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Sex-related exacerbation of injury-induced mechanical hypersensitivity in GAD67 haplodeficient mice

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Abstract

Decreased GABA levels in injury-induced loss of spinal inhibition are still under intense interest and debate. Here, we show that GAD67 haplodeficient mice exhibited a prolonged injury-induced mechanical hypersensitivity in postoperative, inflammatory, and neuropathic pain models. In line with this, we found that loss of 1 copy of the GAD67-encoding gene *Gad1* causes a significant decrease in GABA contents in spinal GABAergic neuronal profiles. Consequently, GAD67 haplodeficient males and females were unresponsive to the analgesic effect of diazepam. Remarkably, all these phenotypes were more pronounced in GAD67 haplodeficient females. These mice had significantly much lower amount of spinal GABA content, exhibited an exacerbated pain phenotype during the second phase of the formalin test, developed a longer lasting mechanical hypersensitivity in the chronic constriction injury of the sciatic nerve model, and were unresponsive to the pain relief effect of the GABA-transaminase inhibitor phenylethylidenehydrazine. Our study provides strong evidence for a role of GABA levels in the modulation of injury-induced mechanical pain and suggests a potential role of the GABAergic system in the prevalence of some painful diseases among females.

Keywords: Spinal GABA levels, Mechanical hypersensitivity, Inflammatory, Postoperative and neuropathic pain, Sex-related differences

1. Introduction

Chronic inflammatory, neuropathic, or postoperative pain gives rise to highly debilitating and long-lasting sensory abnormalities such as hyperalgesia, allodynia, and spontaneous pain.[44] Increasing evidence suggests that decreased spinal inhibition (disinhibition) is believed to underlie several forms of chronic pain.[7,44,51,53] Early studies reported that sciatic nerve transection induces a significant decrease in the number of GABA-immunoreactive cells in laminae I–III of rat spinal cord,[9] with similar changes in the number of cells that express gamma-aminobutyric acid (GABA) or its synthesizing enzyme glutamic acid decarboxylase (GAD) seen after different types of peripheral nerve injury.[9,14,17,33] Other studies went further and proposed that peripheral nerve injury-induced decrease in GABA and GAD expression was due to selective cell death of inhibitory interneurons,[33,45] but this view has been challenged,[6,39–42] leaving open the question regarding GABA levels in the development of injury-induced tactile allodynia or thermal hyperalgesia.

Gamma-aminobutyric acid is the major inhibitory neurotransmitter in the adult brain. It is converted from glutamate by GAD. Glutamic acid decarboxylase occurs in 2 major paralogues gad2 and gad1, respectively encoding, GAD65 and GAD67. In cortical neurons, GAD67 is widely distributed throughout the cell, while GAD65 expressed primarily in axon terminals, and this has led to the suggestion that GAD67 preferentially synthesizes cytoplasmic GABA, whereas GAD65 synthesizes GABA for vesicular release.[28,46] Ablation of the GAD65-encoding gene *Gad2* has no effect on brain GABA contents or animal behavior, except for a slight increase in susceptibility to seizures.[1] However, ablation of the GAD67-encoding gene *Gad1* results in a >90% reduction in basal GABA levels and neonatal lethality.[2,19] *Gad1* heterozygous mice are viable and show largely normal brain morphology.[47] These mice exhibit an overall 40% reduction of GAD67 expression accompanied with a slight reduction (16%) of GABA contents in the young adult brain. Reduced GAD67 expression has been causally linked to signs of neuropsychiatric disorders such as reduced social interaction and social dominance, and increased immobility in the forced swim test.[36,43] It has been reported that spinal levels of GAD65 ipsilateral to nerve injury are reduced 2 weeks after chronic constriction injury (CCI) or spared nerve injury (SNI), whereas GAD67 levels remain unchanged.[33] Although the number of GAD67-expressing cells is apparently

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reduced 4 weeks after SNI,[45] the impact of changes in GAD67 expression (and subsequently spinal GABA levels) in pathological pain has not been thoroughly investigated. To address this, we have used a GAD67 haplodeficient mouse line to determine how reduced GAD67 expression influences postoperative-induced, inflammatory-induced, and CCI-induced mechanical hypersensitivity. In each case, we found that GAD67 haplodeficient mice showed enhanced pain responses compared with wild-type littermates and that these changes in sensitivity were more pronounced in female mice. These phenotypes were due to a significant decrease in the level of spinal GABA content as demonstrated by the inability of GAD67 haplodeficient mice to respond to the analgesic effect of diazepam. These mice had significantly much less spinal GABA levels, exhibited a selective exacerbation of the pain phenotype during the second phase of the formalin test, developed a longer lasting mechanical hypersensitivity in the CCI model, and were unresponsive to the pain relief effect of phenylethylidenehydrazine (PEH).

2. Materials and methods

2.1. Mice

Gad1$^{GFP/+47}$ were generated by crossing Gad1$^{GFP/+}$ males with C57/Bl6J females. Wild type (WT) littermates were used as control. Mice of both sexes (8-12-week-old) were used for all experiments. Mice were maintained under standard housing conditions (21°C, 40% humidity, 12-hour light cycles, and free access to food and water). Particular efforts were made to minimize the number of mice used in this study (the same mice were used for open-field, rotarod, and hot-plate tests) as well as the stress and suffering to which they were subjected.

2.2. Ethical statement

All experiments were conducted in line with European guidelines for the care and use of laboratory animals (Council Directive 86/609/EEC). All experimental procedures were approved by an independent ethics committee for animal experimentation (APAFIS), as required by the French law and in accordance with the relevant institutional regulations of French legislation on animal experimentation, under license numbers #30046-2021062313194207 v1 and #34464-2021122214544963 v3.

2.3. Formalin test

Formalin solution was prepared at 2% in PBS (phosphate-buffered saline) from a 37% formaldehyde solution (Fisher Scientific, Ontario, Canada). Mice were habituated to the room for 30 minutes, then placed individually into Plexiglas observation chambers for another 30 minutes. After subcutaneous injection of formalin (10 μL for a mouse weighing 25 g) in the left hind paw, the animals were immediately placed individually in observation chambers. Pain behavior (shaking, licking, biting, flinching, and lifting of the injected paw) was monitored for 60 minutes. The pain behavior cumulative time of the injected paw was counted for 5-minute intervals. The injected volume varied from 7.6 to 12 μL because mice weighed between 19 and 30 g.

2.4. Paw incision—Brennan test

Paw incision surgery was performed as described in 8. Mice were anesthetized with ketamine/xylazine (40 mg/kg and 5 mg/kg i.p., respectively), and a longitudinal incision was made through the skin and fascia of the right hind paw. Forceps were used to elevate the flexor digitorum brevis muscle longitudinally and an incision was made through the muscle with a scalpel to cut it into 2 halves. The wound was closed with sutures, and the animals were allowed to recover and returned to their cages.

2.5. Chronic constriction injury

Chronic constriction injury was performed by modifying the one described in 4. In brief, 2 ligatures (6-0 Monocryl, Ethicon, Raritan, NJ) tied loosely (with about 1 mm spacing) around the common sciatic nerve of the right paw were performed on anaesthetized mice (ketamine/xylazine). Nerves were constricted to a barely discernible degree, so that circulation through the epineural vasculature was not interrupted. The wound was closed with sutures. Animals are kept on a warm chamber and monitored until waking up before returning to their home cage. Weight, general aspect, feeding, and water drinking are verified regularly.

2.6. Zymosan injection

Twenty microliters of a solution containing 3 mg/mL zymosan-A from *Saccharomyces cerevisiae* (Sigma, St. Louis, MO, prepared in 0.9% NaCl solution) was injected subcutaneously into the plantar surface of the left hind paw of the mouse with a Hamilton syringe.

2.6.1. Behavioral tests

Animals were acclimated for 1 hour to their testing environment before each experiment, and all experiments were performed at room temperature (~22°C). Experimenters were masked to the genotype of the mice during testing. The number of tested animals was indicated in figures. Thermal plates, open-field, and von Frey apparatus were from BioSeb Instruments, Chaville, France.

2.7. Open-field test

The open-field apparatus consists of an empty square arena (40 × 40 × 35 cm), surrounded by walls to prevent animal from escaping. Light inside the arena was uniform and kept at approximately 100 lux throughout the tests. Control and GAD67 haplodeficient mice were individually placed in the center of the arena and their behavior was recorded using the EthoVision XT16 video-tracking system (Noldus, Inc, Leesburg, VA) over a 10-minute period. The time spent grooming and rearing, the total distance traveled, and the total amount of time spent in the peripheral borders and in the center were recorded.

2.8. Hot-plate test

To assess heat sensitivity, control and GAD67 haplodeficient mice were placed individually on a metal surface maintained at 48°, 50°, 52°, or 55°C and the latency to nociceptive responses was measured (licking, shaking of hind paws, or jumping). To prevent tissue damage, mice were removed from the plate immediately after a nociceptive response or a cutoff of 90 s, 60 s, 45 s, and 20 seconds was applied, respectively. Each mouse has been tested 3 times with a 5-minute interval between each test (and only 2 times for 55°C). The withdrawal time corresponds to the mean of the 3 measures.

2.9. Rotarod test

A rotarod apparatus (LSI Letica Scientific Instruments, Barcelona, Spain) was used to explore coordinated locomotor and balance function in mice. Mice were placed on a rod that slowly accelerated from 4 rpm to 44 rpm over 5 minutes and the latency to fall off during this period was recorded. The test was conducted over 3 consecutive days. Each day, the animals were tested 3 times separated by at least a 5-minute resting period.

2.10. von Frey test of mechanical threshold: the up and down method

Mice were placed in plastic chambers on a wire mesh grid and stimulated with von Frey filaments (Bioseb) using the up and down method[10] starting with 1 g and using 2 g and 0.07 g filaments as the cutoff values.

For the zymosan, von Frey tests were performed before the injection (baseline) and 1, 3, 5, 7, 10, 14, and 30 days after the injection. For the paw incision model, von Frey tests were performed before the surgery (baseline) and 1, 3, 5, 7, 10, 15, 25, and 40 days after the surgery. For the CCI model, von Frey tests were performed before the surgery (baseline) and 3, 7, 11, 23, 28, 33, 43, and 60 days after the surgery.

2.11. von Frey test of mechanical threshold: the 10 times method

As for the up and down method, mice were placed in plastic chambers on a wire mesh grid and stimulated with von Frey filaments (Bioseb). For the Zymosan model, mechanical allodynia was assessed using the 10 times method as described (Delfini, 2013) with von Frey hair filaments of 3 bending forces (0.07 g, 0.4 g, and 1 g). For each filament, 5 stimuli were applied 2 times with an interval of 15 seconds alternating between right and left paw. The von Frey test was performed before the injection (baseline) and 1 day after injection.

2.12. Drugs and in vivo administration methods

Diazepam (DZP, Roche, Laval, QC, Canada) was dissolved in 0.9% NaCl solution containing 10% dimethyl sulfoxide (DMSO) to obtain a 5 mg/mL stock solution. 10 μL of 0.09 mg/kg diazepam was administered intrathecally into nonanesthetized mice. Successful placement of the needle was confirmed by a slight flick of the tail.

Muscimol (Tocris Bioscience) was dissolved in PB 0.1 M and 0.16 μg was i.t. administered in 10 μL.

2.13. Tissue preparation for ultrastructural morphology

WT (n = 3 females; n = 3 males) and Gad1$^{GFP/+}$ (n = 3 females; n = 3 males) were deeply anesthetized with sodium pentobarbital (60 mg/100 g) and perfused with Ringer solution, followed by 1% paraformaldehyde + 2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Lumbar spinal cord segments were dissected out, postfixed for 2 hours in the same aldehyde mixture, and washed several times in PB. Coronal sections were cut on a vibratome (Leica VT1000S, Wetzlar, Germany) at a thickness of 100 μm.

2.14. Immunoelectron microscopy embedding

Spinal cord sections were postfixed in osmium ferrocyanide for 1 hour at 4°C; dehydrated in graded acetone; and incubated in acetone/Spurr resin (1:1; 30 minutes), acetone/Spurr resin (1:2; 30 minutes), and Spurr resin overnight at room temperature. Finally, sections were embedded in Spurr resin in 0.5-mL Eppendorf tubes (24 hours, at 70°C). Ultrathin sections were cut with an ultramicrotome (EM UC6, Leica Microsystems, Wetzlar, Germany) and collected on uncoated nickel grids (200 mesh).

2.15. Immunoelectron microscopy postembedding immunostaining

Ultrathin sections were immunostained following a conventional postembedding protocol.[31] Sections were treated for 2 minutes with a saturated aqueous solution of sodium metaperiodate (Sigma), rinsed in 1% Triton X-100 in Tris-buffered saline (TBS) 0.5 M, and incubated for 1 hour in 10% normal serum. Grids were then incubated overnight on drops of a rabbit anti-GABA primary antibody (1:250; Sigma, Cat# A2052). After rinsing in TBS, they were incubated for 1 hour at 37°C in anti-rabbit 20-nm gold-conjugated secondary antibody (1:15; BBI Solutions, Cardiff, United Kingdom). They were then rinsed in TBS, transferred into drops of 2.5% glutaraldehyde in cacodylate buffer 0.05 M, pH 7.4 for 10 minutes and washed in distilled water. Sections were further counterstained with Uranyl Less EM Stain for 30″ and with lead citrate for 30'' (Electron Microscopy Sciences, Hatfield, PA).

Sections were observed with a JEM-1400 Flash transmission electron microscope (JEOL, Tokyo, Japan) and images were acquired with a high-sensitivity scientific CMOS camera.

2.16. Immunoelectron microscopy quantification and statistical analysis

To assess the distribution and expression levels of GABA through lamina II of the dorsal horn in WT and Gad1$^{GFP/+}$ mice, counts of GABA-immunoreactive (IR) profiles were directly performed on randomly selected ultrathin sections within 90 × 90 μm squares of 200-mesh EM grids (mean μm^2 area/animal = 35,951 μm^2) by an operator unaware of the experimental group.

The number of gold particles per area (GPs/mm^2) over different neuronal profiles (axon terminals [At], dendrites [d], vesicle-containing dendrites [V1] engaged in glomerulus of type Ia [GIa] configuration [V1 GIa], vesicle-containing dendrites [V1] engaged in glomerulus of type II [GII] configuration [V1 GII], and axonal peripheral profiles [V2] engaged in GII configuration [V2 GII]) was calculated. Differences in GABA immunolabeling were evaluated with the Mann–Whitney test and considered significant for $P < 0.05$. The density of GPs over resin was determined for each animal by calculating the mean GP density value over 10 randomly selected sections and chosen as an index of background staining (bk index).

To quantify the specific GABA labeling, the bk index was then subtracted from values obtained for the neuronal profiles.

2.17. Quantification and statistical analysis

Results were expressed as mean ± SEM. Quantitative and statistical analyses were performed by using the GraphPad Prism 7 (GraphPad Software, La Jolla, CA) and were indicated in each figure. Statistical significance was set to $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

2.18. Synthesis and administration of phenylethylidenehydrazine

In a 50-mL round-bottom flask equipped with a magnetic stirring bar, the hydrazine hydrate (384 μL, 6 mmol, 1.2 equiv.) was dissolved in THF (5 mL). A solution of 2-phenylacetaldehyde in

THF was added dropwise to the hydrazine. The reaction mixture was stirred for 1 hour at room temperature, then 500 mg of Na_2SO_4 and 500 mg of molecular sieves (4A) were added sequentially. It was stirred for an additional hour at room temperature. The mixture was filtered and evaporated under reduced pressure at room temperature. Eight hundred nine milligrams of yellow pale solid was obtained (PEH). As confirmed by NMR(1H), no purification was needed (supplementary Fig. 3, available at http://links.lww.com/PAIN/B895). Phenylethylidene-hydrazine was dissolved in DMSO at 447 mM and keep in dark at room temperature. Just before the injection, PEH was diluted 20 times in PBS to have a final concentration of 22.35 mM. Mice were injected intraperitoneally daily (100 μL for 10 g) for 14 days either with PEH or with vehicle (5% DMSO in PBS). Von Frey tests were always performed before the daily injection of PEH.

2.19. High-performance liquid chromatography

2.19.1. Tissue preparation

Mice spinal cords were dissected, snap-frozen in liquid nitrogen, and then preserved at −70°C. Frozen tissues were lysed with a Potter homogenizer in cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, and 1% Triton X-100) in the ratio of 50 μL of buffer for 10 mg of tissue. After centrifugation (13,000*g* for 5 minutes at 4°C), supernatants were separated and mixed with the same volume of perchloric acid (0.5 N) for protein precipitation (5 minutes at room temperature). The mixture was centrifuged at 13,000 rcf for 10 minutes at 4°C and the supernatants were stored at −70° before high-performance liquid chromatography (HPLC) analysis.

2.19.2. Separation and quantitative detection of amino acids

Standard stock solutions of amino acids were prepared on a daily basis by dissolving the corresponding amount of each amino acid in distilled water to obtain a 10^{-6} M solution. The OPA derivatization reagent was prepared by dissolving 20 mg of phthaldialdehyde (Sigma-Aldrich, Ontario, Canada) in 0.75 mL of borate buffer 0.1 M pH 10, 25 μL of mercaptoethanol, 0.75 mL of absolute ethanol, and 2.25 mL of distilled water.

Twenty microliters of standard or sample solution was mixed with 20 μL of fluorescent mix for 1 minute and then immediately injected into the HPLC system and analyzed for 35 minutes.

Chromatographic separations of amino acids were performed on a Waters HPLC system consisting of W717 autosampler, W600 pump, and W474 fluorescence detector and controlled by a computerized system (Empower, Waters, Milford, MA). The analytical column was a reversed-phase Spherisorb ODS2 column (C18 column, 150 × 4.6 mm, 5.0 μm, Waters) maintained at 35°C.

The sequential elution of amino acids was performed with a gradient between 2 mobile phases: from phase A (0.1 M acetate buffer and methanol, pH 5.8) to phase B (half buffer A and half methanol, hydrochloric acid). Amino acid-OPA derivatives were detected by fluorescence at λex/λem = 345/440 nm. Analysis was performed thanks to Empower software (Waters).

3. Results

3.1. GAD67 haplodeficiency exacerbates injury-induced mechanical hypersensitivity

Dysfunction of GAD67 has been associated with several neurological disorders, including schizophrenia,[22] bipolar disorders,[3] Parkinson disease,[25] Alzheimer disease,[50] and social behavior.[43] However, the consequences of GAD67 haplodeficiency in somatosensation have never been investigated. We thus sought to investigate whether loss of 1 copy of *gad-1* gene impairs pain sensation in GAD67 haplodeficient male and female mice. Analysis of the general behavior of littermate wild type (hereafter named WT) and GAD67 haplodeficient mice using open-field, rotarod, and hot-plate tests showed that all mice behaved the same way in all these tests regardless of their sexes or genotypes (Fig. S1, available at http://links.lww.com/PAIN/B895). Next, we subjected the mice to a wide range of pain models. We started with the Brennan test as a model of postoperative pain. As shown in **Figure 1A**, the baseline mechanical thresholds of males and females of both genotypes were similar. At day 1 after paw incision, all mice exhibited a significant drop in their mechanical threshold. In WT mice, the mechanical hypersensitivity lasted up to 5 days and had completely resolved by day 15. However, at day 25, GAD67 haplodeficient males and females were still hypersensitive and returned to baseline levels by day 40. Next, we used the intraplantar injection of zymosan A as a model of inflammatory pain.[29] In this pain model, all mice developed a significant mechanical hypersensitivity at day 1 (**Fig. 1B**). Same as in the Brennan test, male and female WT mice recovered their normal mechanical sensitivity as early as day 7, whereas GAD67 haplodeficient males and females were still hypersensitive until day 15 and returned to baseline levels at day 30. Finally, given the critical role of the GABAergic system in modulation of neuropathic pain, we sought to investigate the incidence of GAD67 haplodeficiency in this pathological pain model. We chose the chronic constriction of the sciatic nerve (CCI) being a reversible model of pain. **Figure 1C** shows that CCI-induced mechanical hypersensitivity occurs with the same kinetics in all mice. However, when WT mice of both sexes started recovering normal mechanical sensitivity, both male and female GAD67 haplodeficient mice were still hypersensitive up to day 45 post-CCI. Remarkably, by day 60 post-CCI, GAD67 haplodeficient male's mechanical sensitivity returned to baseline levels, whereas GAD67 haplodeficient females were still hypersensitive.

To test the extent of injury-induced mechanical hypersensitivity in GAD67 haplodeficient mice, we used the intraplantar injection of zymosan-A and monitored mechanical sensitivity using the 2 times 5 stimulations method previously described in 11. We used 3 different von Frey filaments with increasing calibers: 0.07, 0.4, and 1.0 g. At day 1 postinflammation, WT and GAD67 haplodeficient mice of both sexes had a 100% response to 1.0 g von Frey caliber, demonstrating that both genotypes developed pronounced mechanical hyperalgesia (**Figs. 1D and E**). However, GAD67 haplodeficient mice exhibited a stronger response to the intermediate 0.4 g and a much pronounced response to the finest 0.07 g filaments, demonstrating that GAD67 haplodeficient mice developed a stronger mechanical allodynia than their WT littermates (**Figs. 1D and E**).

3.2. Formalin-evoked pain is selectively exacerbated in GAD67 haplodeficient female mice

Activation or positive allosteric modulation of the ionotropic ($GABA_A$) or metabotropic ($GABA_B$) GABAergic signaling reduces formalin-evoked nocifensive behaviors,[5,12,20,34] suggesting that the GABAergic system plays a critical role in this pain model. To test the impact of formalin injection in GAD67 haplodeficient mice, we used our recently developed protocol in which the amount of injected formalin was normalized to the weight of the mice.[5] As shown in **Figure 2**, intraplantar injection of 2% formalin triggers robust first and second pain responses in males and females of both genotypes. In particular, GAD67 haplodeficient male mice displayed the prototypical biphasic response that is identical to that of their WT littermates (**Fig. 2A**) and spent the

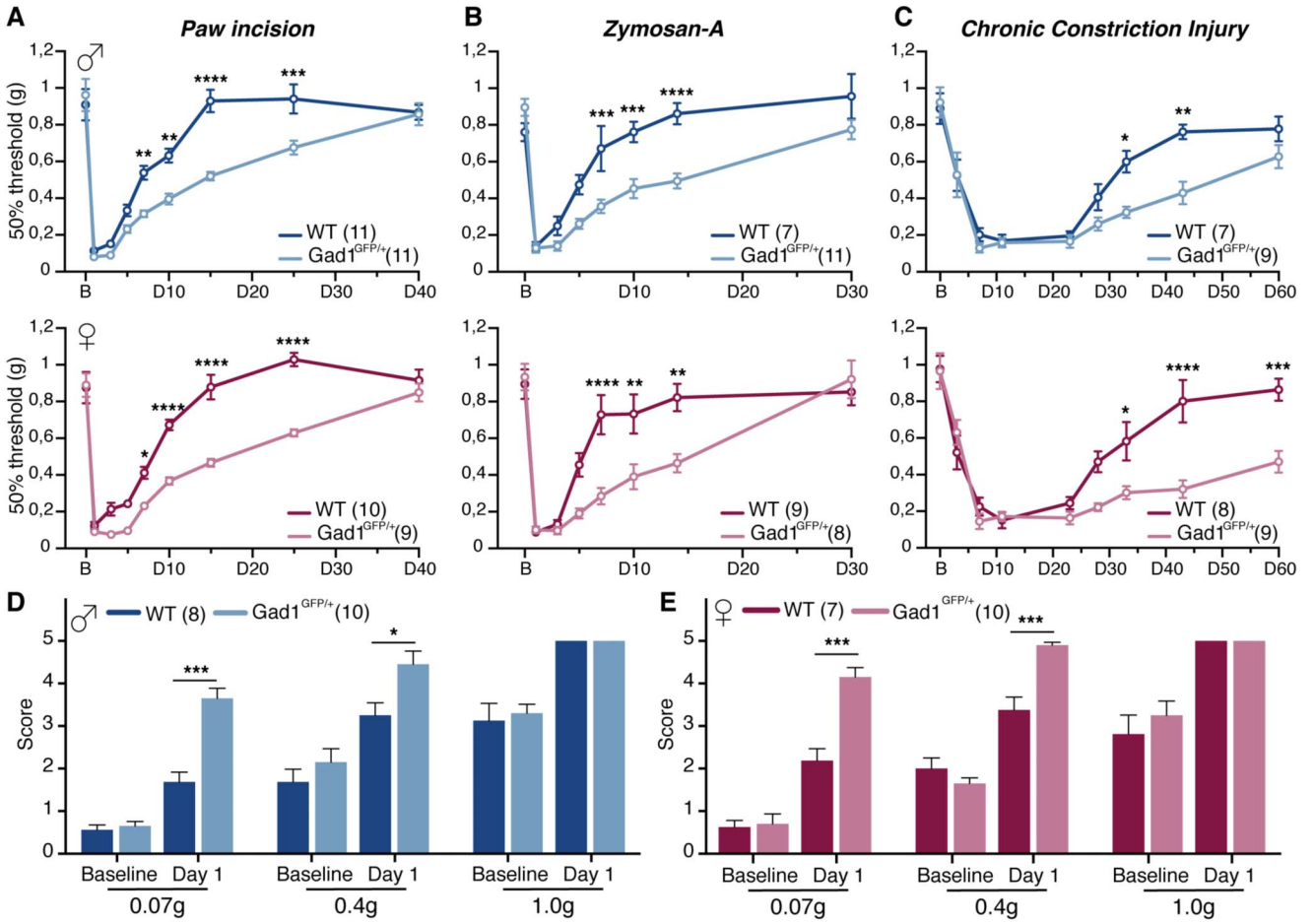

Figure 1. GAD67 haplodeficiency causes prolonged injury-induced mechanical hypersensitivity. Paw withdrawal threshold in response to static mechanical stimulation (von Frey assay) after paw incision (A), intraplantar injection of zymosan-A (B), or chronic constriction of sciatic nerve (C) in WT and GAD67 haplodeficient male (top) and female (bottom) mice. In all pain models, a significant long-lasting hypersensitivity is observed in GAD67 haplodeficient mice regardless of their sex. Mechanical sensitivity of WT and GAD67 haplodeficient male (D) and female (E) mice. Score (number of the paw withdrawl for 5 stimulations) was determined before (baseline) and 1 day after intraplantar injection of zymosan-A (day 1) using 3 different filaments of increasing calibers (0.07, 0.4, and 1 g). Statistical tests: (A, B, C) 2-way repeated-measures ANOVA followed by Bonferroni test and (D and E) 2-way ANOVA followed by Tukey *t* test. Number of animals in each group is indicated on the figure. See also Figure S1, available at http://links.lww.com/PAIN/B895. ANOVA, analysis of variance; GAD, glutamate decarboxylase.

same total amount of time licking, flinching, and shaking their inflamed paw as their WT littermates (**Fig. 2B**). On the other hand, GAD67 haplodeficient females had a completely different behavior. WT females displayed a highly variable second phase, whereas GAD67 haplodeficient females displayed a sharp second phase (**Fig. 2C**). Furthermore, GAD67 haplodeficient females spent significantly more time licking, flinching, and shaking their inflamed paw than their WT littermates (**Fig. 2D**).

Altogether, our data demonstrate that loss of 1 copy of GAD67-encoding gene (*gad-1*) exacerbates postoperative, inflammatory, and neuropathic mechanical pain. The data also show that CCI-induced and formalin-evoked pains were more pronounced in GAD67 haplodeficient female mice.

3.3. The exacerbated pain behavior in GAD67 haplodeficient mice correlates with decreased spinal GABA levels

Next, we sought to provide mechanistic insights into why the injury-induced pain phenotypes were exacerbated in GAD67 haplodeficient mice and why some of these phenotypes were more pronounced in female mice. Because our phenotypes were related to somatosensation, and given the importance of the GABAergic system in this process, we assessed the consequence of GAD67 haplodeficiency on the levels of spinal GABA.

Previous studies reported that ablation of 1 copy of a *Gad1* gene triggered a 40% reduction of the GAD67 protein and a slight reduction (16%) of global GABA in the juvenile brain, with no effect on GAD65 and vesicular GABA transporter in the brain.[47,49] In the spinal cord, immunohistochemical studies have shown that virtually all inhibitory axon terminals express both GAD isoforms.[27] Because the relative intensity of labeling for each isoform was found to vary, it was proposed that axons derived from different neurochemical populations may show specific patterns of expression of GAD65 and GAD67. Inhibitory spinal interneurons comprise a heterogeneous population of cells, and these may correspond to functional groups that serve distinct sensory modalities.[15,16] To address these questions, we assessed the number and density of GABA-expressing neuronal profiles and the levels of GABA through lamina II of the dorsal horn using immunoelectron microscopy (EM). First, we observed that, irrespective of the genotype, GABA was localized in vesicle-containing dendrites (V1) engaged in glomeruli of type Ia configuration (V1 GIa; **Fig. 3**), V1 and/or axonal peripheral profiles (V2) engaged in glomeruli of type II configuration (V1 GII or V2 GII),

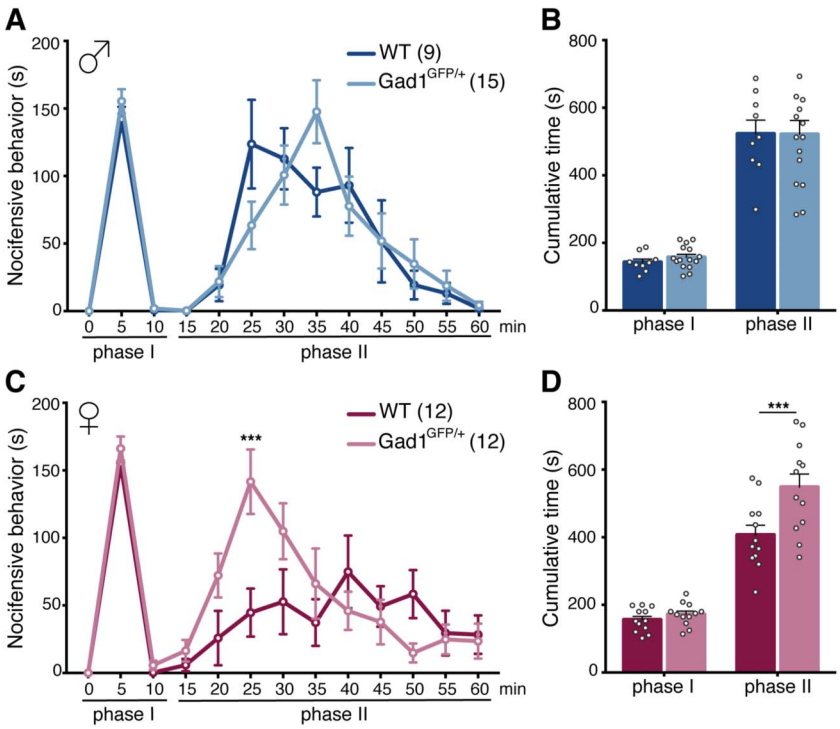

Figure 2. GAD67 haplodeficient female mice exhibit exacerbated nonevoked pain during the second phase of the formalin test. Time course of the nocifensive response: shaking, licking, biting, flinching, and lifting of the injected paw (A), and cumulative nocifensive behavior of WT and GAD67 haplodeficient male mice during the first and the second phase of the formalin test (B). Time course of the nocifensive response: shaking, licking, biting, flinching, and lifting of the injected paw (C) and cumulative nocifensive behavior of WT and GAD67 haplodeficient female mice during the first and the second phase of the formalin test (D). Statistical tests: (A and C) 2-way repeated-measures ANOVA followed by Bonferroni test. (B and D) Multiple unpaired *t* test followed by Sidak–Bonferroni test. Number of animals in each group is indicated on the figure. ANOVA, analysis of variance; GAD, glutamate decarboxylase.

axon terminals (At), and dendrites (d) (Figs. S2A-L, available at http://links.lww.com/PAIN/B895). GABA-immunogold particles were primarily associated with agranular vesicles in V1, V2, and At or distributed in the cytoplasm in d, as already described in literature both in rat[30,48] and mouse.[5,18] Next, we counted the total number of GABA-expressing profiles and calculated their density in lamina II. We found no significant differences between the genotypes or the sexes of the mice (Figs. S2M and S2N, available at http://links.lww.com/PAIN/B895). Then, we quantified the number of gold particles indicative of immunolabeling over the different GABA-expressing neuronal profiles. Our first analysis was performed on vesicle-containing dendrites presynaptic to type I glomeruli (V1 GIa) because they only express GABA,[48] thus suggesting that they come from 1 population of GABAergic interneurons. Using this experimental design, we found no significant difference in GABA levels between males and females in WT mice (**Figs. 3A, B, E**), whereas immunogold labeling in GAD67 haplodeficient mice was significantly lower in both sexes (**Figs. 3C–E**). Strikingly, the extent of GABA reduction was significantly higher in V1 (GIa) of female mice (**Figs. 3C–E**). Extending the same quantification strategy to all the other neuronal profiles, we found no difference in GABA expression between male and female WT mice but significantly lower levels in GAD67 haplodeficient mice (Figs. S2O-R, available at http://links.lww.com/PAIN/B895). Again, immunogold labeling was significantly lower in female mice (Figs. S2O-R, available at http://links.lww.com/PAIN/B895). Together, these data demonstrate that the exacerbated injury-induced phenotype in GAD67 haplodeficient mice is likely due to decreased spinal GABA content, and the female-related pronounced phenotypes in the CCI and formalin pain models can be explained by the greater decrease of GABA contents in GAD67 haplodeficient females.

3.4. Positive allosteric GABAergic modulation is impaired in GAD67 haplodeficient mice

To provide a causal link between GAD67 haplodeficiency, the reduced spinal GABA content, and the impaired injury-induced mechanical pain in GAD67 haplodeficient mice, we sought to use the positive allosteric GABAergic modulator diazepam. Our hypothesis was that GAD67 haplodeficient mice would be less responsive to the positive allosteric modulation of the GABAergic system by diazepam in comparison to their WT littermates. Intraplantar injection of zymosan A induced a significant reduction in paw withdrawal thresholds at day 2 postinjection (**Fig. 4**). Intrathecal injection of diazepam provided significant pain relief in WT but had no effect in GAD67 haplodeficient mice in both sexes (**Fig. 4A**). We then asked whether this phenotype was due to the decreased GABA levels or to an impairment of $GABA_A$ receptor function. Intrathecal injection of the $GABA_A$ receptor agonist muscimol significantly reduced mechanical hypersensitivity in a similar manner in both genotypes and sexes (**Fig. 4B**), demonstrating that the nonresponse to diazepam is due to the decrease of spinal GABA levels in the GAD67 haplodeficient mice.

3.5. Phenylethylidenehydrazine rescues zymosan-A-induced prolonged mechanical pain in GAD67 haplodeficient males but not females

We next sought to rescue our observed phenotypes by using the GABA-transaminase inhibitor 2-phenylethylidenehydrazine (PEH)

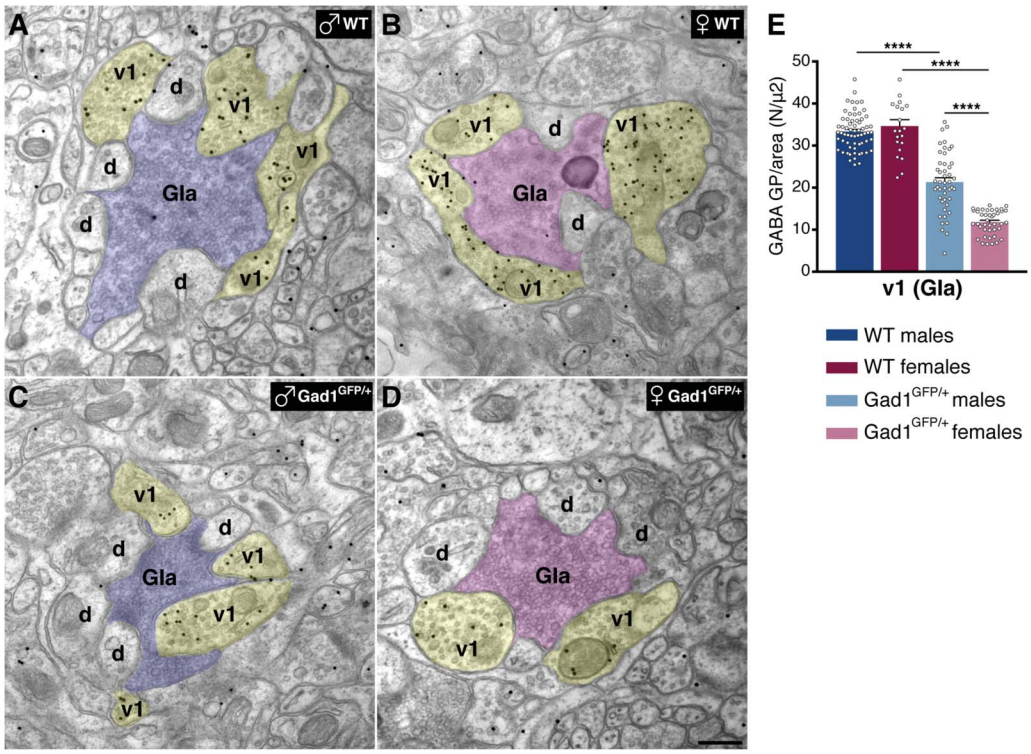

Figure 3. Ultrastructural localization of GABA in lamina II of WT and Gad1$^{GFP/+}$ mice. (A–D) Examples of nonpeptidergic glomeruli of the type Ia (Gla) surrounded by different vesicle-containing dendrites (V1) and plain dendrites (d) in WT males (A), WT females (B), Gad1$^{GFP/+}$ males (C), and Gad1$^{GFP/+}$ females (D). Note that, all V1 profiles are immunoreactive for GABA (20-nm gold particles), while all dendrites are unlabeled. Scale bar, 500 nm. Histogram showing GABA gold particle (GP) mean ± SEM densities in neuronal profiles (GABA GP/area, N/μ^2) in WT and gad1$^{GFP/+}$ male and female mice (E). Note that WT mice show no statistical differences between males and females. Gad1$^{GFP/+}$ mice show a significant reduction of GABA level in both sexes (Mann–Whitney test). See also Figure S2, available at http://links.lww.com/PAIN/B895.

to increase the GABA levels in the haplodeficient mice. Phenylethylidenehydrazine was synthesized in the laboratory of Dr. Jean-Marc PONS, and purity was checked by NMR (Fig. S3E, available at http://links.lww.com/PAIN/B895). This compound has been shown to trigger a much-pronounced increase in spinal GABA levels in females compared with males.[32] Our results show that intraperitoneal (i.p.) injection of PEH induced a significant increase in spinal GABA in WT and GAD67 haplodeficient males but had no effect on females of both genotypes (Figs. S3A and S3B, available at http://links.lww.com/PAIN/B895). Although we did not see much effect on females, we decided to check whether a daily injection of PEH would rescue the prolonged mechanical hypersensitivity after an intraplantar injection of zymosan A. GAD67 haplodeficient mice of both sexes were divided in 2 groups: one receiving a daily injection of PEH and the other receiving the vehicle for 14 days. Mechanical hypersensitivity was monitored at days 3, 5, 7, 10, 15, and 20. As shown in **Figure 4C**, PEH treatment allowed a faster recovery of GAD67 haplodeficient males but had no effect on females. Phenylethylidenehydrazine treatment has no effect on the mechanical thresholds of the uninjured contralateral paw, demonstrating no motor or cognitive defects (Figs. S3C and S3D, available at http://links.lww.com/PAIN/B895). Together, our data demonstrate strong sex-related differences between males and females in relation to the ionotropic GABAergic neurotransmission in the spinal cord.

4. Discussion

In this study, we found that GAD67 haplodeficient mice show exacerbated pain phenotypes in postoperative, inflammatory, and neuropathic models, with female mice showing more pronounced phenotypes. These findings provide an additional layer of evidence supporting a role of spinal GABA in the modulation of pathological pain. The study also provides new evidence for a sex-related difference regarding the role of spinal GABA levels in pathological pain in mice. We showed that, regardless of their sex, GAD67 haplodeficient mice exhibited a prolonged injury-induced mechanical hypersensitivity in several pain models: paw incision-induced postoperative pain, zymosan-A-induced inflammatory pain, and CCI-induced neuropathic pain. We showed that these phenotypes were causally linked to a significant decrease in the spinal GABA content. In line with the pronounced reduction of spinal GABA levels, GAD67 haplodeficient female mice displayed a sex-specific phenotype in 2 pain models in which the role of the GABAergic system is well established: formalin-evoked pain and CCI neuropathic pain.

Intraplantar injection of 2% formalin triggers similar pain responses in WT and GAD67 haplodeficient male mice. However, injection of formalin to females revealed 2 distinct phenotypes: (1) the magnitude of the second-phase response was significantly higher in GAD67 haplodeficient females and (2) WT females displayed a widespread second-phase response, whereas GAD67 haplodeficient females had a sharp second response. We recently showed that the widespread second-phase response in WT female mice represents 2 categories of females at a specific estrus cycle. F1 females, most of which are at proestrus/estrus phase, have a short interphase and F2 females, most of which are at metestrus phase, have a much longer interphase.[38] In line with this, studies in female rats demonstrate differences in nociception across the estrus cycle. Nociceptive sensitivity peaks

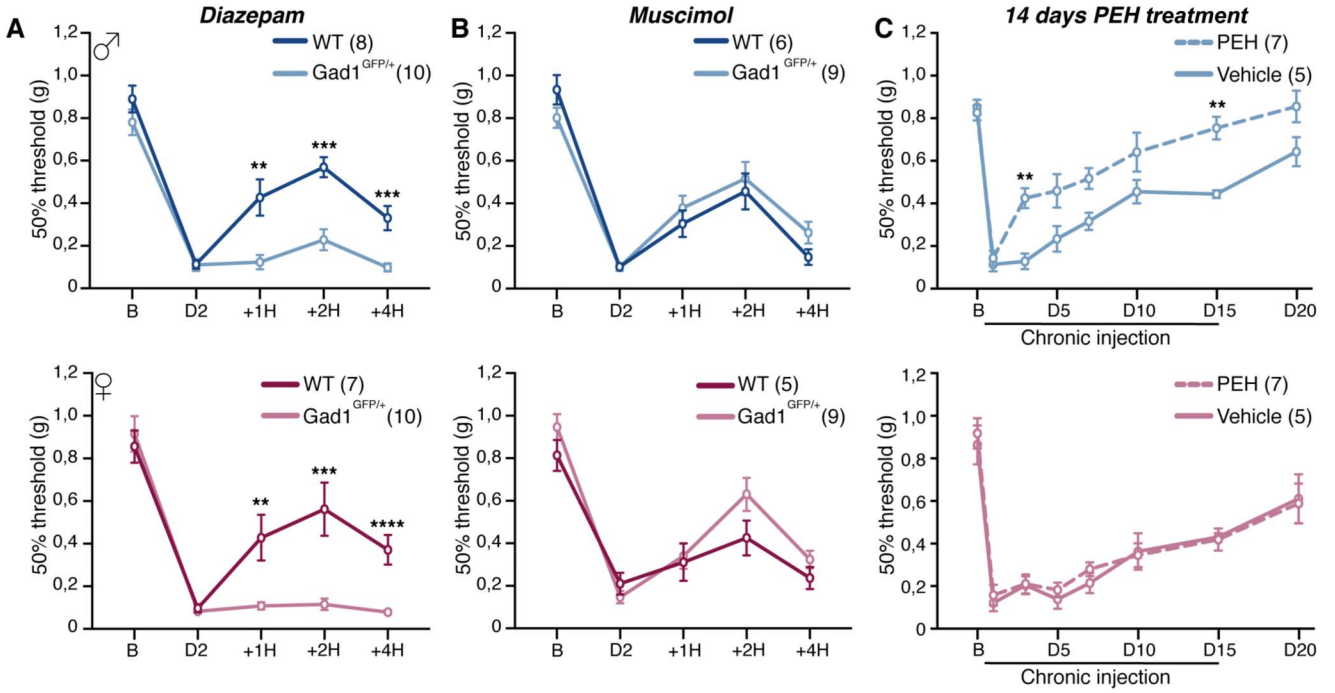

Figure 4. Pharmacological modulation of mechanical sensitivity in GAD67 haplodeficient mice. Antinociceptive effects of diazepam (A) and muscimol (B) were measured 1, 2, and 4 hours after injection by paw withdrawal threshold in response to static mechanical stimulation in the inflammatory model (2 days after intraplantar injection of zymosan-A). Compared with the WT mice, GAD67 haplodeficient mice male (top) and female (bottom) have an expected response to muscimol (B) but not to diazepam (A). Chronic injection of PEH for 14 days after intraplantar injection of zymosan-A restores significantly long-lasting hypersensitivity observed in GAD67 haplodeficient male (top) but not in female mice (bottom) (C). Statistical tests: (A and B) Multiple *t* test; (C) 2-way repeated measures ANOVA followed by Bonferroni test. Number of animals in each group is indicated on the figure. See also Figure S3, available at http://links.lww.com/PAIN/B895. ANOVA, analysis of variance; GAD, glutamate decarboxylase; PEH, phenylethylidenehydrazine.

during the estrus cycle stage of sexual receptivity (proestrus) and is reduced during the cycle stage of sexual nonreceptivity (metestrus).[13,21,23,26] Regarding formalin-evoked pain, Kuba et al.[23] showed that administration of estradiol to ovariectomized rats significantly reduced the number of flinches during phase II, whereas administration of progesterone attenuated phase I. In rodents, the level of progesterone is high during the metestrus phase.[35] Progesterone is known to be metabolized in neurons to 5α-dihydroprogesterone, which itself is converted by the enzyme 3α-hydroxysteroid oxidoreductase (3α-HSOR) to 3α,5α-tetrahydroprogesterone (also known as allopregnanolone). Allopregnanolone is a potent positive allosteric modulator of the $GABA_A$ receptor. Its systemic administration significantly reduced the second phase of the formalin test in a $GABA_A$ receptor–dependent manner.[37] Given that the spinal GABAergic system is known to tightly control the interphase and the magnitude of the second phase of formalin-evoked pain,[5,20,34] we hypothesize that in WT female mice, the metestrus-related high levels of progesterone promote the increase of allopregnanolone, which in turn contributes to increasing the spinal GABAergic tone, thus contributing to a reduced first phase and to the prolonged interphase. In GAD67 haplodeficient females, the amount of spinal GABA is too low to allow such positive modulation by allopregnanolone. As a consequence, all GAD67 haplodeficient females display a short interphase and an exacerbated formalin-evoked second phase, demonstrating a tight functional link between sex steroid hormones and the GABAergic system.

The second pain model in which we also found a female-biased phenotype is the CCI model. We showed that CCI-mediated mechanical hypersensitivity was prolonged in both male and female GAD67 haplodeficient mice. However, at day 60 post-CCI, mechanical sensitivity of GAD67 haplodeficient male mice returned to baseline levels, whereas GAD67 haplodeficient females were still hypersensitive. These results further demonstrate that GABA levels play a critical role in the modulation of CCI-induced neuropathic pain in males and females and provide an additional layer of knowledge why a variety of painful diseases are more prevalent among females.

Several studies have provided evidence that there is loss of GABAergic inhibition in the dorsal horn after peripheral nerve injury. Primary afferent depolarization (a measure of presynaptic inhibition) was reduced after CCI.[24] Moore et al.[33] demonstrated a drastic reduction in the GABAergic components of primary afferent-evoked IPSCs in lamina II neurons 2 weeks after CCI. However, still under debate whether there is also a loss of GABAergic interneurons in addition to a reduction in GABA synthesis and release in different types of peripheral nerve injury.[9,16,33,39,40,42] Here, we showed that loss of 1 copy of *Gad1* gene led to a significant reduction of immunogold labeling in all GABA-expressing neuronal profiles, and this is likely to reflect a reduction in GABA expression in inhibitory interneurons. The consequence of this GABA reduction is a prolonged mechanical hypersensitivity in all the pain models tested, including the CCI model in GAD67 haplodeficient mice. Instead, we did not observe a loss of GABA-expressing profiles in lamina II, thus supporting the hypothesis that nerve injury-induced loss of inhibition likely involves the reduction of transmitter release in GABAergic interneurons. Why did GAD67 haplodeficient females develop a longer lasting mechanical hypersensitivity than males in the CCI model? To address this question, we reanalyzed the electrophysiology data recently published in the study by Yoo et al.[52] in which we used the *Gad1*$^{GFP/+}$ mice. Because we found no

difference in the painkilling effect of TAFA4 between males and females, the electrophysiological data obtained from both sexes were pooled. We showed that spared nerve injury induced a significant decrease in I_A amplitudes in lamina II outer excitatory interneurons (L-IIo-ExIN) and a significant increase in I_A amplitudes in lamina II inner inhibitory interneurons (L-IIi-InhIN). The increase in I_A amplitudes in L-IIi-InhIN was accompanied by a decrease in I_h amplitude. Because we showed here that GAD67 haplodeficient females behaved differently than their male littermates, we reanalyzed the data by separating the electrophysiological recordings obtained from females vs those obtained from males. Fig. S4A (available at http://links.lww.com/PAIN/B895) shows that the SNI-mediated increase in I_A amplitudes in L-IIo-ExIN was observed in GAD67 haplodeficient females but not males. In L-IIi-InhIN, SNI-mediated increase in I_A amplitudes occurred in both sexes, whereas the effect on I_h only occurs in females (Figs. S4B and S4C, available at http://links.lww.com/PAIN/B895). The concomitant I_A increase and I_h decrease in L-IIi-InhIN and the I_A decrease in L-IIo-ExIN are likely to explain why GAD67 haplodeficient females develop a much-prolonged mechanical hypersensitivity in the CCI model and why PEH failed to rescue the zymosan-A-induced prolonged mechanical hypersensitivity.

In conclusion, our study demonstrates that reduction of spinal GABA levels predisposes the mice to develop long-lasting injury-induced pain. This deleterious effect is more pronounced in females, highlighting why females in general are more prone to develop chronic pain.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Inclusion and diversity: The authors worked to ensure sex balance in the selection of nonhuman subjects.

Authors' contributions: A.C. performed all the behavioral experiments, made all the figures and wrote the Materials and Methods section. L.P. generated the formalin test data and contributed with A.C. to perform the general behavior of mice. P.M. managed the mouse colonies used in this study. F.C. supervised the work of A.C. and taught her how to make high-quality figures. C.S. performed all the EM experiments and wrote the corresponding Materials and Methods section, results, and Figure legends. A.M. conceived the project and wrote the manuscript.

Supplemental digital content

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