A Glra3 phosphodeficient mouse mutant establishes the critical role of protein kinase A–dependent phosphorylation and inhibition of glycine receptors in spinal inflammatory hyperalgesia

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Abstract
Glycinergic neurons and glycine receptors (GlyRs) exert a critical control over spinal nociception. Prostaglandin E2 (PGE2), a key inflammatory mediator produced in the spinal cord in response to peripheral inflammation, inhibits a certain subtype of GlyRs (α3GlyR) that is defined by the inclusion of α3 subunits and distinctly expressed in the lamina II of the spinal dorsal horn, ie, at the site where most nociceptive nerve fibers terminate. Previous work has shown that the hyperalgesic effect of spinal PGE2 is lost in mice lacking α3GlyRs and suggested that this phenotype results from the prevention of PGE2-evoked protein kinase A (PKA)-dependent phosphorylation and inhibition of α3GlyRs. However, direct proof for a contribution of this phosphorylation event to inflammatory hyperalgesia was still lacking. To address this knowledge gap, a phospho–deficient mouse line was generated that carries a serine to alanine point mutation at a strong consensus site for PKA-dependent phosphorylation in the long intracellular loop of the GlyR α3 subunit. These mice showed unaltered spinal expression of GlyR α3 subunits. In behavioral experiments, they showed no alterations in baseline nociception, but were protected from the hyperalgesic effects of intrathecally injected PGE2 and exhibited markedly reduced inflammatory hyperalgesia. These behavioral phenotypes closely recapitulate those found previously in GlyR α3-deficient mice. Our results thus firmly establish the crucial role of PKA-dependent phosphorylation of α3GlyRs in inflammatory hyperalgesia.

Keywords: Prostaglandin, Inflammation, Central sensitization, Phosphorylation, Knock-in, Dis-inhibition, Dorsal horn, Spinal cord, Pain, Mouse, von Frey, Hargreaves test, Heat hyperalgesia, Alloodynia

1. Introduction
Chronic pain is a growing concern worldwide, affecting roughly 20% of the population.26 It is a complex disorder associated with alterations in the peripheral nervous system and central nervous system. In most patients, chronic pain is maintained either by inflammatory processes (inflammatory pain) or results from damage to the somatosensory nervous system (neuropathic pain). In both conditions, compromised functioning of inhibitory circuits of the spinal cord crucially contributes to the development and maintenance of chronic pain.2,32,41 At this site, inhibitory neurotransmission is mediated by both glycine and γ-aminobutyric acid (GABA).30 Several reports from different groups have provided compelling evidence for a critical role of glycinergic neurons and glycinergic neurotransmission in the spinal control of nociception. Blockade of spinal GlyRs with strychnine induces hyperalgesia, allodynia and signs of spontaneous discomfort at subconvulsive doses.6 Local ablation or silencing of dorsal horn glycinergic neurons results in mechanical, heat, and cold hypersensitivity,11 and patients with hyperekplexia who carry disease-causing mutations in glycine receptors or glycine transporters also exhibit heightened pain sensitivity.33 Moreover, recently developed positive allosteric GlyR modulators and glycine transport inhibitors reduce hyperalgesia in mouse models of chronic inflammatory and neuropathic pain.1,16,24,38,39 For a recent review, see Ref. 40.

Although both inflammation and neuropathy compromise neuronal inhibition in the spinal cord, underlying mechanisms are rather different. Peripheral nerve damage has been shown to lead to a microglia-dependent disruption of the transmembrane chloride gradient that
Compromises both GABAergic and glycine inhibition, while other work has demonstrated that changes that specifically interfere with the responsiveness of superficial dorsal horn glycineergic neurons and glycine receptors (GlyRs) are major contributors to chronic inflammatory pain. The spinal dorsal horn harbors an abnormally sparse expression of GlyR α3 subunits in the channel complex. Mice lacking these GlyR α3 subunits (gene name Glra3) show normal baseline nociception, but are resistant to the hyperalgesic effect of intrathecally injected PGE2 and show markedly reduced hyperalgesia evoked by peripheral inflammatory insults. Whole-cell recording experiments in superficial dorsal horn neurons have shown that PGE2 inhibits glycineergic postsynaptic currents in a protein kinase A (PKA)-dependent manner, and additional electrophysiological experiments on recombinant glycine receptors expressed in HEK293T cells indicate that PKA phosphorylates a serine residue (S346) in the long intracellular loop of GlyR α3 subunits between transmembrane segments 3 and 4.

Although these in vitro experiments suggested a critical role of phosphorylation of GlyR α3 subunits at S346 in inflammation-induced hyperalgesia, its relevance for in vivo inflammatory hyperalgesia has not yet been directly demonstrated. Here, we report the generation, and electrophysiological and behavioral analysis of a GlyR α3 phospho-deficient (S346A point-mutated) mouse line. The behavioral phenotypes observed in these mice resemble those previously described for the GlyR α3-deficient mice and firmly establish the critical role of PKA-dependent phosphorylation and inhibition of α3GlyR in inflammatory hyperalgesia.

2. Methods

2.1. Mouse breeding and maintenance

Experiments were performed in wild-type mice (C57BL/6), GlyRx3(S346A) point-mutated mice (Glra3tm1.1Umu), GlyR α3 subunit-deficient mice (Glra3tm1.1Umu) and vGAT::ChR2 (Tg[Tg26260.COP-1.3::EYFP-APH(3′)NeoR]J28) bac transgenic mice. All mice were maintained on C57BL/6 genetic background. For experiments involving optogenetic stimulation, the vGAT::ChR2 transgene was crossed into wild-type and GlyRx3 (S346A) point-mutated mice. Mice were kept group-housed under intermediate barrier conditions and under a 12/12-hour light/dark cycle with ad libitum access to food and water. Permissions for all animal experiments reported here were obtained from the Canton of Zurich (licenses ZH011/2019, ZH031/2016, and ZH231/2017). All animal experiments were conducted in compliance with the relevant ethical regulations. Genotyping of GlyRx3(S346A) point-mutated mice was performed with two separate PCR reactions involving primers (1) and (2) for the wild-type allele and primers (1) and (3) for the point-mutated allele.

(1) Glra3_wt_antisense: GTCTTGCTGCTGATGAATGTCTTCATG; (2) Glra3_wt_sense: CAGGATGATGAGGTGaggGAGag; (3) Glra3_mut_sense: CAGGATGATGAGGTGagaGAGgc.

2.2. Electrophysiological recordings in spinal cord slices

Transverse lumbar spinal cord slices were prepared from 3 to 5 week-old vGAT::ChR2 bac transgenic mice and from vGAT::ChR2; GlyRx3(S346A) double transgenic mice of both sexes (for details, see Ref. 1). In brief, 400 μm-thick transverse slices were prepared from the lumbar spinal cord. Immediately after cutting, slices were transferred to oxygenated ACSF at a flow rate of 1.5 to 2.0 mL/min. Superficial dorsal horn neurons were visually identified using infrared gradient contrast equipment. Recordings were made from presumed excitatory interneurons located in lamina II identified by the absence of a blue light–induced photocurrent. Whole-cell patch-clamp recordings were made at room temperature at a holding potential of −60 mV using a HEKA EPC-10 amplifier and PatchMaster v2.11 software (HEKA Elektronik, Ludwigshafen/Rhein, Germany). Patch pipettes (3.5 to 4.5 MΩ resistances) were prepared from borosilicate glass capillaries and filled with internal solution containing (in mM): 120 CsCl, 10 EGTA, 4 MgCl2, 0.5 GTP, 2 ATP, and 10 HEPES (pH 7.30, adjusted with CsOH). QX-314 (5 mM) was added to the internal solution to block voltage-activated Na+ currents of the recorded cell. Light-evoked inhibitory postsynaptic currents (IPSCs) were induced by whole-field blue light (473 nm) illumination of the left or right dorsal horn with a Polychrome V monochromator (473 nm, 4 ms, 2.7 mW, Thermo Fisher Scientific, Waltham, MA).

Glycine-evoked light-IPSCs were isolated using bicuculline (10 μM (−)-bicuculline methochloride, Tocris, Bristol, United Kingdom). After 5 min of baseline recording, slices were superfused with ACSF containing 1 μM PGE2 (Tocris) for 5 to 10 minutes. At the end of each recording, strychnine (0.5 μM) was added to confirm the glycine receptor nature of the recorded IPSCs. Access resistance was continuously monitored. Recordings were discarded if the access resistance changed by more than 25%. The decay of IPSCs was fitted to a dual-exponential function using IgorPro software (Wavemetrics Inc., Oregon).

2.3. Behavioral analyses

All behavioral experiments were conducted in 7 to 12 week-old sex-matched mice by the same female experimenter blinded to the genotypes or treatments of the mice. Mice were assigned to treatment groups in a randomized manner. All behavioral experiments were performed during light phase (10 AM - 3 PM; ZT03-08). The effect of the S346A phospho-mutation on acute nociception and on spinal PGE2-induced or peripheral zymosan A-induced hyperalgesia was studied in 7 to 12 week-old sex-matched mice. Spinal PGE2-induced hyperalgesia was studied in mice injected intrathecally with PGE2 (0.4 nmol/mouse in 5 μL ACSF, from 10 mM stock dissolved in ethanol) through the L5/L6 intervertebral space under brief (2-3 min) anesthesia with 2% isoflurane using a 10 μL Hamilton syringe and a 30-gauge needle. Inflammatory hyperalgesia was evoked with zymosan A (0.06 mg in 20 μL saline) subcutaneously injected into the plantar side of the left hind paw. To study the effects of the phospho-mutation on neuropathic pain, mice were subjected to a chronic constriction injury (CCI) of the left sciatic nerve. In brief, 3 loose (5-0 surgical silk) ligatures were put around the left sciatic nerve proximal to the trifurcation. None of the mice showed signs of paralysis. Mechanical withdrawal threshold and thermal withdrawal latency were assessed using electronic von Frey filaments (IITC, Woodland Hills, CA) and a Hargreaves test apparatus (IITC, Woodland Hills, CA), respectively.

To assess motor coordination, animals were placed onto a rotarod setup (IITC, Woodland Hills, CA). The rod was set to accelerate from 4 to 40 rpm over a period of 300 s. Two training sessions on the same accelerating rod were performed 3 days before the actual test. In the test session, the latency to fall from the rod was recorded at 5 times per mouse with 10-min intervals between tests, during which the mouse was placed back into its home cage. Averages of the test runs were calculated for each mouse.
2.4. Immunohistochemistry

Three mice per genotype were anaesthetized with sodium pentobarbital and intracardially perfused with ice-cold ACSF at room temperature for 2 min (2 mM CaCl₂, 2 mM MgCl₂, 30 mM glucose, 5 mM KCl, 119 mM NaCl, 25 mM HEPES, pH 7.4). The lumbar spinal cord was rapidly dissected, frozen on powdered dry ice, and stored at −80°C until used. Approximately 20 μm-thick cryostat-cut sections mounted on SuperFrost Plus glass slides (Thermo Fisher Scientific, Reinach, Switzerland) were fixed with 4% PFA in PBS for 20 min at room temperature. For antigen retrieval, the sections were incubated in 10 mM citrate buffer pH 6.0, 0.05% Tween 20 for 30 min at 90°C. After cooling to room temperature, the sections were washed in PBS and incubated in blocking/permeabilization solution (TBS containing 10% normal goat serum and 0.5% Triton X-100) for 30 min. Subsequently, the solution was removed, and the sections were incubated overnight at 4°C with a rabbit polyclonal antiserum directed against the GlyR α3 subunit diluted 1:100 in TBS containing 10% NGS and 0.5% Triton X-100. After extensive washing (5 times for 5 min in TBS, 0.05% Tween 20), the sections were incubated with fluoresce-fluoresce labeled secondary antibody (donkey anti-rabbit Alexa Fluor Plus 488, Thermo Fisher Scientific, Reinach, Switzerland) diluted 1:2000 in TBS containing 10% NGS and 0.05% Tween 20 for 1 hour at room temperature. The sections were then extensively washed and coverslipped in DAKO Fluorescence Mounting Medium. Images of the labeled sections were acquired using a Zeiss LSM 800 microscope equipped with a 25x objective.

GlyR α3 immunofluorescence intensity was quantified using the ImageJ software (https://imagej.nih.gov/ij). Raw fluorescence intensity values were determined by dividing the sum of fluorescence grey values in each pixel of laminae I and II of the dorsal horn gray matter by the number of the analyzed pixels. Nonspecific fluorescence, measured ventral of the immunopositive lamina II, was subtracted to obtain the GlyR α3 immunofluorescence. The same regions were analyzed in all sections.

2.5. Statistics

Statistical analyses of electrophysiological data were performed with 2-tailed unpaired t tests. Behavioral data were analyzed with 2-tailed unpaired t tests or with 2-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post hoc tests. Quantifications of the morphological data were performed with 1-way ANOVA with the Tukey multiple comparison test.

3. Results

3.1. Generation of S346A GlyR α3 phospho-deficient mice and baseline morphological and electrophysiological characterization

GlyRα3(S346A) point-mutated mice were generated using CRISPR technology. The serine (S) residue 346, which constitutes the phosphorylation site within a strong consensus sequence for PKA-dependent phosphorylation (RESIR) in the long intracellular loop of the GlyR α3 subunit (Fig. 1A), was mutated into an alanine (A) by changing the serine encoding base triplet AGT to GCT (Fig. 1B). A mixture containing active guide RNA molecules, a single stranded oligodeoxynucleotide donor, and qualified Cas-9 mRNA was injected into the cytoplasm of C57BL/6N embryos that were subsequently implanted into pseudopregnant female mice. Genotyping of pups was performed by genomic DNA sequencing (Fig. 1B) or using a PCR with mutation-specific primers (Fig. 1C). No differences in the levels of GlyR α3 subunit expression were found in transverse sections of the lumbar spinal cord of wild-type mice and homozygous α3 GlyR (S346A) point-mutated mice (Fig. 1D). Specificity of the antibody was confirmed in sections prepared from GlyR α3-deficient mice.

3.2. Electrophysiological characterization of inhibitory synaptic transmission in the superficial spinal dorsal horn

We next performed a first electrophysiological characterization of inhibitory synaptic transmission in the GlyRα3(S346A) point-mutated mice and combined electrophysiological whole-cell patch-clamp recordings in transverse lumbar spinal cord slices with an optogenetic approach to trigger synaptic neurotransmitter release from inhibitory neurons (Fig. 2A). To this end, we used wild-type mice and homozygous GlyRα3(S346A) point-mutated mice that carried the vGAT:ChR2 bacterial artificial chromosome transgene. In spinal cord slices prepared from these mice, a 1-s blue light (473 nm) pulse induced a depolarizing photocurrent in voltage-clamped inhibitory neurons, while exposure to shorter (4 ms) blue light pulses in current-clamp evoked single action potentials in these neurons and triggered IPSCs in sympathetically connected cells. Targeted whole-cell recordings were performed from photocurrent-negative (presumed excitatory) neurons. Excitatory interneurons of the dorsal horn have previously been shown to serve an important role in pathological pain states. Brief wide-field blue light exposure of slices of wild-type and homozygous GlyRα3(S346A) point-mutated mice evoked IPSCs in all recorded neurons (n = 28 and 22, for wild-type and GlyR α3(S346A) point-mutated, respectively) (Figs. 2B–D). IPSC amplitudes were measured, and their decay kinetics were determined by calculating a weighted τw from a double exponential fit of the falling phase of the IPSC. Significant difference in the amplitudes of total IPSCs and of their glycinergic components (gly-IPSC) were found between wild-type and GlyRα3(S346A) point-mutated mice (Figs. 2B and D). Total IPSC amplitudes were −580 ± 62 pA in wild-type mice (n = 28) vs −1228 ± 108 pA in S346A point-mutated mice (n = 22) (P < 0.0001, unpaired t test). Average gly-IPSC amplitudes were −288.5 ± 31.50 pA in wild-type mice (n = 15) vs −632.0 ± 155.7 in S346A point-mutated mice (n = 13) (P = 0.029, unpaired t test). No difference was observed in the decay time course of the total IPSCs and the gly-IPSCs between wild-type and GlyRα3(S346A) point-mutated mice. Average decay time constants of the total IPSC 161.5 ± 30.7 ms for wild-type mice (n = 28) vs 119.1 ± 34.1 ms in S346A point-mutated mice (n = 22) (P = 0.36, unpaired t test). Average decay time constants of the gly-IPSC were 68.7 ± 12.8 ms in wild-type mice (n = 15) vs 55.0 ± 8.2 ms in S346A point-mutated mice (n = 13) (P = 0.39, unpaired t test) (Fig. 2D).

3.3. Effects of prostaglandin E2 on glycinergic synaptic transmission in the superficial spinal dorsal horn

Previous work has shown that PGE₂ reduces the amplitudes of gly-IPSCs in superficial dorsal horn neurons of wild-type mice and homozygous α3 GlyR (S346A) point-mutated mice and combined electrophysiological whole-cell patch-clamp recordings in transverse lumbar spinal cord slices with an optogenetic approach to trigger synaptic neurotransmitter release from inhibitory neurons (Fig. 2A). To this end, we used wild-type mice and homozygous GlyRα3(S346A) point-mutated mice that carried the vGAT:ChR2 bacterial artificial chromosome transgene. In spinal cord slices prepared from these mice, a 1-s blue light (473 nm) pulse induced a depolarizing photocurrent in voltage-clamped inhibitory neurons, while exposure to shorter (4 ms) blue light pulses in current-clamp evoked single action potentials in these neurons and triggered IPSCs in sympathetically connected cells. Targeted whole-cell recordings were performed from photocurrent-negative (presumed excitatory) neurons. Excitatory interneurons of the dorsal horn have previously been shown to serve an important role in pathological pain states. Brief wide-field blue light exposure of slices of wild-type and homozygous GlyRα3(S346A) point-mutated mice evoked IPSCs in all recorded neurons (n = 28 and 22, for wild-type and GlyR α3(S346A) point-mutated, respectively) (Figs. 2B–D). IPSC amplitudes were measured, and their decay kinetics were determined by calculating a weighted τw from a double exponential fit of the falling phase of the IPSC. Significant difference in the amplitudes of total IPSCs and of their glycinergic components (gly-IPSC) were found between wild-type and GlyRα3(S346A) point-mutated mice (Figs. 2B and D). Total IPSC amplitudes were −580 ± 62 pA in wild-type mice (n = 28) vs −1228 ± 108 pA in S346A point-mutated mice (n = 22) (P < 0.0001, unpaired t test). Average gly-IPSC amplitudes were −288.5 ± 31.50 pA in wild-type mice (n = 15) vs −632.0 ± 155.7 in S346A point-mutated mice (n = 13) (P = 0.029, unpaired t test). No difference was observed in the decay time course of the total IPSCs and the gly-IPSCs between wild-type and GlyRα3(S346A) point-mutated mice. Average decay time constants of the total IPSC 161.5 ± 30.7 ms for wild-type mice (n = 28) vs 119.1 ± 34.1 ms in S346A point-mutated mice (n = 22) (P = 0.36, unpaired t test). Average decay time constants of the gly-IPSC were 68.7 ± 12.8 ms in wild-type mice (n = 15) vs 55.0 ± 8.2 ms in S346A point-mutated mice (n = 13) (P = 0.39, unpaired t test) (Fig. 2D).
3.4. Baseline nociceptive sensitivities of GlyRα3(S346A) point-mutated mice

As the next step, we compared the somatosensory and nociceptive sensitivity of naive wild-type and homozygous GlyRα3(S346A) point-mutated mice (Fig. 4). No significant difference in response latencies were found between these mouse lines upon exposure to noxious heat (wild-type (n = 10): 20.0 ± 1.4 s vs S346A point-mutated mice (n = 14): 21.3 ± 0.9 s; unpaired t test; P = 0.47) and noxious cold (wild-type (n = 10): 5.2 ± 0.3 s vs S346A point-mutated mice (n = 9): 5.4 ± 0.3 s; unpaired t test; P = 0.72) (Figs. 4A and B). Similarly, no differences were detected in response frequencies upon noxious mechanical stimulation (pin-prick response scores; Fig. 4C) (wild-type (n = 5): 0.8 ± 0.06 vs S346A point-mutated mice (n = 7): 0.81 ± 0.05; P = 0.91, unpaired t test), in response thresholds upon exposure to punctuate mechanical stimuli applied with von Frey filaments (Fig. 4D) (wild-type (n = 10): 4.2 ± 0.2 g vs S346A point-mutated mice (n = 12): 4.2 ± 0.1 g; P = 0.87, unpaired t test). Similarly, no differences were observed in response frequencies (scores) upon brush stimulation (wild-type (n = 7): 0.88 ± 0.051 vs S346A point-mutated mice (n = 6): 0.92 ± 0.053; P = 0.64, unpaired t test) (Fig. 4E). In addition, no difference was found in the rota-rod performance between genotypes (wild-type (n = 5): 117.7 ± 15.1 s vs S346A point-mutated mice (n = 8): 92.0 ± 10.8 s; P = 0.19, unpaired t test) (Fig. 4F).

3.5. Lack of hyperalgesic effects of intrathecal prostaglandin E2 in GlyRα3(S346A) point-mutated mice

Previous work has shown that GlyR α3-deficient mice were protected from the behavioral pain sensitization elicited by
Figure 2. Inhibitory synaptic transmission in GlyRα3(S346A) point-mutated mice. (A) Experimental set-up for combined electrophysiological optogenetic experiments. (B) Left: Average traces of total (mixed GABAergic/glycinergic) IPSCs recorded from wild-type and homozygous GlyRα3(S346A) point-mutated mice. Blue lines indicate time points of optogenetic blue light stimulation. Right: statistical comparison. Scattered plot, data points represent 28 and 22 individual neurons from 21 wild-type and 15 homozygous S346A/S346A point-mutated mice, respectively. Lines indicate mean ± SEM. P < 0.01, unpaired two-sided t test. (C) Same as (B) but decay kinetics of total IPSCs. Left: scaled average traces (black) with double exponential fits superimposed (red). Quantification of decay kinetics in wild-type and GlyRα3(S346A) point-mutated mice. (D) Analysis of amplitudes and decay kinetics of the glycinergic IPSC component isolated with bicuculline (10 μM). Fifteen and 13 neurons from 13 wild-type and 9 homozygous S346A/S346A point-mutated mice, respectively. Quantification of glycinergic decay kinetics in wild-type and GlyRα3(S346A) mice. IPSC, inhibitory postsynaptic current.
spinal PGE₂.¹³,²⁷ We repeated these experiments in GlyRa3(S346A) point-mutated mice and injected PGE₂ (0.4 nmoles in 5 μL ACSF) intrathecally (i.e., into the subarachnoid space) at the level of the lumbar spinal cord. As expected, in wild-type mice, PGE₂ induced a transient mechanical hyperalgesia lasting for several hours. No such hyperalgesia was observed in GlyRa3(S346A) point-mutated mice (Figs. 5A and B). Two-way repeated measures ANOVA revealed significant time × genotype interactions for both sensory tests (Hargreaves: F(5,80) = 31.41, P < 0.0001; von Frey: F(5,80) = 13.05, P < 0.0001). Bonferroni post hoc tests identified significant differences between the 2 genotypes at 30, 60, 90, and 120 min after PGE₂ injection (P < 0.001 for 30, 60, 90, and 120 min for both Hargreaves and von Frey tests). No statistically significant hyperalgesia was detected in homozygous GlyRa3(S346A) point-mutated mice (1-way repeated measures ANOVA; F(5,40) = 1.30; the Dunnett multiple comparison test P ≥ 0.06 for all time points).

3.6. Reduced inflammatory hyperalgesia but retained neuropathic sensitization after peripheral nerve injury

We next used the zymosan A model to induce peripheral inflammation in the left hind paw. Previous work has shown that in this model, heat hyperalgesia and mechanical hyperalgesia depend on spinal PGE₂ formation and that GlyR α3-deficient mice exhibit less hyperalgesia in this model than wild-type mice.²⁷ We therefore tested GlyRa3(S346A) point-mutated also in the zymosan A model and injected zymosan A.
(0.06 mg in 20 μL saline) subcutaneously into the left hind paw. Relative to their wild-type littermates, GlyRα3(S346A) point-mutated developed significantly less inflammatory hyperalgesia. In case of the Hargreaves test, 2-way repeated measures ANOVA revealed a significant time * genotype interaction (F(6,84) = 5.93; P < 0.0001; n = 9 and 7 for wild-type and S346 point-mutated mice, respectively). Significant differences between genotypes were found for day 1 (P < 0.0001), day 2 (P < 0.01), day 3 (P < 0.001), and day 4 (P < 0.01) (Fig. 5C). A similar time * genotype interaction was also found for the von Frey test (F(6,84) = 7.27; P < 0.0001; n = 9 and 7 for wild-type and S346A point-mutated mice). In this test, significant differences between genotypes were again found for day 1 (P < 0.0001), day 2 (P < 0.0001), day 3 (P < 0.0001), and day 4 (P < 0.01) (Fig. 5D). We used 1-way repeated measures ANOVA to test whether in GlyRα3(S346A) point-mutated mice developed significant heat or mechanical hyperalgesia. No such heat sensitization was detected in either the Hargreaves test (1-way repeated measures ANOVA; F(6,36) = 2.6, the Dunnett multiple comparison test P $\geq$ 0.14 for all time points) or the von Frey test (F(6,36) = 3.53, the Dunnett multiple comparison test P $\geq$ 0.08 for all time points).

We also tested whether wild-type mice and GlyRα3(S346A) point-mutated mice would differ in their mechanical or thermal hypersensitivity developed in response to a peripheral nerve injury induced by a chronic ligation of the sciatic nerve. In these experiments, we found indistinguishable heat and mechanical hyperalgesia 7 days after chronic ligation of the sciatic nerve (Figs. 5E and F) (Hargreaves: wild-type (n = 9); 5.48 ± 0.41 s; S346A (n = 7); 4.35 ± 0.39 s; P = 0.07, unpaired t test. von Frey: wild-type (n = 8); 1.33 ± 0.06 g; S346A (n = 7); 1.41 ± 0.09 g; P = 0.46, unpaired t test), again consistent with previous reports showing that α3GlyRs are not directly involved in the neuropathic pain development or maintenance.14,15

4. Discussion

In this study, we have used morphological, electrophysiological, and behavioral methods to assess the contribution of the S346 phosphorylation site of the GlyR α3 subunit to the inhibition of glycnergic neurotransmission by PGE2 and to inflammatory or neuropathic hyperalgesia. The results of the electrophysiological experiments indicate that the introduction of the phospho-deficient S346A point mutation into the GlyR α3 subunit of mice rendered dorsal horn glycine receptors insensitive to the inhibitory action of PGE2. Behavioral experiments demonstrate that naive GlyRα3(S346A) point-mutated mice exhibit unaltered nociceptive, somatosensory, and gross motor functions. However, unlike wild-type mice, GlyRα3(S346A) α3GlyRs are mice failed to develop nociceptive sensitization after intrathecal PGE2 injection. These mice also showed less sensitization than wild-type mice in the zymosan A model of inflammatory hyperalgesia. Thus, GlyRα3(S346A) point mutated mice closely recapitulate the phenotypes previously described for GlyR α3-deficient mice.13,27 Importantly, unaltered GlyR α3 immunoreactivity in wild-type and GlyRα3(S346A) point-mutated mice ruled out that the similarity of the phenotypes was caused by reduced expression of point-mutated GlyR α3 subunits. The results presented here hence provide compelling support for a major contribution of PKA-
dependent phosphorylation and inhibition of α3 GlyRs to centrally mediated inflammatory hyperalgesia.

The rate-limiting step in the formation of prostaglandins is the activity of prostaglandin H synthase, better known as cyclooxygenase (COX), which exist in 2 isoforms (COX-1 and COX-2). Prostaglandin formation in the CNS depends mainly on COX-2 whose spinal expression increases after peripheral inflammation.4,27,28 The rise in spinal PGE2 that occurs after peripheral insults depends mainly, if not exclusively, on the induction of COX-2 expression.4,27,28 Our results therefore allow insights into the role of spinal COX-2 and PGE2 in hyperalgesia of different etiologies. Although a contribution of COX-2 and PGE2 to inflammatory hyperalgesia is universally accepted, their role in neuropathic hyperalgesia is less clear. Some evidence suggests a role of COX-2 and PGE2 through macrophages infiltrating the peripheral injured nerve,7,22 but a contribution of spinal COX-1 or COX-2 has also been proposed.7,22

In a previous report,15 we have addressed the susceptibility to neuropathic hyperalgesia in GlyR α3-deficient mice. This study analyzed neuropathic sensitization at short intervals over a 20-day period after chronic constriction injury (CCI) surgery and found no differences in sensitization between wild-type mice and GlyR α3-deficient mice at any time point. In this study, normal development of neuropathic sensitization was observed in addition in the GlyR α3(S346A) point-mutated mice. Both studies indicate that COX-2/PGE2-evoked PKA-dependent phosphorylation of α3GlyRs is dispensable for the development of neuropathic hyperalgesia after peripheral nerve injury. This result is consistent with several previous reports that found only a minor induction of spinal COX-2 in the spared nerve injury model8 and no antihyperalgesic effect of the selective COX-2 inhibitors celecoxib and rofecoxib in rats with a spared nerve or chronic constriction injury of the sciatic nerve.7,8 It should however be noted that COX-2 may have functions different from prostaglandin production, eg, in the metabolism of endocannabinoids18; hence, antihyperalgesic effects of COX-2 inhibitors that are unrelated to the block of prostaglandin formation cannot be excluded based on the present results.29

The unaltered development of neuropathic hyperalgesia in GlyR α3-deficient and GlyR α3(S346A) point-mutated mice does not exclude that α3GlyRs still control neuropathic pain. Previous reports have in fact demonstrated that superficial dorsal horn excitatory interneurons...
are part of a circuit that is required for complex behaviors in neuropathic pain states. Although the electrophysiological recordings in 2 of our previous studies were made from unidentified lamina II neurons, in this study, targeted recordings were made from identified excitatory neurons in lamina II. The new results indicate that PGE₂-mediated inhibition of glycinerergic neurotransmission occurs in excitatory interneurons. Efficacy of GlyR modulators in rodent neuropathic pain models is supported by a study that tested a recently developed positive allosteric modulator of glycine receptors (AM-1488) and a second study that investigated dehydroxy-cannabidiol (DH-CBD), a cannabinoïd derivative with no activity at classical cannabinoïd CB1 and CB2 receptors but potentiating actions at GlyR α3 receptors.

It is tempting to speculate about implications of our findings for pain therapy and pain genetics in humans. The phenotype of the GlyRx3(S346A) point-mutated mice in models of inflammatory pain may suggest that COX inhibitors that reach COX-inhibiting concentrations in the CNS should be superior to drugs that do not penetrate the blood-brain barrier. This concept remains to be thoroughly tested in humans. On the other hand, the lack of a phenotype in GlyRx3(S346A) point-mutated and GlyRx3-deficient mice corresponds well with the low efficacy of COX inhibitors against neuropathic pain in patients.

Given that human genetic mutations can cause pronounced changes in pain sensitivity, we also felt tempted to investigate whether genetic variants exist in the human GLRA3 gene potentially recapitulating the effects of the murine Glra3 gene deletion or the S346A point mutation. The human GLRA3 (UniprotKB-entry Q100811) and the corresponding murine Glra3 (UniprotKB-entry Q01668) share 98.9% of their amino acids (459 of the 464). In the large intracellular loop spanning positions 336 to 430, the homology is 95.8% with a complete conservation of the phosphoserine sites, suggesting that at a structural and functional level, the roles of these channel is most likely conserved across the 2 species. We therefore searched the NCBI variation database (www.ncbi.nlm.nih.gov/variation/) for nonsense mutations causing a premature translational stop, for small insertion/deletion mutations that cause a shift in the reading frame, and for missense mutations in the RESR consensus site for PKA dependent phosphorylation. We found 3 missense mutations in the RESR motif (rs1179069123: S → N amino acid exchange, rs15601082476: S → R, and rs145802010; R → Q in the fourth position of the RESR consensus motif). All 3 variants are extremely rare with minor allele frequencies of 8 × 10⁻⁶ to 2 × 10⁻⁴ (www.ncbi.nlm.nih.gov/snp/). Even in the case of the most frequent variant (rs145802010), the whole human population very likely includes less than 200 persons homozygous for the R → Q mutation. Fourteen nonsense mutations (premature stop codons) and 8 frame-shift mutations were identified, of which all but 1 occurred with minor allele frequencies < 1 × 10⁻². One frame-shift mutation (rs200339054) has a minor allele frequency of 1.6 × 10⁻⁴ suggesting a frequency of homozygous carriers of 2.4 × 10⁻⁸ (equivalent to 25 homozygous carriers per 1 billion persons). Hence, mutations in the human GLRA3 gene occur with frequencies far too low for systematic analyses in clinical studies. Furthermore, given the generally mild phenotypes observed in heterozygous carriers of loss-of-function alleles, most de novo mutations in GLRA3 should not lead to apparent symptoms in humans.

An unexpected finding in the electrophysiological experiments was a change in baseline inhibitory synaptic transmission, namely an increase in gly-IPSC (and also total IPSC) amplitudes of GlyRx3(S346A) point mutant mice. Such an increase was not observed in previous experiments on GlyRx3-deficient mice. Part of this increase might be explained by the slightly (about 10%) higher open probability of S346A point-mutated α3GlyRs observed in experiments on recombinant receptors. Block of constitutive GlyR α3 phosphorylation at S346A and subsequent prevention of inhibition of glycine receptors in the point-mutated mice may also contribute. However, these 2 potential mechanisms can still not fully explain the observed changes, as an increase in the IPSC amplitude explains only half of the increase in the total IPSC amplitude, suggesting that GABA-IPSCs were also increased. At present, we can only speculate about the underlying processes. Presynaptic mechanisms might be involved. GlyRs have been found on presynaptic terminals of spinal neurons where they modulate transmitter releases through the activation of a depolarizing CI⁻ current. Because GABA and glycine are coreleased in the spinal cord from the same presynaptic vesicles, enhanced presynaptic GlyR activity might increase synaptic release of both glycine and GABA. Given the supralinear dependence of transmitter release on presynaptic depolarization and Ca²⁺ influx, a relatively small increase in GlyR activity in the S346A point-mutated mice, eg, by the mechanisms discussed above, may trigger disproportional larger increase in IPSC amplitudes. However, postsynaptic mechanisms are also possible. Phosphorylation of glycine and GABA_receptors has been shown to impact their lateral diffusion into and out of postsynaptic receptor clusters by changing their interaction with the postsynaptic scaffolding protein gephyrin. In addition, secondary activity-dependent changes in the gephyrin scaffold may alter GABA_receptor recruitment or residence time in the GlyRx3(S346A) point-mutated mice. In summary, our results provide direct evidence for a critical contribution of GlyR α3 phosphorylation to inflammatory hyperalgesia and further support α3GlyRs as targets for novel analgesics.

Conflict of interest statement

J. Gingras has been an employee of Amgen Inc during the time when the work was performed. The remaining authors have no conflicts of interest to declare.

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Supplemental video content

A video abstract associated with this article can be found at http://links.lww.com/PAIN/B308.

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