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Pregabalin produces analgesia in males but not females in an animal model of chronic widespread muscle pain

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Abstract

Introduction: Pregabalin, which acts on the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels, relieves ≥50% of pain in a third of individuals with fibromyalgia. Thus far, preclinical studies of pregabalin have predominantly used male animals.

Objectives: The purpose of our study was to investigate potential sex differences in the analgesic efficacy of pregabalin that may contribute to disparities in human outcomes.

Methods: We used a mouse model of chronic widespread muscle pain (CWP) to test the effects of pregabalin on muscle hyperalgesia, nonreflexive pain, and motor behaviors. The CWP pain model combines 2 pH 4.0 saline injections, spaced 5 days apart, into the gastrocnemius muscle and produces bilateral muscle hyperalgesia. Furthermore, we explored sex differences in the mRNA and protein expression of the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels in the dorsal horn of the spinal cord and dorsal root ganglia after development of CWP.

Results: Pregabalin fully attenuated muscle hyperalgesia bilaterally in male but not female mice with equal motor deficits produced in both sexes. In addition, using the conditioned place preference test, mice of both sexes with CWP spent significantly more time in the pregabalin-paired chamber compared with baseline, but not significantly greater than pain-free controls. Chronic widespread muscle pain produced no changes in $\alpha_2\delta$-1 subunit mRNA or protein expression in the dorsal horn of the spinal cord or dorsal root ganglia in either sex.

Conclusion: Overall, these findings indicate pregabalin may be more effective in treating CWP in males, but the factors leading to these differences are not fully understood.

Keywords: Pregabalin, Sex differences, Musculoskeletal pain, $\alpha_2\delta$-1, Fibromyalgia

1. Introduction

Musculoskeletal pain conditions, including fibromyalgia, are a leading contributor to disability, affecting approximately 20% to 33% of the world's population.[7] Pregabalin is a commonly used pharmacological treatment for neuropathic and fibromyalgia pain.[6,8] Pregabalin's effectiveness in alleviating pain is limited, with only 30% of individuals with fibromyalgia experiencing a pain reduction of 50% or more after 8 weeks of treatment (numbers needed to treat = 9.7).[1,10,17] Although most pain conditions have a higher prevalence in women, most animal studies investigating pregabalin's analgesic effects have not included both sexes.[3,15] In addition, mechanistic studies of pregabalin's analgesic actions have been conducted primarily in male animals.[4,11,13,14,16,23,30] Moreover, the evaluation of mechanical pain testing in animals treated with pregabalin is complicated by pregabalin's adverse effects on motor function when administered at analgesic doses.[50] Thus, further exploration of pregabalin's efficacy in alleviating reflexive and nonreflexive musculoskeletal pain in both sexes is necessary.

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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The molecular mechanism of pregabalin-induced analgesia remains unclear; however, it binds to $\alpha_2\delta$-1 subunits of voltage-gated calcium channels.[16] This binding leads to a decrease in calcium-mediated neurotransmitter release at presynaptic terminals in the spinal cord.[16,19,23] After neuropathic injury, there is upregulation of $\alpha_2\delta$-1 subunit in the dorsal root ganglia (DRG) and dorsal horn of the spinal cord.[4,9,28,36,49,51] Furthermore, $\alpha_2\delta$-1 knockout mice have reduced sensitivity to mechanical stimuli and delayed onset of pain, whereas $\alpha_2\delta$-1 overexpression causes tactile allodynia and thermal hyperalgesia, and treatment with pregabalin inhibits increased trafficking of the $\alpha_2\delta$-1 subunit.[4,29,38] These findings suggest that $\alpha_2\delta$-1 subunit contributes to neuropathic pain and pregabalin reduces pain by inhibiting the heightened excitability of neurons. We previously demonstrated pregabalin reduces paw and muscle hyperalgesia after induction of widespread muscle pain in male mice.[35,45,50] However, effects in females and changes in expression of $\alpha_2\delta$-1 subunit in this model are unknown. Therefore, this study aimed to investigate sex differences in effectiveness of pregabalin using an animal model of chronic widespread pain. The objectives of this study were twofold: (1) to evaluate analgesic effectiveness of pregabalin in both sexes using reflexive and nonreflexive pain testing and (2) to explore potential sex-specific differences in $\alpha_2\delta$-1 subunit expression in DRG and spinal cord after induction of muscle pain.

2. Materials and methods

2.1. Animals

A total of 172, 8-week-old C57BL/6J mice with equal numbers of males and females were used for this study. Mice were kept on a 12:12 hour light/dark cycle with food and water available ad libitum. Animals were housed in groups of 4 and randomly allocated to treatment groups. Experimenters were blinded to group assignment during behavioral testing and tissue sample assessments. All behavior tests were completed in the morning except for conditioned place preference, which based on the experimental design had to be completed in the morning and afternoon. All experiments were conducted in accordance with the National Institutes of Health's Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

2.2. Widespread muscle pain model

The chronic widespread muscle pain (CWP) model, which combines 2 intramuscular (i.m.) acidic saline injections spaced 5 days apart, was induced as previously described.[45] On day 0, the left gastrocnemius muscle was injected with 20 μL of pH 4.0 ± 0.1 acidic saline. Five days later, animals received a second i.m. injection of pH 4.0 saline into the left gastrocnemius muscle. Pain-free controls received i.m. injections of 20 μL of pH 7.2 ± 0.1 saline into the left gastrocnemius muscle on days 0 and 5. The CWP model produces bilateral muscle hyperalgesia, visceral hyperalgesia, mechanical hypersensitivity, and altered cardiovascular autonomic balance without damage to the tissue and models fibromyalgia with a similar pharmacological treatment profile.[12,21,33,34,37,41,45,46]

2.3. Drug administration

Pregabalin (Y0001805, Millipore Sigma) was dissolved in deionized water and delivered at a dose of 30 or 60 mg/kg through intraperitoneal (i.p.) injection. These doses were chosen based on previous literature showing an analgesic effect on muscle hyperalgesia in males.[50]

2.4. Behavioral assessments

2.4.1. Muscle withdrawal threshold

To test for muscle hyperalgesia, we assessed muscle withdrawal thresholds (MWTs) of the left (ipsilateral) and right (contralateral) gastrocnemius muscle. Before testing, animals were acclimated to a gardener's glove 2 times a day for 2 days. Muscle withdrawal threshold was determined by squeezing the gastrocnemius muscle using custom-built, force sensitive tweezers until the animal withdrew its hind limb. Three trials spaced 5 minutes apart were averaged to obtain 1 reading for each assessment point. The presence of muscle hyperalgesia is indicated by a decrease in MWT.

2.4.2. Conditioned place preference

Conditioned place preference (CPP) was used to test nonreflexive pain after induction of CWP. The CPP apparatus consisted of 3 chambers, 2 outer pairing chambers (10.75″ × 8.25″) and 1 connecting chamber (4.75″ × 8.25″). One pairing chamber had black and white striped walls and smooth surface floors, and the other pairing chamber had solid black walls and ridged flooring. The middle chamber was solid black with a smooth surface and contained an overhead light. On day 6 after induction of CWP, animals were placed in the CPP apparatus with access to all 3 chambers, and time spent in each was recorded. In the morning on days 7 to 10, animals were injected with pregabalin vehicle (ddH_2O, i.p.) and placed in a pairing chamber for 60 minutes. Four hours later, animals were injected with pregabalin (60 mg/kg, i.p.) and confined to the opposite pairing chamber for 60 minutes. The assignment to pairing chamber was randomized and counterbalanced. On day 11, animals were granted access to all 3 chambers for 30 minutes, and time spent in each was recorded. Raw and change scores for time spent in pregabalin-paired chambers were analyzed. One male was removed from the analysis because it spent ≤80% of baseline testing in 1 chamber.

2.4.3. Rotarod

Rotarod was used to test for motor behavior deficits as previously described.[50] Briefly, animals were acclimated on the rotarod for 2 days with 3 sessions per day. The rotarod acclimation protocol involved increasing the rotarod speed from 13 to 17 rpm over different time intervals in 3 sessions on consecutive days. On the test day, the rotarod ramped from 13 to 17 rpm over 120 seconds and maintained 17 rpm for 30 seconds. Only animals who were able to stay on the rotarod during the baseline test session were included in the data analysis (1 male excluded). After baseline testing, animals received the CWP model. Animals were then injected with pregabalin (30 or 60 mg/kg, i.p.) or vehicle and tested on the rotarod 90 minutes after injection.

2.4.4. Balance beam

To further assess motor deficits, a balance beam test was performed as previously described.[32] In brief, the balance beam had 2, raised rectangular 1-meter beams (6 mm in width). A 700-lumen light was placed at the starting end and a dark box at the finishing end. All mice underwent 2 acclimation days, which included crossing balance beam 3 times each day, and baseline measures were obtained before induction of CWP. Time to cross

the balance beam and number of hind leg foot slips while crossing were analyzed. Markings were made at the 10-cm point and 90-cm point on each beam, and a stopwatch was used to record crossing time between the 2 points. Two successful passes across each beam (no stalling) were recorded, and the fastest time was used for analysis. All passes were recorded by video. The total number of left and right foot slips were tallied and summed by a blinded reviewer for the 2 successful passes (used for time to cross). No more than 3 trials were performed to obtain 2 successful passes for analysis on each beam. A 10-minute rest was given between rotarod and balance beam testing.

2.5. Quantitative polymerase chain reaction

Mice were euthanized with CO_2, and L4-6 DRG and dorsal horn of the spinal cord were collected and placed in RNA later (Invitrogen, Thermo Fisher Scientific, Waltham, MA). RNA was isolated using a fibrous tissue mini kit (Qiagen, Hilden, Germany) and further purified with an RNA clean and concentrate kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. Samples of isolated total RNA were stored at −80°C until analysis. Reverse transcription was performed with 200 ng of RNA using an Affinity Script Quantitative PCR (qPCR) cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer's instructions. The cDNA was stored at −20°C until qPCR analysis was performed. cDNA was used as a template for qPCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). All forward and reverse primers were developed using NCBI Primer-BLAST software to ensure no off-target amplification (http://www-ncbi-nlm-nih-gov.proxy.lib.uiowa.edu/tools/primer-blast/), manufactured by Integrated DNA Technologies (Coralville, IA) and are listed in **Table 1**. Analysis of qPCR amplifications was performed with QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) using the 2-ΔΔCT method with 36B4 serving as an internal control. Samples were run in duplicate, and the cycle threshold was averaged between the 2 samples. Reactions were performed under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C.

2.6. Immunohistochemistry

Animals were anesthetized with ketamine/xylazine (87.5/12.5 mg/kg, i.p.) and perfused with heparinized saline followed by freshly prepared 4% paraformaldehyde. L4-6 DRG and spinal cord were extracted and placed in 4% paraformaldehyde overnight. Tissue was placed in increasing concentrations of sucrose (10%-30%) then frozen in OCT (Tissue Tek; Thermo Fisher Scientific) over dry ice and stored at −20°C. Tissues were cut into 20-μM sections and stained using an antibody for the $\alpha_2\delta$-1 subunit. Tissue sections were incubated for 30 minutes in 5% normal goat serum (S-1000, Vector Labs, Burlingame, CA). Tissues were washed in an avidin-biotin blocking wash (Vector Labs) before incubation in the $\alpha_2\delta$-1 antibody overnight. Sections were then incubated with a biotin-goat anti-mouse IgG secondary antibody for 1 hour and then incubated for an hour in streptavidin Alexa Fluor-568 antibody before coverslipping with Vectashield (Vector Labs) (antibody details in **Table 2**). 20× images were taken on a BX-61 light microscope (Diagnostic Instruments, Sterling Heights, MI) in the Central Microscope Facility at the University of Iowa. For each location and animal, 3 to 5 sections were quantified and averaged. Optical density of $\alpha_2\delta$-1 staining was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) as previously described.[20] For the dorsal horn of the spinal cord, the optical density in the superficial (I-II) and deep (III-VI) lamina were analyzed separately. For the ventral horn of the spinal cord, 1 optical density reading was recorded for the ipsilateral and contralateral sides. Animals were only included in the immunohistochemical analysis if 3 or more tissue sections could be quantified; therefore, 2 males and 4 females were excluded from the spinal cord analysis and 3 males and 1 female from the DRG analysis.

2.7. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (version 27; IBM, Armonk, NY). All statistics are reported as mean ± SEM. Muscle withdrawal threshold data were analyzed with a 2-way analysis of variance (ANOVA) with fixed effects for drug and sex at the 90-minute MWT timepoint. If a significant interaction between drug and sex was found, data were separated by sex, and a 1-way ANOVA with a Sidak post hoc test was performed. Cohen's *d* effect size was calculated for the 90-minute MWT timepoint. Time spent in the pregabalin-paired chamber was analyzed with a mixed model, repeated measures ANOVA for time, group, and sex. For CPP change scores of time spent in the pregabalin-paired chamber, data were analyzed using a 2-way ANOVA. For rotarod, balance beam, qPCR, and protein data, a 2-way ANOVA with Sidak multiple comparisons was used. If a significant interaction between group and sex was found, data were separated by sex, and a 1-way ANOVA with a Sidak post hoc test was used. For all experiments, a *P*-value of <0.05 was

Table 1

Primer design for quantitative PCR experiments.

Gene name	Accession number		Primer sequence	Product size
CACNA2D1 ($\alpha_2\delta$-1)	NM_001110843.1	F R	CCCTTCGCCCGTCACTATCA AGTTGGCGTGCATTGTTGGG	153 bp
CACNA1C ($Ca_v1.2$)	NM_009781.4	F R	CACCATTGCCTCCGAACATTAC GGCTTTATTGGCTGTGTCTTGC	70 bp
CACNA1D ($Ca_v1.3$)	NM_001083616.2	F R	TGACGCCTGGAACACGTTTG ACCAACCGCATCACTCGGAA	138 bp
CACNA1B ($Ca_v2.2$)	NM_001042528.3	F R	GACACCGAATGCCGGGAGTA GTGTTGCCAGCCGCATCATT	152 bp
CACNA2D2 ($\alpha_2\delta$-2)	NM_001174047.1	F R	CTGCCGCCTCTGTTGCTTTT TGCACGCCTCCAAAAATCCG	143 bp
36B4 (housekeeping)	NM_007475.5	F R	GCAGGTGTTTGACAACGGCA CACAGACAATGCCAGGACGC	190 bp

Table 2

Antibodies used in immunohistochemistry experiments.

Antibody	Company	Product number	Concentration
CACNA2D1	Invitrogen	MA3-921	1:500
Biotin-goat anti-mouse IgG	Life Technologies	B11027	1:500
Streptavidin Alexa Fluor 568	Invitrogen	S11226	1:500

considered statistically significant. Sample sizes were determined based on previously published data from our laboratory showing significant differences between group and sex.[18,26,27]

3. Results

3.1. Experiment 1: acute injection of pregabalin alleviates muscle hyperalgesia in males only

To determine whether pregabalin alleviated muscle hyperalgesia, we induced CWP, administered pregabalin, and tested MWT 90 minutes and 24 hours after treatment (**Fig. 1A**). There was a significant interaction of drug by sex on the ipsilateral ($F_{1,40} = 15.52$, $P < 0.01$) and contralateral ($F_{1,40} = 25.10$, $P < 0.01$) limb; thus, MWT values were analyzed separately for each sex. In males, there was a significant drug interaction on the ipsilateral ($F_{2,20} = 28.66$, $P < 0.01$) and contralateral ($F_{2,20} = 39.6$, $P < 0.01$) limb. In males, when compared with vehicle administration, pregabalin increased MWT values on the ipsilateral (30 mg/kg: $P < 0.01$, Cohen's $d = 2.76$; 60 mg/kg: $P < 0.01$, Cohen's $d = 3.54$) and contralateral limb (30 mg/kg: $P < 0.01$, Cohen's $d = 3.51$; 60 mg/kg: $P < 0.01$, Cohen's $d = 4.06$; **Fig. 1B and C**). There was no significant difference in the MWT values between males receiving 30- and 60-mg/kg pregabalin on either limb ($P = 0.10$-0.11). In females, pregabalin showed no significant effect on the MWT values on the ipsilateral ($F_{2,20} = 0.28$, $P = 0.76$) and contralateral limb ($F_{2,20} = 0.48$, $P = 0.63$; **Fig. 1D and E**). Thus, systemic doses of pregabalin acutely reversed muscle hyperalgesia bilaterally in male mice only.

3.2. Experiment 2: pregabalin does not impact nonreflexive pain in males or females

Next, we used CPP to investigate the effects of pregabalin on nonreflexive pain (**Fig. 2A**). Mice received CWP or were pain-free and underwent 4 consecutive days of pairing after pregabalin administration. A mixed effects repeated measures ANOVA comparing time spent in pregabalin-paired chamber at baseline and after pairing revealed a significant effect of time ($F_{1, 27} = 50.88$, $P < 0.01$) but not time by group ($F_{1, 27} = 3.50$, $P = 0.07$), time by sex ($F_{1, 27} = 2.22$, $P = 0.15$), or time by group by sex ($F_{1,}$

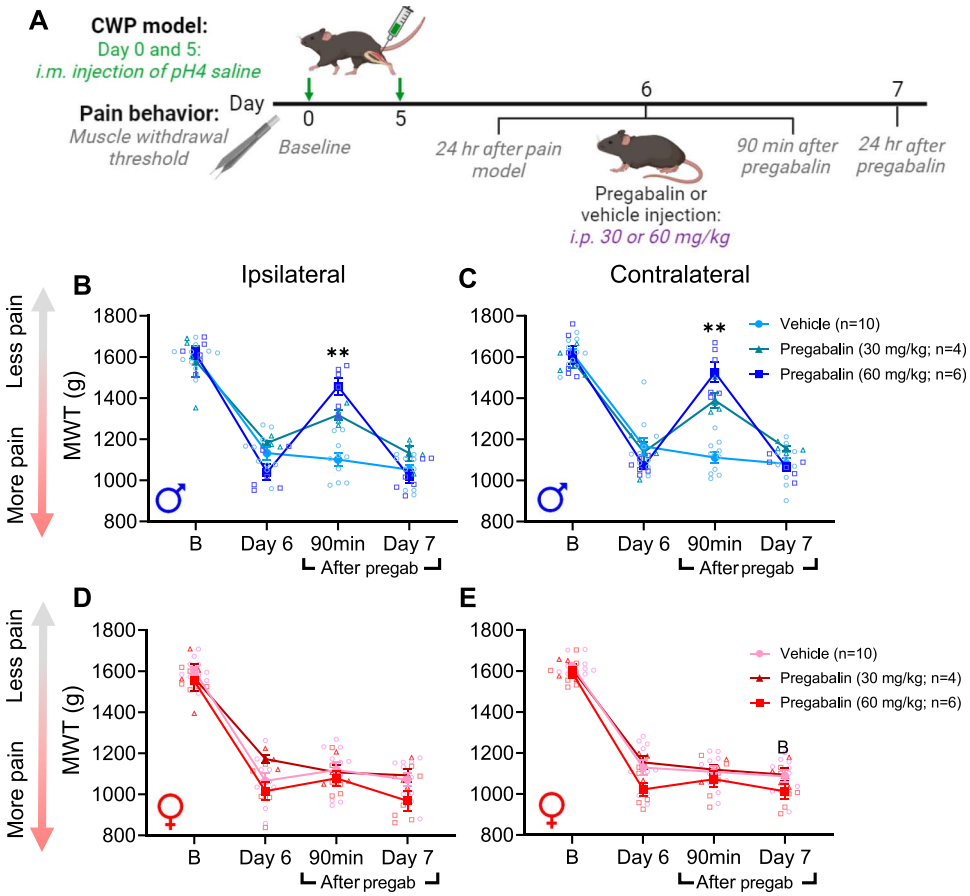

Figure 1. Sex differences in muscle withdrawal thresholds (MWT) at 90 minutes after injection of pregabalin (30 or 60 mg/kg, intraperitoneal [i.p.]). (A) Graphical depiction of experimental protocol of testing analgesic effects of pregabalin on MWT in male and female mice after induction of chronic widespread pain. (B and C) MWT for male mice. Injection of pregabalin (30 and 60 mg/kg) after induction of pain model significantly alleviates hyperalgesia on the ipsilateral (B) and contralateral (C) limb when compared with animals receiving vehicle injections. There was no significant difference in the MWT values between animals receiving 30- and 60-mg/kg doses of pregabalin. (D and E) MWT for female mice. Injection of pregabalin (30 and 60 mg/kg) after induction of pain model did not alter hyperalgesia on the ipsilateral (D) or contralateral (E) limb when compared with animals receiving vehicle administration. **$P < 0.01$ compared with vehicle control; graphs are mean ± SEM; images made on BioRender.

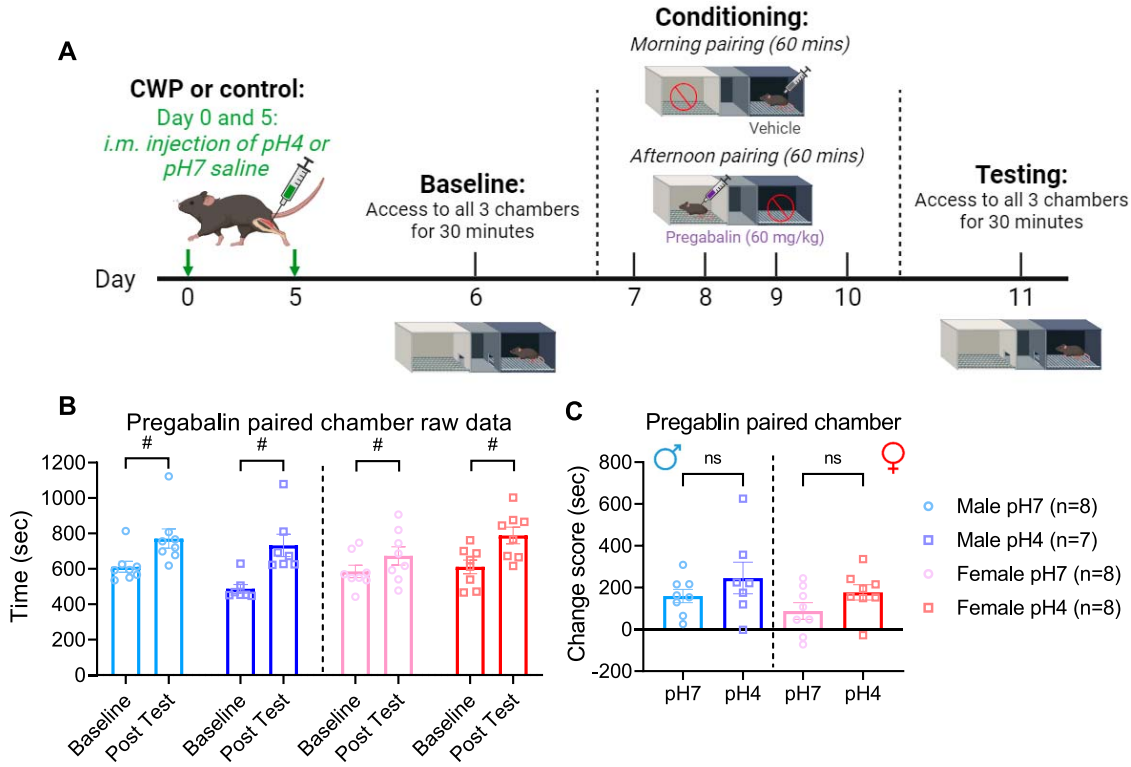

Figure 2. Conditioned place preference testing with pregabalin after induction of chronic widespread pain. (A) Graphical depiction of experimental protocol testing the analgesic effects of pregabalin using conditioned place preference. Mice received injection of vehicle (60 mg/kg volume, i.p.) in the morning and pregabalin (60 mg/kg, i.p.) in the afternoon for 4 days during the pairing phase. (B) Time spent in the pregabalin-paired chamber at baseline and postpairing test. Males and females in the pain condition increased the time spent in the pregabalin-paired chamber during the postpairing test compared with baseline. (C) Difference between baseline and postpairing tests in the amount of time spent in the pregabalin-paired chamber. There are no significant differences in the baseline to postpairing test change scores between group or sex. #$P < 0.01$ for effect of time; graphs are mean ± SEM; images made on BioRender.

$_{27} < 0.01$, $P = 0.92$) indicating an increase in time spent in the pregabalin-paired chamber in all groups regardless of sex (**Fig. 2B**). A 2-way ANOVA comparing the change score between time spent in the pregabalin-paired chamber showed no significant difference for sex, group, or sex by group (**Fig. 2C**), suggesting that pregabalin did not alleviate nonreflexive pain.

3.3. Experiment 3: pregabalin produces motor deficits in males and females

We used rotarod and balance beam behavioral assays to examine motor deficits produced by pregabalin (**Fig. 3A**). For rotarod, analysis of latency to first fall revealed a significant effect of drug ($F_{2,47} = 170.10$, $P < 0.01$) and drug by sex ($F_{2,47} = 3.79$, $P = 0.03$; **Fig. 3B**). Post hoc testing revealed that in males, the latency to first fall was significantly different between animals receiving both doses of pregabalin and those receiving vehicle injection ($P < 0.01$); however, there was no difference between animals receiving 30 and 60 mg/kg of pregabalin ($P = 0.42$). In females, there was a significant difference between all 3 treatment conditions with both doses of pregabalin having a quicker latency to first fall compared with those receiving vehicle ($P < 0.01$) and a significant difference between animals receiving 30 and 60 mg/kg of pregabalin ($P < 0.01$; **Fig. 3B**). Similarly, analysis of the number of falls revealed a significant effect of drug ($F_{2,47} = 45.95$, $P < 0.01$) but not drug by sex ($F_{2,47} = 1.10$, $P = 0.34$; **Fig. 3C**). Post hoc testing revealed that animals receiving both doses of pregabalin had a significantly higher number of falls compared with vehicle treatment ($P <$ 0.01); however, there was no significant difference between the 30 and 60 mg/kg of pregabalin groups ($P = 0.06$).

For the change score of time to cross the balance beam, there was a significant main effect of drug ($F_{1,28} = 7.96$, $P < 0.01$) but not sex ($F_{1,28} = 0.02$, $P = 0.89$) or sex by drug ($F_{1,28} = 1.45$, $P =$ 0.24; **Fig. 3D**). This suggests that pregabalin impaired motor behavior equally in both sexes. Similarly, for the change score on the number of foot slips, there was a significant main effect of drug ($F_{1,28} = 8.72$, $P < 0.01$) and sex by drug interaction ($F_{1,28} =$ 5.84, $P = 0.02$) but not sex ($F_{1,28} = 0.04$, $P = 0.84$; **Fig. 3E**). Post hoc testing showed pregabalin caused a significant increase in number of foot slips in females ($P < 0.01$) but not in males ($P = 0.71$).

3.4. Experiment 4: widespread muscle pain does not affect $\alpha_2\delta$-1 subunit mRNA in either sex

Next, we examined mRNA expression of *CACNA2D1* ($\alpha_2\delta$-*1*) subunit of calcium channels in the DRG and dorsal horn of the spinal cord after induction of CWP (**Fig. 4A**). There was no significant difference in mRNA expression of *CACNA2D1* ($\alpha_2\delta$-*1*) in the ipsilateral or contralateral DRG or in the dorsal horn when explored for group, sex, or group by sex interactions (**Fig. 4B**; statistics in **Table 3**). Because the $\alpha_2\delta$-1 subunit is known to increase trafficking of calcium channels, we also examined specific calcium channels in the DRG and expression of another calcium channel subunit ($\alpha_2\delta$-2) as a control.[4,5,40,48] Again, there were no significant differences in mRNA expression of *CACNA2D2* ($\alpha_2\delta$-*2*), *CACNA1C* ($Ca_V1.2$), *CACNA1D* ($Ca_V1.3$), or

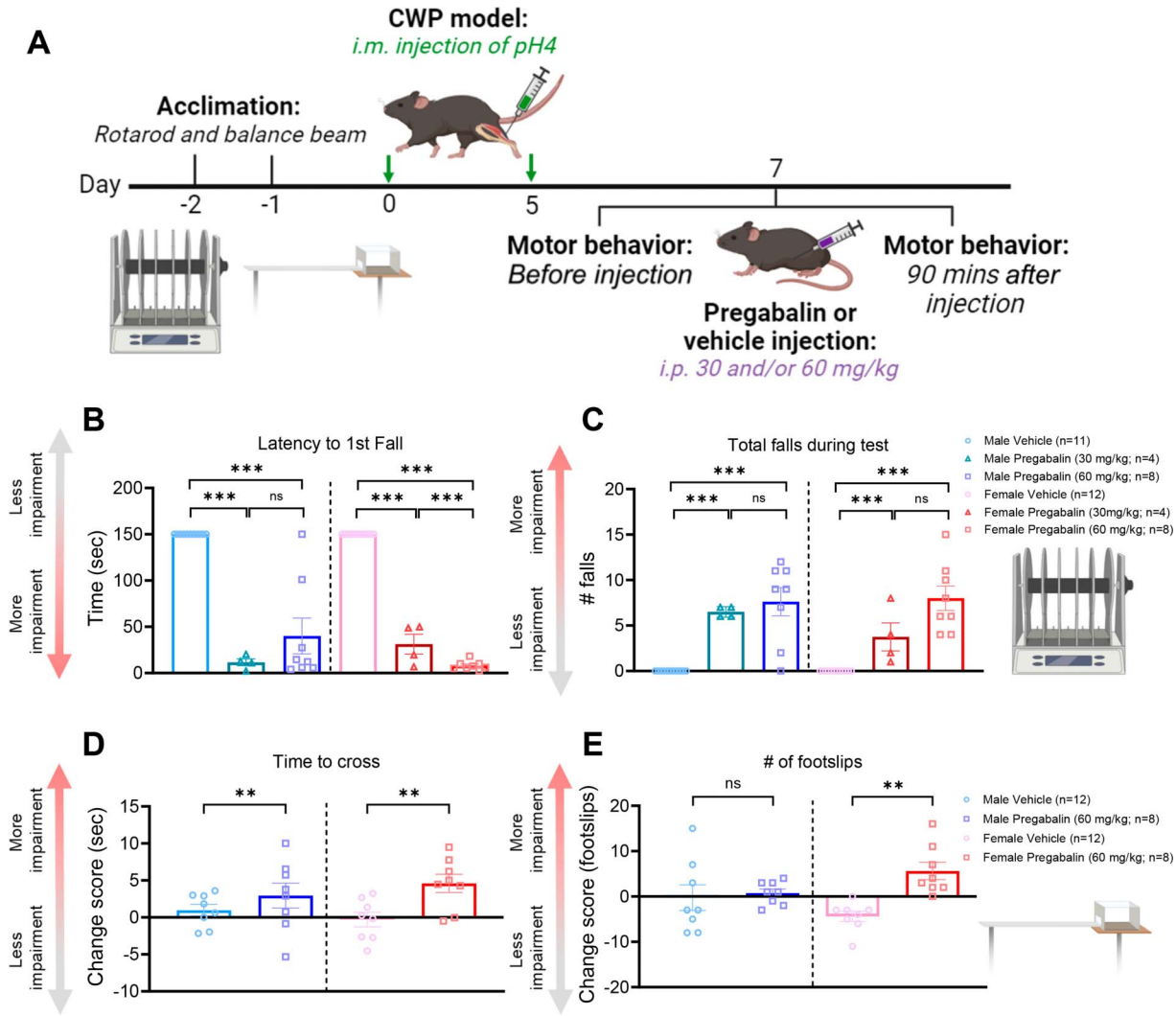

Figure 3. Motor behavior testing using rotarod (30 or 60 mg/kg, i.p.) and balance beam (60 mg/kg) 90 minutes after injection of pregabalin (i.p.). (A) Graphical depiction of experimental protocol testing the motor deficits of pregabalin with rotarod and balance beam. (B) Latency to first fall on rotarod 90 minutes after injection of pregabalin or vehicle. Pregabalin (30 and 60 mg/kg) significantly increased the latency to first fall in both males and females compared with vehicle control. In females, there was a significant increase in latency to first fall between animals receiving 30- and 60-mg/kg pregabalin. (C) Number of falls on rotarod 90 minutes after injection of pregabalin or vehicle. Pregabalin (30 and 60 mg/kg) significantly increased number of falls in both males and females compared with vehicle control. (D) Difference in time to cross the balance beam between before and after injection of pregabalin. Pregabalin significantly increased the amount of time to cross the balance beam in both sexes. (E) Difference in number of foot slips before and after injection of pregabalin. Pregabalin significantly increased the number of foot slips in female but not male mice. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle control; graphs are mean ± SEM; images made on BioRender.

CACNA1B (Ca$_V$2.2) for group, sex, or group by sex interaction (**Fig. 4C**; statistics in **Table 3**). Thus, induction of CWP did not alter $\alpha_2\delta$-1 subunit or calcium channel mRNA in the DRG or spinal cord.

3.5. Experiment 5: widespread muscle pain does not affect protein expression in the DH of the spinal cord in either sex

We further examined protein expression of $\alpha_2\delta$-1 subunit of calcium channels in the ipsilateral DRG and spinal cord after induction of CWP (**Fig. 5A**). In the ipsilateral DRG, a 2-way ANOVA revealed a significant effect of sex ($F_{1,28} = 9.07$, $P < 0.01$), suggesting females have greater expression of the $\alpha_2\delta$-1 subunit. However, there was no significant effect observed for group or group by sex interaction (**Fig. 5B**; statistics in **Table 4**). In the ipsilateral and contralateral dorsal and ventral horn, there was no significant effect of group, sex, or group by sex in protein expression of the $\alpha_2\delta$-1 subunit (**Fig. 5C and D**; statistics in **Table 4**).

4. Discussion

This study demonstrated that pregabalin exhibits sex-specific, analgesic effects on muscle hyperalgesia, observed exclusively in male mice. It is unknown whether pregabalin alleviated non-reflexive pain, as all animals displayed a preference for the pregabalin-paired chamber, suggesting the presence of rewarding-like behavior with pregabalin administration. Pregabalin impaired motor behaviors in both male and female mice, as indicated by increased falls and decreased latency to first fall on the rotarod and increased time to cross and foot slips on the balance beam for females. The current study also found no changes in mRNA or protein expression of the $\alpha_2\delta$-1 subunit of voltage-gated calcium channels in the DRG or dorsal horn of the spinal cord after induction of CWP, suggesting that these factors do not explain the sex differences of pregabalin analgesia.

Our study replicated previous findings in a chronic muscle pain model, showing reduced muscle hyperalgesia in male

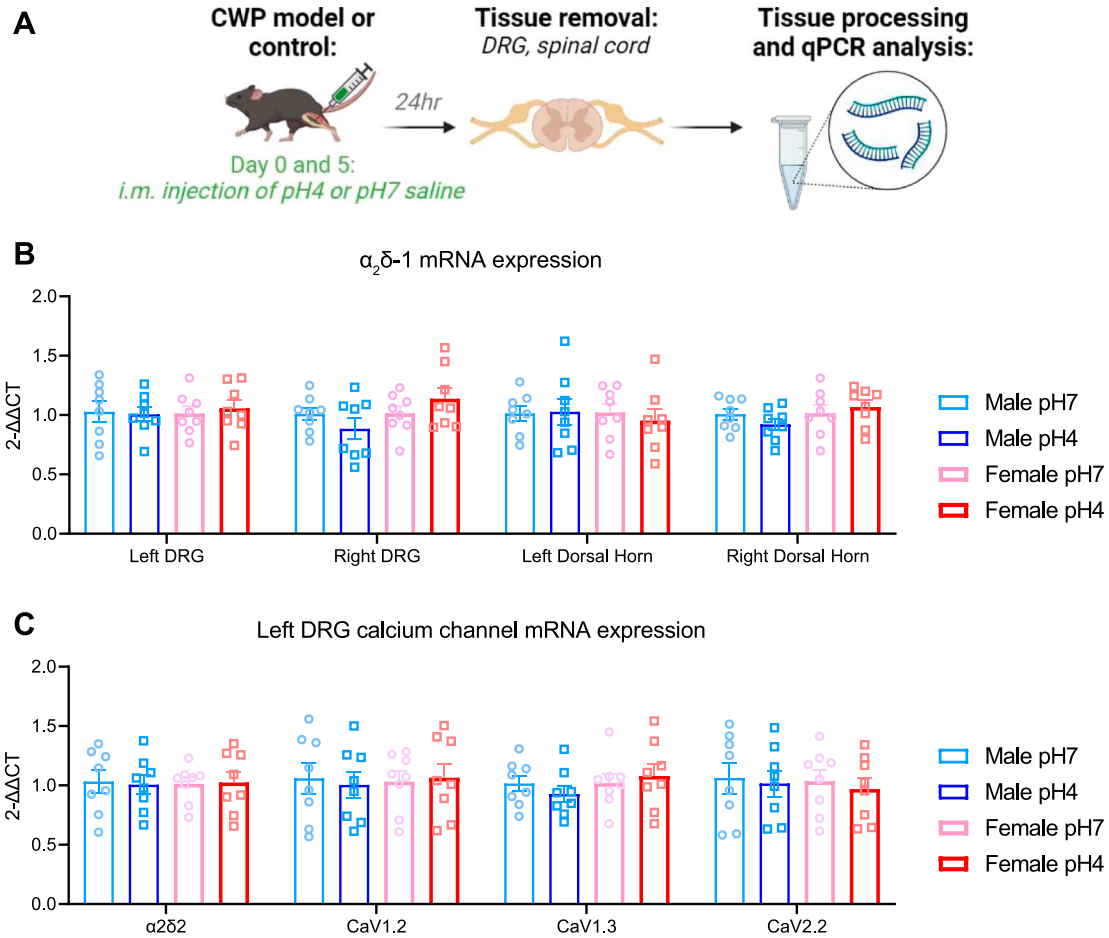

Figure 4. Quantitative PCR analysis of mRNA expression of $\alpha_2\delta$-1 subunit in the ipsilateral and contralateral dorsal root ganglia (DRG) and dorsal horn of the spinal cord and expression of $\alpha_2\delta$-2, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v2.2$ in the ipsilateral DRG. (A) Graphical depiction of the experimental protocol testing the mRNA expression of different calcium channels $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunit in the DRG and dorsal horn of the spinal cord. (B) mRNA expression of $\alpha_2\delta$-1 subunit in the ipsilateral and contralateral DRG and dorsal horn of the spinal cord. There was no significant difference in expression in the different regions between group or sex. (C) mRNA expression of $\alpha_2\delta$-2, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v2.2$ in the ipsilateral DRG. There was no significant difference in expression of $\alpha_2\delta$-2, $Ca_v1.2$, $Ca_v1.3$, and $Ca_v2.2$ in DRG ipsilateral to the muscle insult. This is consistent between males and females. Graphs are mean ± SEM, n = 8 for all groups; images made on BioRender.

animals after acute administration of pregabalin or mirogabalin.[35,42,50] By contrast, no sex differences in pregabalin analgesia were found when it was injected directly into the central nucleus of the amygdala after chronic muscle pain; however, this study did not disaggregate or analyze the data by sex.[31] Consistent with our results, a previous study observed sex differences in sciatic cuff-induced neuropathic pain with male, but not female, rats exhibiting analgesia after pregabalin treatment.[47] By contrast, no sex differences in pregabalin analgesia were found after induction of neuropathic pain.[22] Although pain assessment for pregabalin has primarily relied on stimulus-evoked responses, our study was unable to demonstrate a clear analgesic effect of pregabalin on nonreflexive pain. This ambiguity arises from the observation that animals, regardless of muscle pain, displayed a preference for the pregabalin-paired chamber, making it challenging to determine whether this preference is due to analgesic effects or reward-like behavior. It has previously been shown that the CWP model produces spontaneous pain behavior as shown by increases in facial grimace and CPP after pairing with clonidine.[24,25] In

Table 3

Statistical results of quantitative PCR experiments.

Gene name	Location	Effect for group	Effect for sex	Effect for group × sex
CACNA2D1 ($\alpha_2\delta$-1)	Left DRG	$F_{1,28} = 0.02$, $P = 0.88$	$F_{1,28} = 0.06$, $P = 0.81$	$F_{1,28} = 0.21$, $P = 0.64$
	Right DRG	$F_{1,28} = 0.00$, $P = 0.99$	$F_{1,28} = 2.92$, $P = 0.10$	$F_{1,28} = 2.76$, $P = 0.11$
	Left dorsal horn	$F_{1,28} = 0.09$, $P = 0.76$	$F_{1,28} = 0.15$, $P = 0.70$	$F_{1,28} = 0.22$, $P = 0.64$
	Right dorsal horn	$F_{1,28} = 0.11$, $P = 0.74$	$F_{1,28} = 1.98$, $P = 0.17$	$F_{1,28} = 1.42$, $P = 0.23$
CACNA1B ($Ca_v2.2$)	Left DRG	$F_{1,28} = 0.03$, $P = 0.86$	$F_{1,28} = 0.07$, $P = 0.79$	$F_{1,28} = 0.16$, $P = 0.69$
CACNA1D ($Ca_v1.3$)	Left DRG	$F_{1,28} = 0.03$, $P = 0.85$	$F_{1,28} = 0.08$, $P = 0.78$	$F_{1,28} = 0.15$, $P = 0.70$
CACNA2D2 ($\alpha_2\delta$-2)	Left DRG	$F_{1,28} = 0.04$, $P = 0.85$	$F_{1,28} = 0.08$, $P = 0.78$	$F_{1,28} = 0.14$, $P = 0.72$
CACNA1C ($Ca_v1.2$)	Left DRG	$F_{1,28} = 0.04$, $P = 0.85$	$F_{1,28} = 0.08$, $P = 0.78$	$F_{1,28} = 0.14$, $P = 0.70$

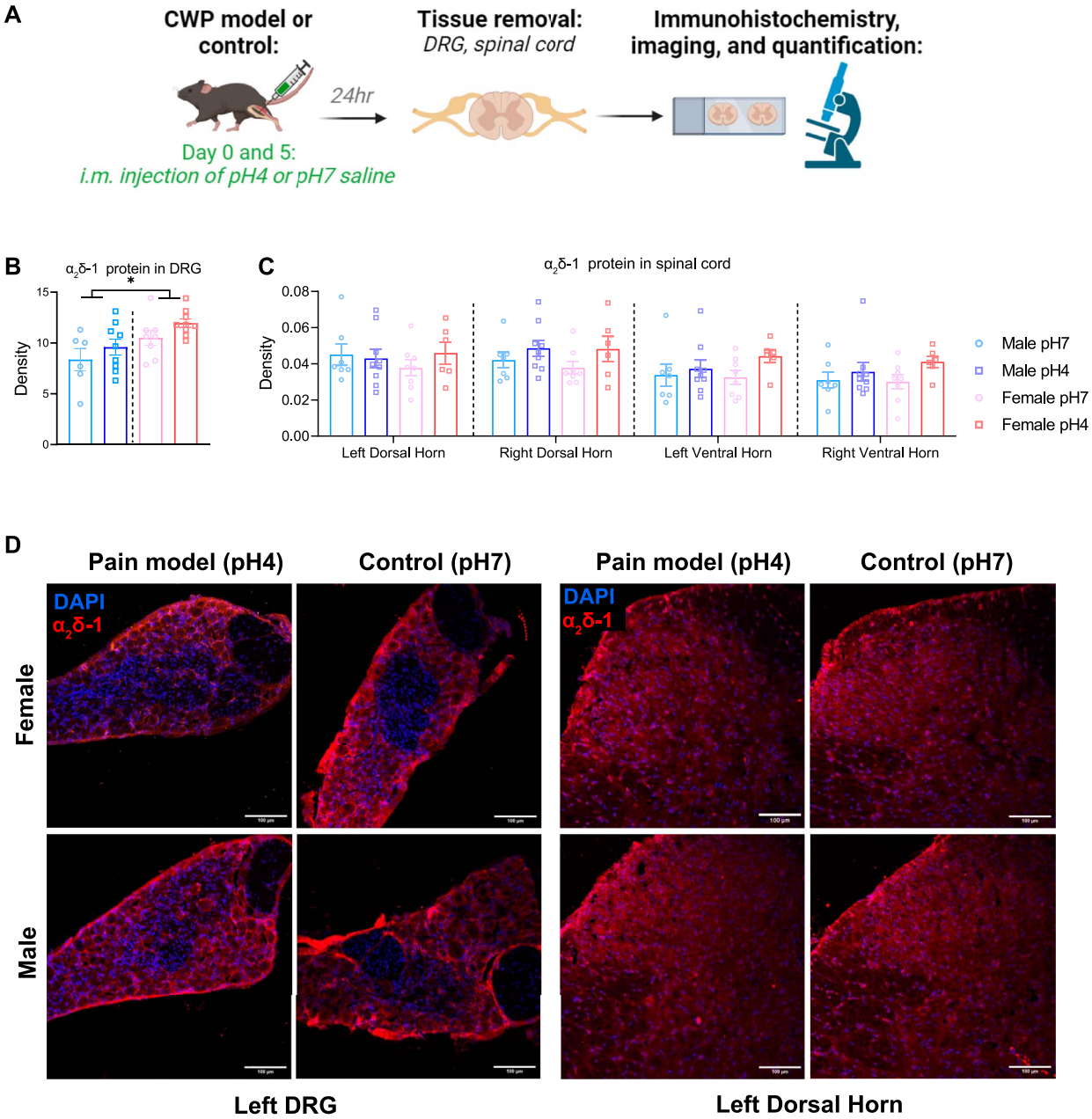

Figure 5. Immunohistochemistry analysis of protein expression of $\alpha_2\delta$-1 subunit in the ipsilateral dorsal root ganglia (DRG) and dorsal and ventral horn of the spinal cord. (A) Graphical depiction of the experimental protocol examining the protein expression of $\alpha_2\delta$-1 subunit in the ipsilateral DRG and dorsal and ventral horn of the spinal cord. (B) $\alpha_2\delta$-1 subunit expression in the ipsilateral DRG. There was a significant main effect of sex but no significant difference in pain condition or sex by pain condition interaction. (C) $\alpha_2\delta$-1 subunit expression in the spinal cord. There was no difference in $\alpha_2\delta$-1 subunit protein expression in ipsilateral or contralateral dorsal or ventral horn between pain condition or sex. (D) Representative images of the left DRG and dorsal horn of the spinal cord in both sexes and groups. *$P <$ 0.05 for a significant effect of sex; graphs are mean ± SEM; images made on BioRender.

agreement with our findings, in the absence of pain, pregabalin produces rewarding behavior in both sexes at a similar dose (60 mg/kg, i.p.).[2]

A systematic review highlighted a significant underrepresentation of female rodents in preclinical research of pregabalin with only 16.2% of experiments testing both sexes of 532 experiments

Table 4

Statistical results of immunohistochemistry experiments.

Gene name	Location	Effect for group	Effect for sex	Effect for group × sex
CACNA2D1 ($\alpha_2\delta$-1)	Left dorsal horn	$F_{1,26} = 0.32$, $P = 0.58$	$F_{1,26} = 0.16$, $P = 0.69$	$F_{1,26} = 0.89$, $P = 0.35$
	Right dorsal horn	$F_{1,26} = 3.21$, $P = 0.09$	$F_{1,26} = 0.25$, $P = 0.62$	$F_{1,26} = 0.17$, $P = 0.69$
	Left ventral horn	$F_{1,26} = 2.50$, $P = 0.13$	$F_{1,26} = 0.34$, $P = 0.57$	$F_{1,26} = 0.74$, $P = 0.40$
	Right ventral horn	$F_{1,26} = 3.02$, $P = 0.09$	$F_{1,26} = 0.27$, $P = 0.61$	$F_{1,26} = 0.51$, $P = 0.48$
	Left DRG	$F_{1,28} = 3.21$, $P = 0.08$	$F_{1,28} = 9.07$, $P < 0.01$	$F_{1,28} = 0.03$, $P = 0.87$

across 204 articles.[15] Although the meta-analysis found that pregabalin was analgesic regardless of pain model, assessment method, or sex, it was constrained by the limited inclusion of mixed-sex (12) and female-only (5) experiments because of inadequate reporting elements.[15] Furthermore, the review was unable to compare the diverse characteristics of these studies by sex such as species, age, administration schedule, pain etiology, or pain measure, and none of these mixed-sex studies explored chronic muscle pain.[15] In human research, systematic reviews indicate the efficacy of pregabalin in treating fibromyalgia with 30% to 50% of individuals in these trials experiencing >30% reduction in pain and 20% to 30% achieving a 50% reduction in pain.[10] However, the predominance of females with fibromyalgia hinders comparisons between the sexes with >90% of participants in clinical trials being women, limiting the ability to evaluate potential sex differences in treating chronic muscle pain in the clinical setting. Our study reveals a potential unique sex-specific pain alleviation response to pregabalin treatment for muscle pain, which requires further research.

Both male and female mice displayed equal motor deficits on the rotarod, whereas only females displayed a motor deficit on the balance beam. Interestingly, we observed qualitative differences in the performance on the balance beam between males and females that suggest sexual dimorphism in coping strategies for the motor impairment. Specifically, male mice demonstrated a unique compensatory strategy for managing motor impairments, resting their posterior body on the balance beam and dragging themselves across, thus avoiding the need for precise foot placement and hind limb weight-bearing. This strategy likely reflects the sex differences in balance beam motor behavior because of the greater adaptive demand for males because of their larger size. Regardless, nonsignificant differences before and after pregabalin administration in both time to cross and number of foot slips in males were a function of increased stability obtained because of their compensatory strategy. Previous preclinical studies have lacked reporting of motor deficits after pregabalin administration, which limits the interpretation of its analgesic efficacy as these motor deficits could create a false positive during pain testing that requires a motor response.[15] Future studies should include the use of motor behavior tests to further tease out the analgesic vs sedative effects of pregabalin. Overall, the similar motor deficits induced by pregabalin between the sexes suggest that the sex differences observed in muscle hyperalgesia are more likely due to mechanistic differences in pain mechanism rather than differences in the pharmacokinetics of pregabalin between the sexes. We have repeatedly shown that muscle pain has mechanistic sex differences further supporting this hypothesis.[18,26,27]

Pregabalin, by antagonizing voltage-gated calcium channels through its binding to $\alpha_2\delta$ auxiliary subunits ($\alpha_2\delta$-1 and $\alpha_2\delta$-2), reduces Ca^{2+}-mediated neurotransmitter release at presynaptic terminals in the dorsal horn of the spinal cord.[16,19] Knockdown of the $\alpha_2\delta$-1 subunit reduces sensitivity to mechanical stimuli and delays onset of neuropathic injury, whereas its overexpression in healthy mice induces neuropathic pain phenotypes.[4,29,38] Our study found no differences in mRNA or protein of the *CACNA2D* ($\alpha_2\delta$-*1*) subunit of voltage-gated calcium channels after induction of CWP in the DRG. This contrasts with studies of neuropathic injury, which exhibit increased mRNA expression in the DRG and protein expression in both the DRG and dorsal horn of the spinal cord.[4,10,28,36,49,51] The $\alpha_2\delta$-1 subunit associates with the pore-forming $Ca_V\alpha_1$ subunit and enhances the localization of Ca_V1 and Ca_V2 channels to the plasma membrane. In our study, mRNA of *CACNA1C* (*$Ca_V1.2$*), *CACNA1D* (*$Ca_V1.3$*), and *CACNA1B* (*$Ca_V2.2$*) were not increased after induction of CWP. We have previously shown heightened glutamate release in the spinal cord after CWP and an NMDA glutamate receptor antagonist reduces muscle hyperalgesia.[43,44] Since pregabalin has been shown to decrease glutamate release, future studies should investigate whether pregabalin administration affects glutamate release in the spinal cord after induction of CWP.[23] Such research may provide insight into the potential mechanism underlying the behavioral sex differences shown in this model.

Overall, these findings contribute to our understanding of pregabalin's analgesic effects on muscle pain and highlight the importance of considering sex differences in pain research and clinical practice. Over the past decade, preclinical pain studies have increased the use of both sexes; however, both clinical and preclinical research lack reporting of data disaggregated by sex.[39] This omission limits meta-analyses to explore sex differences in treatment outcomes in preclinical research. A limitation of this work is the lack of mechanistic insight surrounding pregabalin's sex-specific effects; thus, further research using unbiased approaches is necessary to explore the underlying mechanisms behind the observed sex-specific effects. This knowledge could lead to more tailored and effective pain management strategies that address sex-based differences.

Disclosures

The authors have no conflicts of interest to declare.

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