecular studies have shown that a mutation leading to the substitution of a single amino acid, arginine at position 217 with alanine (R217A) on the α2-δ-1 subunit (15, 16) to form the complete channel complex.

Results

Construction of R217A Mutant Mice. A mouse expressing an α2-δ-1 protein with a mutation at residue 217 was successfully generated...

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confirmed that there was no change in the expression of membranes from HEK293 cells overproducing human respectively (Fig. 1).

Analysis was 1.06 and mutant mice. The relative density as shown by Western blot mutant in Cos-7 cells (Fig. 1).

graphic localization of [3H]pregabalin in spinal cord and brain of mutant mice.

generated by RT-PCR using RNA extracted from whole brain of et al.

mice (see Fig. 6 and Supporting Text).

Effect of Single-Point Mutation on α2-δ-1 Protein Levels and Pregabalin Binding. Western blot analysis of brain tissue (neocortex) extracts showed that α2-δ-1 protein was expressed at similar levels in WT and mutant mice. The relative density as shown by Western blot analysis was 1.06 ± 0.14 and 0.94 ± 0.03 for WT and R217A mice, respectively (Fig. 1C). Pregabalin binding in R217A mouse neocortex was markedly reduced. The binding activity in R217A cortical membranes was 49 ± 3 as compared with 353 ± 48 fmol/mg protein for WT control (P < 0.001) (Fig. 1E). Similar results were obtained by using transiently expressed porcine α2-δ-1 R217A mutant in Cos-7 cells (Fig. 1D and F). In addition, RT-PCR analysis confirmed that there was no change in the expression of α2-δ-1 and α2-δ-2 mRNA in whole brain RNA isolated from WT and mutant mice (see Fig. 6 and Supporting Text, which are published as supporting information on the PNAS web site).

Pregabalin Autoradiography in Mutant and WT Mice. Autoradiographic localization of [3H]pregabalin in spinal cord and brain of the WT mouse showed a differential anatomical distribution (Fig. 2A–D) that was essentially identical to that of [3H]gabapentin (20, 21). The highest levels of binding were found in outer layers of the cerebral cortex, followed by the molecular layer of the cerebellum.
Formalin Test in Mutant and WT Mice. After injection of 20 μl of 2% formalin, both mutant and WT mice displayed a typical biphasic nocifensive response. The first (early) phase was seen in the first 10 min where the animals lick and bite the hind paw resulting in ~75 s of nocifensive activity (Fig. 4A). The second period (late phase) started after ~15 min, peaked at ~20–35 min, and was largely complete by the 45-min time point resulting in ~160 s of nocifensive activity (Fig. 4A).

Pregabalin (30 mg/kg) and morphine (3 mg/kg) produced a significant blockade of the late-phase nocifensive response in the WT mice (Fig. 4B). Notably, pregabalin was ineffective in blocking pain behavior in the mutant mice, whereas morphine produced a similar analgesic effect in both mutant and WT mice (Fig. 4B).

Discussion

The key finding of this study is that the analgesic action of pregabalin and gabapentin are mediated through the α2δ-1 subunit of VDCCs. The mutation at position 217 prevents pregabalin from binding to the α2δ-1 subunit, without affecting the level of protein expression. The mutation decreases the barium flux through calcium channels expressed in vitro, as supporting information on the PNAS web site).

data from two experiments with similar expression levels have been averaged, the current at +5 mV was enhanced 3.22 ± 0.42-fold by WT α2δ-1 (n = 15). The R217A α2δ-1 produced a smaller enhancement of CaV2.2 currents at this potential (1.93 ± 0.35-fold, n = 14, P = 0.043 compared with WT α2δ-1). This shows that the reduced ability of R217A α2δ-1 to enhance CaV2.2/B1 currents does not depend on the expression system. In further studies, robust calcium currents were recorded from DRG neurons isolated from R217A mice, which were qualitatively and quantitatively similar to those recorded from WT mouse DRG (see Fig. 7 and Supporting Text, which are published as supporting information on the PNAS web site).

Chronic Constriction Injury (CCI)-Induced Punctate Alldynia in Mutant and WT Mice. After CCI of the sciatic nerve, both mutant and WT mice developed punctate allodynia as measured by the application of von Frey filaments. This reached a peak 7 days after injury and was maintained for at least 3 weeks (Fig. 5A). A similar onset and magnitude of pain behavior was seen in both the mutant and WT mice (Fig. 5A).

Pregabalin (30–100 mg/kg), gabapentin (100–300 mg/kg), and amitriptyline (4–12 mg/kg) all produced dose-dependent inhibition of the CCI-induced punctate allodynia in the WT mice (Fig. 5B–D). Amitriptyline also produced a similar blockade of allodynia in the mutant mice (Fig. 5D). However, pregabalin and gabapentin showed no efficacy in the mutant mice, which maintained pain behaviors (punctate allodynia) similar to vehicle-treated controls (Fig. 5B and C).

No abnormal phenotype was noted for the R217A mice during these studies (see Fig. 8 and Supporting Text, which are published as supporting information on the PNAS web site).

Fig. 3. Effect of WT α2δ-1 and R217A α2δ-1 on CaV2.2/B1 currents. CaV2.2/B1 was expressed either alone or with WT α2δ-1 or R217A α2δ-1 in tsA-201 cells (A and B) or Xenopus oocytes (C) and recorded by using 10 mM Ba2+ as charge carrier. (A) Representative current traces elicited by steps to test potentials (TP) between −30 and +15 mV in 10-mV steps from a holding potential of −90 mV for CaV2.2/B1 (Left), CaV2.2/B1/α2δ-1 (Center), and CaV2.2/B1/R217A α2δ-1 (Right). The scale bar refers to all traces. (B) I–V relationships for the three experimental conditions following expression in tsA-201 cells. Open triangles, CaV2.2/B1 (n = 11); open squares, CaV2.2/B1/α2δ-1 (n = 9); filled circles, CaV2.2/B1/R217A α2δ-1 (n = 11). (C) I–V relationships for the three experimental conditions following expression in Xenopus oocytes. Open triangles, CaV2.2/B1 (n = 9); open squares, CaV2.2/B1/α2δ-1 (n = 15); filled circles, CaV2.2/B1/R217A α2δ-1 (n = 14).

Fig. 4. Formalin test in mutant and WT mice. (A) Effect of formalin induced nocifensive response in knockin and WT mice. (B) Effect of pregabalin and morphine in the formalin test in mutant and WT mice. Results are expressed as mean ± SEM time spent licking and biting the hind paw for late phase (n = 6–8 for each group). *P < 0.05; **P < 0.01 (one-way ANOVA followed by Dunnett’s t test vs. vehicle at each time point).
per group (vertical bars represent first and third quartiles).

Median force (g) required to induce a paw withdrawal in six to seven animals were reassessed for up to 4 h. The punctate allodynia data are expressed as olds (PWT) to von Frey hairs were assessed. After drug administration, PWTs allodynia in the mutant and WT mice. Baseline (BL) paw withdrawal thresholds (22), at least in part, because of a reduction in trafficking and subcortical regions including the thalamus.

In expression systems examined to date, calcium channel complexes lacking the α2-δ subunit have markedly reduced current densities (22), at least in part because of a reduction in trafficking (23). The present study shows that the mutation at position 217 results in a significant decrease in divalent cation current through the pore forming α1 complex. However, although the effect of R217A α2-δ-1 was reduced, the enhancement remained statistically significant in two different expression systems. It is possible that the role of α2-δ subunits on calcium current expression in native systems is less marked than suggested from expression studies. For example, in da/du and dr2q/dr2q, the complete loss of α2-δ-2 results in only about a 30% reduction in Purkinje cell calcium channel currents, although there is no compensation by other α2-δ subunits (24). Furthermore, there is no reduction in somatic calcium channel currents in heterozygotes, despite a partial loss of α2-δ-2 protein. In contrast, in all expression systems examined, an 8-fold enhancement by α2-δ-2 was observed (23, 24). Therefore, it is possible that whatever the nature of the dysfunction in R217A α2-δ-1, it retains sufficient functionality such that in vivo it appears normal.

In this context, it is interesting to note that robust calcium currents were recorded from DRG neurons isolated from R217A mice, which were qualitatively and quantitatively similar to those recorded from WT mouse DRG (see Fig. 7 and Supporting Text) and exhibited properties as described previously for mouse DRG neurons (25). Although we performed no detailed pharmacological survey of calcium channel subtypes, the current–voltage relationships, thresholds, and kinetics of the currents suggested the presence of (at least) T-, N-, and L-type channels in both R217A and WT cells (in accordance with published data from mouse DRG neurons—see ref. 26). The presence of the mutation did not seem to impair calcium channel function, and we were unable to distinguish any differences in the Ca2+ currents between the two populations of cells (see Fig. 7 and Supporting Text).

A number of recent gene targeting studies knocking out various subunits of VDCCs demonstrate distinct changes in nociceptive phenotype. Deletions of the β3, α1B, and α1E subunits result in a distinctly different pain phenotype in the knockout as compared with WT controls (27–29). Ducky mice, which contain a rearranged α2-δ-2 gene, have significant behavioral deficits including absence epilepsy and cerebellar ataxia (24). However, no differences were observed in the general behavioral phenotype between the R217A mutant mice and WT mice, with no differences noted in weight gain or in terms of general and fine motor coordination (see Fig. 8 and Supporting Text). Unlike the calcium channel knockout mice, R217A mutant mice demonstrate the same pain phenotype in models of acute/tonic and chronic pain as their WT littermates and contain the same levels of α2-δ-1 protein in brain. Moreover, formalin and sciatic nerve injury induced the same intensity of pain-related behaviors in both mutant and WT cohorts. Formalin typically produces a biphasic pain response with the first phase thought to be mediated by direct activation of nociceptors and a second phase mediated by the development of central sensitization due to the ongoingafferent fiber activity and localized inflammation (30). Injury to the sciatic nerve by application of loose ligations induces punctate allodynia, a symptom that is also described by patients with neuropathic pain (31). The onset of alldynia and degree of hypersensitivity seen after sciatic nerve injury were similar in both the mutant and WT mice.

Pregabalin possessed no analgesic activity in the R217A mutant mice against either chemically induced tonic pain or nerve injury-induced chronic pain (similar behavioral data were produced for gabapentin in the nerve-injury model). However, typical analgesic responses were demonstrated in littermate WT controls. Pregabalin and gabapentin are known to interact with both the α2-δ-1 and α2-δ-2 subunits (17) (T.-Z.S., unpublished data). It has been shown that α2-δ-1 is up-regulated in DRG neurons after nerve injury (36, 37) and that this correlates with the onset and duration.
of pain behavior. However, activity of pregabalin has been demonstrated in acute preparations (e.g., ref. 38) so that the functional consequence of the up-regulation and its importance in clinical pain states remains unknown. Previous pharmacological investigations have provided evidence that analgesic activity of pregabalin and gabapentin is through the α2-δ subunit. Thus, enantiomeric pairs of α2-δ ligands show affinity-related potency in animal models of pain (13, 39), and structure–activity relationships in models of epilepsy, anxiety, and pain, further demonstrate the importance of α2-δ binding to activity for pregabalin and its analogues (40). The current data are consistent with and add to these findings, demonstrating that it is the α2-δ-1 subunit that provides the requirement for the analgesic action of pregabalin and gabapentin.

Neuropathic pain represents a significant therapeutic challenge, and gabapentin shows clinical efficacy in a wide range of neuropathic pain conditions (41). Pregabalin, the more potent new-generation compound is the most widely studied medicine in controlled clinical trials for neuropathic pain and represents an important new therapy in this area. These data demonstrate that the analgesic action of pregabalin is mediated through the α2-δ-1 subunit of voltage-gated calcium channels and firmly establish this subunit as a target for pain control.

Methods

Binding Studies. Materials. Porcine α2-δ-1 WT and R217A expression constructs and GKS07, a stable cell line overproducing human α2-δ-2, are described in ref. 18. [3H]Pregabalin and the ECL kit were from Amersham Biosciences. The α2-δ-1 monoclonal antibody was purchased from Affinity BioReagents (Golden, CO). Cell culture reagents were from Invitrogen. Other chemicals were from Sigma.

Cell culture and transfection. Cos-7 and GKS07 cells were cultured in DMEM, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FBS, in a humidified incubator with (95% air/5% CO2) at 37°C. For transient transfection into COS-7 cells, 20 μg of plasmid DNA was incubated with 60 μl of Lipofectamine. The mixture was overlayed onto the cells in 15 ml of serum-free medium and incubated for 5 h after which FBS was added to the dishes to a final concentration of 10%. The cells were harvested 48 h after transfection and used for membrane preparation.

Membrane preparation. Membranes were prepared from cultured cells and mouse tissues by homogenization in TE buffer containing 5 mM Tris (pH 7.5), 5 mM EDTA, 0.1 mM PMSF, 0.02 mM leupeptin, and 0.02 mM pepstatin. The resulting homogenates were centrifuged for 10 min at 5,000 × g, and the resultant supernatants were centrifuged at 100,000 × g for 30 min. Pellets were resuspended in the same buffer and stored at −80°C.

Binding assay. Membrane proteins (15 μg) were incubated in the presence of 20 nM [3H]pregabalin and 10 nM Hepes (pH 7.5) for 90 min at 22°C, filtered onto prewetted GF/C membranes, and then quickly washed three times with 3 ml of ice-cold 50 mM Tris buffer (pH 7.5). The filters were dried and counted in a liquid scintillation counter. Nonspecific binding was defined by using 10 μM pregabalin. Specific binding was obtained by subtracting nonspecific binding from total binding. All experiments were carried out in triplicate and values were expressed as mean ± SD.

Western blot analysis. Cell membranes (15 μg proteins for brain tissues and 2 μg for cultured cells) were resolved by 4–20% SDS/PAGE and transferred to nitrocellulose membranes. Transferred membranes were incubated with mouse anti-α2-δ-1 antibody at 4°C overnight, followed by washing with 1× TBST. Blots were incubated with HRP-conjugated anti-mouse IgG for 1 h and developed with ECL reaction according to the procedure recommended by the manufacturer. Western blot images were analyzed by using the Laboratory Imaging and Analysis System (UVP, Upland, CA).

For details on the generation of the R217A mutant mouse, see Supporting Text.

 Autoradiography Studies. Animals and tissue preparation. Three (one male and two female) WT and four (three male and one female) R217A knockin 6-week-old animals were killed with CO2 and perfused transcardially with 500 ml of PBS (pH 7.4) followed by 500 ml of paraformaldehyde (4% wt/vol, in 0.1 M Sorensens phosphate buffer, pH 7.4, 4°C). The lumbar spinal cord (L3–L6) was removed, postfixed overnight at 4°C, and transferred to sucrose in PBS (20% wt/vol) for at least 48 h. The tissues were frozen in OTC embedding medium (Tissue-Tek) to −15°C and the entire lumbar segment sectioned at 15 μm by using a Leica CM3050 cryostat. Sections saved in batches of 15 were thaw-mounted onto Plus slides (Fisher) and stored at −80°C until required.

 Autoradiography. Slides were allowed to air-dry at room temperature for 1 h and preincubated in Hepes/KOH (10 mM, pH 7.4) for 15 min at room temperature. Adjacent sections were then incubated for 30 min at room temperature in the same buffer containing 40 nM [3H]pregabalin (78 Ci/mmol (1 Ci = 37 GBq); Amersham Biosciences; custom synthesis) in the presence or absence of gabapentin (10 μM) to define nonspecific binding. After the incubation, the slides were washed three times for 1 min each in NaCl (100 mM at 4°C), dipped in ice-cold distilled water to remove buffer salts, and air-dried overnight at room temperature.

Dried sections were exposed to tritium Hyperfilm (Amersham Biosciences) for 1 month together with [3H] microscales (ranging from 0.10–109.4 nCi/mg). Quantitative densitometry analysis of autoradiographs was performed by using the Microcomputer Imaging Device (Imaging Research).

Effect of WT α2-δ-1 and R217A α2-δ-1 on CaV2.2/β1b currents. The tsA-201 cells were transfected with cDNAs for rabbit Cav2.2 and rat β1b, both in the vector pMT2 (42), and either WT or R217A α2-δ-1 in pcDNA3.1. The cDNA for green fluorescent protein (mut3 GFP) (43) was included in the transfection to identify transfected cells from which electrophysiological recordings were made. Transfection was performed essentially as described in ref. 44, using a ratio of 3:2:0:2 for Cav2.2, β1b, α2-δ-1, and GFP, respectively. When α2-δ-1 was omitted, it was substituted by pMT2. Calcium-channel expression in tsA-201 cells was investigated by whole-cell patch clamp recording, essentially as described in ref. 45. For details, see ref. 50.

Xenopus oocytes were prepared, injected, and used for two-electrode voltage clamp electrophysiology as described in ref. 23, with the following exceptions. Plasmid cDNAs for the different VDCC subunits Cav2.2, α2-δ-1, and β1b were mixed in equivalent weight ratios at 1 μg/μl−1, and 9 nl was injected intranuclearily. The recording solution for Cav2.2-injected oocytes contained 10 mM Ba(OH)2, 70 mM NaOH, 2 mM CsOH, and 5 mM Hepes (pH 7 with methanesulfonic acid).

Behavioral protocols. All studies were completed by an observer blinded to genetic background of the mice and drug treatments and in accordance with the United Kingdom Animals (scientific procedures) Act (1986) and the internal Pfizer ethical review policy. No abnormal phenotype was noted during these studies (see Fig. 8 and Supporting Text).

Formalin test. R217A α2-δ-1 mutant mice and WT mice were administered pregabalin or gabapentin 1 h before testing and morphone 30 min before testing. Animals were habituated to the perspex observation boxes 15 min before the administration of formalin (20 μl of 5% wt/vol) into the plantar surface of the left hind paw. The time spent licking and biting the paw was recorded in 5-min periods for 45 min. R217A α2-δ-1 mutant mice and WT mice were randomly divided into three subgroups receiving various treatments. All groups were administered drug treatments by s.c. injection at a volume of 10 ml/kg.

CCI model. CCI of the sciatic nerve was induced in R217A α2-δ-1 mutant mice and (n = 24 of each) WT mice based on methods described in ref. 32. Briefly, animals were placed in an anesthetic.
chamber and anesthetized with a 2% isoflurane O2 mixture. The right hind thigh was shaved and swabbed with 1% iodine. Animals were then transferred to a homeothermic blanket for the duration of the procedure, and anesthesia was maintained during surgery via a nose cone. After an incision was made along the line of the thigh bone, the common sciatic was exposed, and three suture ligatures (4-0 silk) were tied around the nerve in a double knot. The incision was closed in layers, and the wound was treated with topical antibiotics.

The development of punctate allodynia was assessed over a period of up to 20 days after surgery, and pharmacological studies were carried out from 10 days after surgery once a stable allodynia had been achieved. Animals were habituated to the test cages (for 1 h) and to punctate stimulus before the start of each experiment. On the test day, baseline readings of punctate allodynia were recorded before drug treatment. Animals were then randomly divided into three subgroups receiving vehicle, pregabalin, or amitriptyline treatments. All groups were administered drug treatments. The experiment was carried out over 2 days, and the data were collated at the end of the experiment.

Punctate allodynia was evaluated by application of von Frey filaments (Stoelting, Wood Dale, IL) in ascending order of force (0.008, 0.02, 0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, and 6.0 g) to the plantar surface of hind paws. Each filament was applied to the paw for a maximum of 6 s or until a withdrawal response occurred. Once a withdrawal response was established, the paw was retested, starting with the filament below the one that produced a withdrawal, and subsequently with the remaining filaments in descending order of force until no withdrawal occurred. The highest force of 6 g lifted the paw in addition to eliciting a response and was taken as the cut off point. Both hind paws of each animal were tested in this manner. The lowest amount of force required to elicit a response was recorded as PWT in grams, with ranges expressed as medians and quartiles and plotted on a log scale. A withdrawal response to a stimulus of 0.04 g or less was defined as punctate allodynia.

Drugs. Pregabalin (PD144723-0000 Lot-G) and gabapentin (PD0087842-0000 Lot-N) were synthesized at Pfizer (Ann Arbor). Morphine and amitriptyline were purchased from Sigma (Poole, U.K.). All compounds were dissolved in water and administered in a dose volume of 10 ml/kg. Doses were selected based on previous studies (32–34) and on pilot studies using the mutant and WT mice.

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