Mice lacking spinal 2GABAA receptors: Altered GABAergic neurotransmission, diminished GABAergic antihyperalgesia, and potential compensatory mechanisms preventing a hyperalgesic phenotype

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Abstract: Diminished synaptic inhibition in the superficial spinal dorsal horn contributes to exaggerated pain responses that accompany peripheral inflammation and neuropathy. 2GABAA receptors (2GABAAR) constitute the most abundant GABAAR subtype at this site and are the targets of recently identified antihyperalgesic compounds. Surprisingly, hoxb8- 2−/− mice that lack 2GABAAR from the spinal cord and peripheral sensory neurons exhibit unaltered sensitivity to acute painful stimuli and develop normal inflammatory and neuropathic hyperalgesia. Here, we provide a comprehensive analysis of GABAergic neurotransmission, of behavioral phenotypes and of possible compensatory mechanisms in hoxb8- 2−/− mice. Our results confirm that hoxb8- 2−/− mice show significantly diminished GABAergic inhibitory postsynaptic currents (IPSCs) in the superficial dorsal horn but no hyperalgesic phenotype. We also confirm that the potentiation of dorsal horn GABAergic IPSCs by the 2-preferring GABAAR modulator HZ-166 is reduced in hoxb8- 2−/− mice and that hoxb8- 2−/− mice are resistant to the analgesic effects of HZ-166. Tonic GABAergic currents, glycinergic IPSCs, and sensory afferent-evoked EPSCs did not show significant changes in hoxb8- 2−/− mice rendering a compensatory up-regulation of other GABAAR subtypes or of glycine receptors unlikely. Although expression of serotonin and of the serotonin producing enzyme tryptophan hydroxylase (TPH2) was significantly increased in the dorsal horn of hoxb8- 2−/− mice, ablation of serotonergic terminals from the lumbar spinal cord failed to unmask a nociceptive phenotype. Our results are consistent with an important contribution of 2GABAAR to spinal nociceptive control but their ablation early in development appears to activate yet-to-be identified compensatory mechanisms that protect hoxb8- 2−/− mice from hyperalgesia.

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Research report

Mice lacking spinal α2GABA$_A$ receptors: Altered GABAergic neurotransmission, diminished GABAergic antihyperalgesia, and potential compensatory mechanisms preventing a hyperalgesic phenotype

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HIGHLIGHTS

- α2 GABA$_A$Rs are the predominant GABA$_A$R isoform in the dorsal horn.
- Here, we investigated mice lacking these receptors from the spinal cord.
- We found reduced GABAergic inhibition and a lack of GABA$_A$R-mediated analgesia.
- Unexpectedly, acute nociception was not altered in these mice.
- No compensatory up-regulation of glycinergic inhibition was detected.

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ABSTRACT

Diminished synaptic inhibition in the superficial spinal dorsal horn contributes to exaggerated pain responses that accompany peripheral inflammation and neuropathy. α2GABA$_A$ receptors (α2GABA$_A$R) constitute the most abundant GABA$_A$R subtype at this site and are the targets of recently identified antihyperalgesic compounds. Surprisingly, hoxb8-α2$^{-/-}$ mice that lack α2GABA$_A$R from the spinal cord and peripheral sensory neurons exhibit unaltered sensitivity to acute painful stimuli and develop normal inflammatory and neuropathic hyperalgesia. Here, we provide a comprehensive analysis of GABAergic neurotransmission, of behavioral phenotypes and of possible compensatory mechanisms in hoxb8-α2$^{-/-}$ mice. Our results confirm that hoxb8-α2$^{-/-}$ mice show significantly diminished GABAergic inhibitory postsynaptic currents (IPSCs) in the superficial dorsal horn but no hyperalgesic phenotype. We also confirm that the potentiation of dorsal horn GABAergic IPSCs by the α2-preferring GABA$_A$R modulator HZ-166 is reduced in hoxb8-α2$^{-/-}$ mice and that hoxb8-α2$^{-/-}$ mice are resistant to the analgesic effects of HZ-166. Tonic GABAergic currents, glycinergic IPSCs, and sensory afferent-evoked EPSCs did not show significant changes in hoxb8-α2$^{-/-}$ mice rendering a compensatory up-regulation of other GABA$_A$R subtypes or of glycine receptors unlikely. Although expression of serotonin and of the serotonin producing enzyme tryptophan hydroxylase (TPH2) was significantly increased in the dorsal horn of hoxb8-α2$^{-/-}$ mice, ablation of serotonergic terminals from the lumbar spinal cord failed to unmask a nociceptive phenotype. Our results are consistent with an important contribution of α2GABA$_A$R to spinal nociceptive control but their ablation early in development appears to activate yet-to-be identified compensatory mechanisms that protect hoxb8-α2$^{-/-}$ mice from hyperalgesia.

1. Introduction

Inhibitory interneurons of the spinal dorsal horn and their fast inhibitory neurotransmitters GABA and glycine play critical roles in maintaining a physiological level of pain sensitivity. Local ablation or silencing of inhibitory dorsal horn neurons through cell type-specific expression of diphtheria toxin or tetanus toxin, evokes exaggerated responses to acute nociceptive stimulation, abnormal withdrawal

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responses to light touch, and spontaneous aversive behaviors (Foster et al., 2015). Earlier studies reported similar changes with $GABA_A$ or glycine receptor blockers injected locally into the spinal canal of rats (Beyer et al., 1985; Roberts et al., 1986; Yaksh, 1989; Sivilotti and Woolf, 1994). Work from several laboratories has shown that a loss of inhibitory neurotransmission occurs endogenously in neuropathic or inflammatory pain states (Ahmadi et al., 2002; Moore et al., 2002; Coull et al., 2003; Harvey et al., 2004; Zhang et al., 2011). A critical contribution of diminished inhibition to exaggerated pain sensitivity in inflammatory and neuropathic disease states is also supported by pharmacological studies that have demonstrated antihyperalgesic efficacy of compounds that facilitate dorsal horn inhibitory neurotransmission (Knabl et al., 2008; Huang et al., 2017) and by the antihyperalgesic efficacy of GABAergic neuron precursor transplantation into the spinal cord (Braz et al., 2012). Studies employing compounds acting on $GABA_A$Rs have pointed to a particularly relevant role for $GABA_A$Rs containing α2 subunits (α2$GABA_A$Rs), which are enriched in the superficial dorsal horn, i.e. in the termination area of nociceptive fibers (Bohlhalter et al., 1994; Bohlhalter et al., 1996; Paul et al., 2012). To demonstrate that the antihyperalgesic effect of α2$GABA_A$R modulators originates from a spinal site and does not involve supraspinal CNS areas, hoxb8-$\alpha2^{-/-}$ mice have been generated, which lack α2$GABA_A$Rs from the spinal cord up to the spinal segment C4 (Paul et al., 2014). These mice exhibited the expected reduction in $GABA_A$R-mediated membrane currents and, in line with this change, a reduced antihyperalgesia by the α2-preferring $GABA_A$R modulator HZ-166 (Rivas et al., 2009; Di Lio et al., 2011). An unexpected result of these experiments was however the absence of a pronociceptive phenotype in hoxb8-$\alpha2^{-/-}$ mice.

To gain more insight into this paradox, we performed an in-depth analysis of inhibitory GABAergic neurotransmission in the dorsal horn of hoxb8-$\alpha2^{-/-}$ mice and of potential compensatory mechanisms. These experiments confirmed a reduction in the amplitude of GABAergic IPSCs by about half and an almost complete loss of the antihyperalgesic effects of HZ-166 in hoxb8-$\alpha2^{-/-}$ mice. Our results exclude increases in glycinergic inhibition and an up-regulation of other $GABA_A$R subtypes as likely compensatory mechanisms. Although our immunohistochemical analyses revealed an increased abundance of serotoninergic markers in the dorsal horn, ablation of serotonergic terminals from the spinal dorsal horn failed to unmask a pronociceptive phenotype. Our results thus confirm a critical role of α2$GABA_A$Rs in spinal nociceptive control. However, a loss of α2$GABA_A$R expression early in development appears to be compensated by a still unknown process that prevents the development of a hyperalgesic phenotype.

2. Results

2.1. Hoxb8α2$^{-/-}$ mice lack α2$GABA_A$Rs from the spinal cord and exhibit reduced GABAergic synaptic inhibition but do not show a hyperalgesic phenotype

We first verified that α2$GABA_A$Rs were completely lost from the lumbar spinal cord of hoxb8α2$^{-/-}$ mice. To this end, we quantified expression of the $GABA_A$R α2 subunits in transverse sections of lumbar spinal cord of $\alpha2^{fl/fl}$, hoxb8α2$^{-/-}$ and global α2$^{-/-}$ mice (Fig. 1A,B). $\alpha2^{fl/fl}$ mice showed the previously observed expression of α2$GABA_A$R subunits in the dorsal horn with highest density in the superficial layers, the main termination area of nociceptive fibers. α2$GABA_A$R subunit immunoreactivity in lumbar spinal cord sections from hoxb8α2$^{-/-}$ mice was virtually indistinguishable from that of global α2$^{-/-}$ mice. This result is in line with the reported expression pattern of hoxb8-cre in spinal cord neurons and astrocytes, and in DRG neurons up to about cervical level C4 (Witschi et al., 2010). The absence of any detectable α2 subunit reactivity indicates that most, if not all, α2 subunits present in the lumbar spinal cord are expressed by intrinsic spinal neurons or peripheral sensory neurons and do not reside on axons descending from supraspinal CNS areas.

In order to assess the consequences of α2$GABA_A$R subunit ablation for spinal inhibitory neurotransmission, we combined electrophysiological whole-cell recordings and optogenetics in transverse slices of the lumbar spinal cord. We crossed the vGAT::ChR2-YFP allele (Zhao et al., 2011) into $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice. In spinal cord slices of these double and triple transgenic mice, IPSCs were robustly evoked by brief (4 ms) wide field illumination of the dorsal horn with blue light (see also Foster et al., 2015). We restricted our analyses to photocurrent negative, presumed excitatory, interneurons, which are required for the full behavioral expression of pain behaviors (Wang et al., 2013) and constitute critical elements of the dorsal circuitry involved in different forms of pathological pain (Peirs et al., 2015). The restriction of our analyses to photocurrent negative cells also allowed avoiding possible contaminations of IPSCs with photocurrents. Slices were continuously superfused with the glycine receptor antagonist strychnine (0.5 μM) to isolate the GABAergic IPSC component. GABAergic IPSC amplitudes were about 50% smaller in the hoxb8-$\alpha2^{-/-}$ mice (-151 ± 21 pA, n = 16 cells) compared to $\alpha2^{fl/fl}$ mice (-267 ± 51 pA, n = 17 cells) (Fig. 1C, D). Decay time constants (τ_{decay}) of GABAergic IPSCs showed a trend towards slower decay in neurons of hoxb8-$\alpha2^{-/-}$ mice (83.4 ± 9.0 ms) relative to $\alpha2^{fl/fl}$ mice (65.0 ± 8.2 ms) but the difference did not reach statistical significance (Fig. 1E).

We also confirmed that baseline nociceptive sensitivity was unchanged in hoxb8α2$^{-/-}$ mice. To this end, we compared the sensitivities of hoxb8α2$^{-/-}$ and $\alpha2^{fl/fl}$ mice in a battery of somatosensory and nociceptive tests including the von Frey test of light punctate mechanical sensitivity, the Hargreaves plantar test for heat sensitivity, the pin-prick test for noxious mechanical stimulation and the cold plantar test. We did not detect significant differences between hoxb8-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice in any of the four tests (Fig. 1F).

2.2. Hoxb8-α2$^{-/-}$ mice exhibit reduced potentiation of GABAergic IPSCs by HZ-166 and diminished analgesia by HZ-166

The lack of an apparent baseline nociceptive phenotype in hoxb8-α2$^{-/-}$ mice (Paul et al., 2014) contrasts with previous studies in $GABA_A$R receptor point-mutated mice that have suggested a critical role of α2$GABA_A$Rs in spinal pain control (Knabl et al., 2008). We therefore readdressed whether deletion of spinal α2$GABA_A$Rs would have an impact on the efficacy of α2-$GABA_A$R-preferring benzodiazepine site ligands with known analgesic activity. For these experiments, we chose the α2$GABA_A$R-preferring and less sedating benzodiazepine site agonist HZ-166 (Rivas et al., 2009; Di Lio et al., 2011). As a prerequisite for these experiments, we analyzed the *in vitro* pharmacological profile of HZ-166 in HEK 293 cells transiently transfected with different combinations of $GABA_A$R subunits (α1β2γ2, α2β3γ2, α3β3γ2 and α5β2γ2). In agreement with a previous publication (Rivas et al., 2009), we found that both potency and efficacy of GABA current (I_{GABA}) potentiation by HZ-166 were higher for α3$GABA_A$R and α2$GABA_A$R than for α1$GABA_A$R and α5$GABA_A$R subtypes (Fig. 2A). We then characterized the sensitivity of GABAergic IPSCs to HZ-166 in slices prepared from $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice. We used a saturating concentration of HZ-166 (10 μM) and analyzed changes in τ_{decay}. After application of HZ-166, τ_{decay} of light-evoked GABAergic currents increased by 94 ± 12% (n = 17 cells) of control values in $\alpha2^{fl/fl}$ mice. In hoxb8-$\alpha2^{-/-}$ mice, τ_{decay} increased only by 57 ± 12% (n = 13) (Fig. 2B). Finally, we employed a mouse pain model to test whether the decreased sensitivity of GABAergic IPSCs to HZ-166 would translate to diminished analgesia. In these experiments we included global $\alpha2^{R/R}$ point-mutated mice in addition to hoxb8-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice. These mice carry a histidine to arginine point mutation in the α2$GABA_A$R gene (*Gabra2*) that renders α2$GABA_A$R insensitive to most benzodiazepine site agonists including HZ-166 (Benson et al., 1998; Paul et al., 2014). For these experiments, we used the formalin model of tonic pain and injected 4%

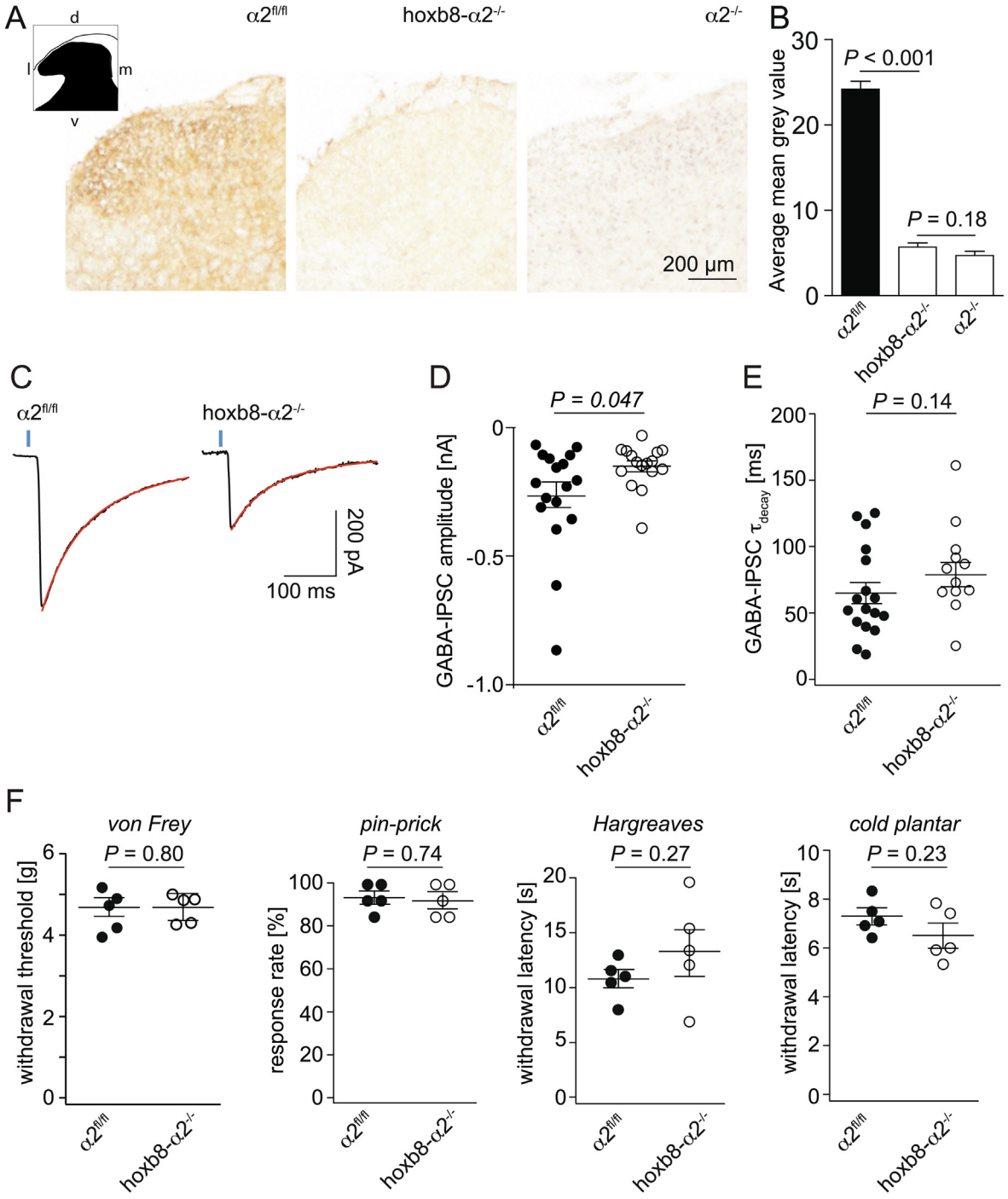


Fig. 1. Baseline morphological, electrophysiological and behavioral analysis of hoxb8-α2$^{-/-}$ mice. A-B, α2GABA$_A$R expression. A, Transverse lumbar spinal dorsal horn sections stained for GABA$_A$R α2 subunits in α2$^{fl/fl}$, hoxb8-α2$^{-/-}$ and global α2$^{-/-}$ mice. d, dorsal; v, ventral; l, lateral; m, medial. B, Quantification of immunoperoxidase intensities (n = 16 sections from 2 mice, unpaired *t*-test). C–E, GABAergic IPSCs. C, Example traces of light-evoked GABA-IPSCs (vertical blue bars represent 4 ms light pulse) recorded in the presence of strychnine (500 nM). Hoxb8-α2$^{-/-}$ mice show reduced GABAergic IPSC amplitudes compared to α2$^{fl/fl}$. Decay time constants were determined from double exponential fits, shown in red. D, Amplitudes of light-evoked GABAergic IPSCs in hoxb8-α2$^{-/-}$ and α2$^{fl/fl}$ mice (n = 17 cells from 14 mice, and n = 16 cells from 14 mice, for α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, respectively, unpaired *t*-test). Data are presented as mean ± SEM. E, Scatter plot shows no significant change in decay time constants between the two genotypes (unpaired *t*-test). F, Somatosensory and nociceptive response thresholds of naïve α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice. Each data point indicates an individual mouse. Data are presented as mean ± SEM, n = 5 mice for all genotypes, *P* values indicate α errors from unpaired t-tests.

formalin into one hind paw and examined the analgesic effect of HZ-166 in the three genotypes (Fig. 2C). HZ-166 (16 mg/kg, i.p.) significantly reduced the number of formalin-induced nocifensive reactions (assessed as time spent licking of the injected paw) in α2$^{fl/fl}$ mice, but failed to do so in hoxb8α2$^{-/-}$ and α2$^{R/R}$ mice. In mice treated with vehicle instead of HZ-166, no differences were found in the formalin-induced pain behavior between the three genotypes. These experiments confirm an important role of α2GABA$_A$Rs in dorsal horn pain control. Since these findings contrast with the apparent lack of a hyperalgesic phenotype in hoxb8-α2$^{-/-}$ mice, we next sought to identify possible

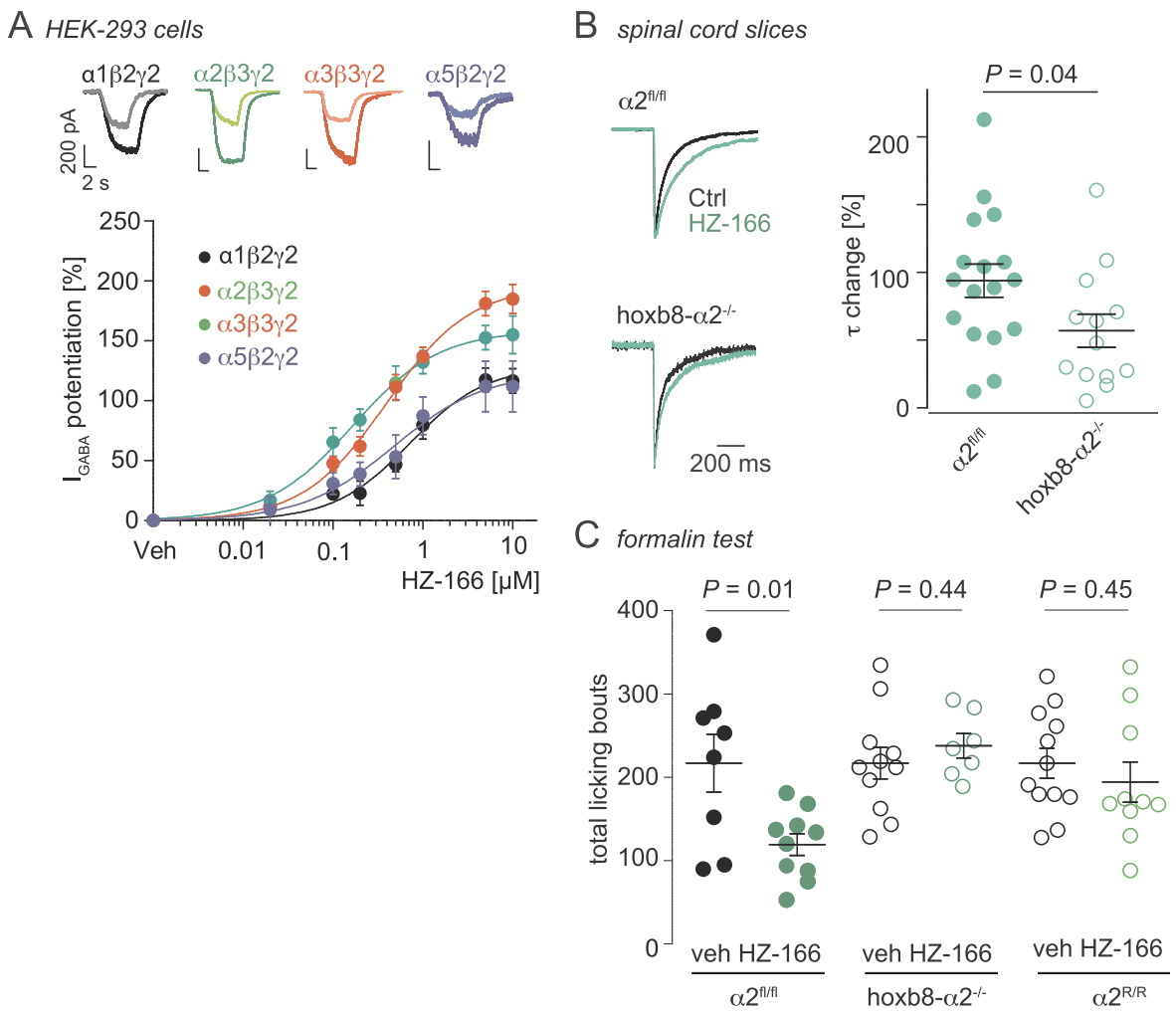


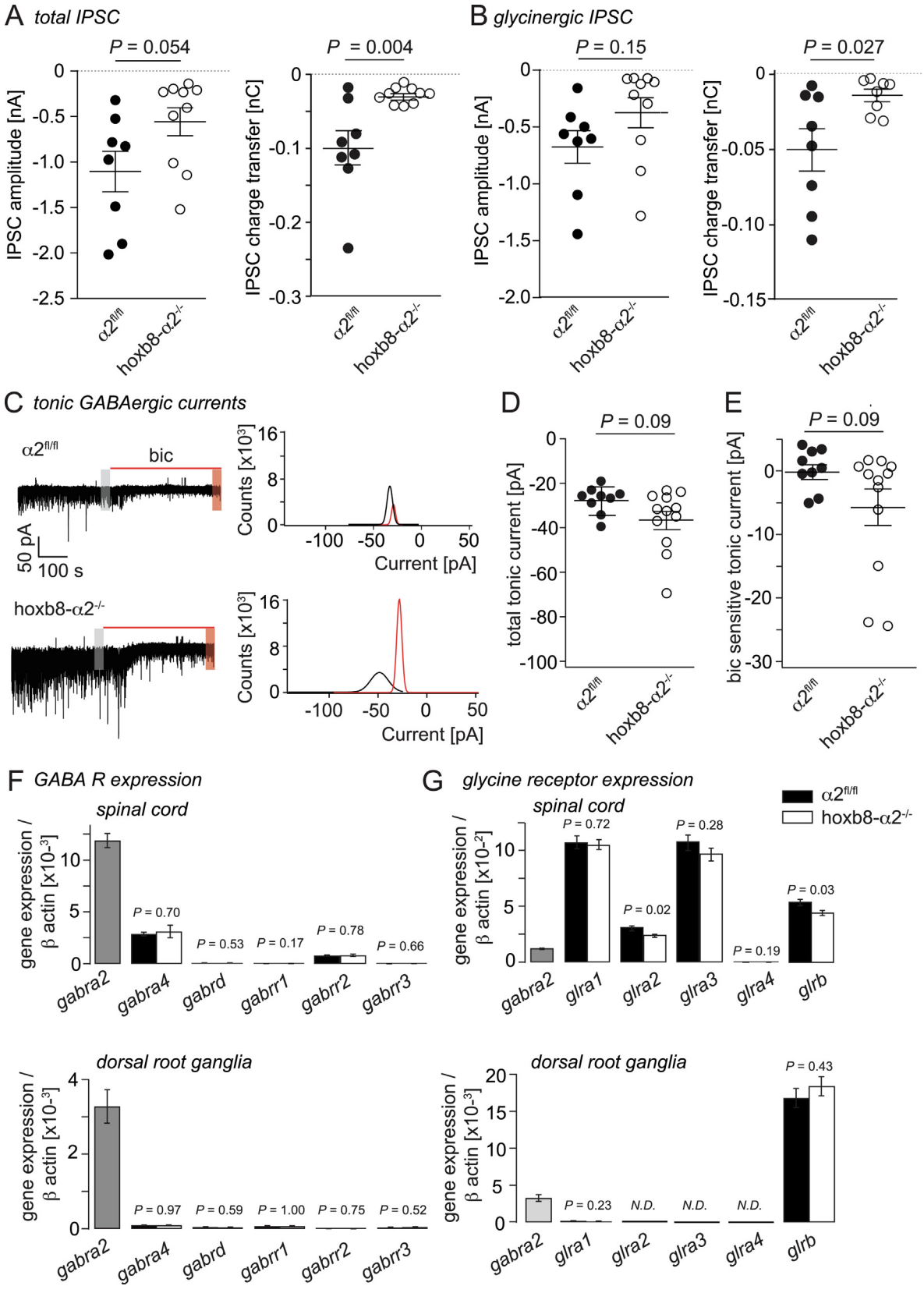
Fig. 2. Diminished analgesia by HZ-166 in hoxb8-α2$^{-/-}$ mice. A, GABA-evoked membrane currents were measured in HEK 293 cells transiently transfected with recombinant α1β2γ2, α2β3γ2, α3β3γ2 and α5β2γ2 $GABA_A$Rs. Example traces on top show current responses evoked by GABA before (light trace) and during application of saturating concentrations of HZ-166 (10 μM, dark trace). Bottom graph representing dose–response curves of the four different $GABA_A$R subtypes at increasing HZ-166 concentrations. GABA was applied at EC_{10} (1 μM, 5 μM, 8 μM, and 1 μM for α1β2γ2, α2β3γ2, α3β3γ2 and α5β2γ2 $GABA_A$Rs, respectively). B, Potentiating actions of HZ-166 on light-evoked GABAergic IPSCs recorded from superficial dorsal horn neurons of α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice. Normalized example traces recorded in the presence of strychnine (500 nM) in α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice before (black) and after (green) application of HZ-166 (10 μM). HZ-166-evoked prolongation of IPSC decay kinetics was significantly smaller in hoxb8-α2$^{-/-}$ than in α2$^{fl/fl}$ mice (n = 17 and 13, for α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, respectively, unpaired *t*-test). C, HZ-166-mediated analgesia. Total number of licking bouts evoked by subcutaneous formalin injection into one hindpaw of α2$^{fl/fl}$, hoxb8-α2$^{-/-}$ and α2$^{R/R}$ mice treated wither with HZ-166 (16 mg/kg, i.p.) or vehicle.

compensatory mechanisms explaining this apparent paradox.

2.3. Potential compensatory processes preventing a hyperalgesic phenotype in hoxb8-α2$^{-/-}$ mice

In addition to GABA, glycine is a second fast inhibitory neurotransmitter in the spinal cord. An increase in glycinergic inhibition might thus be a straightforward mechanism to compensate for diminished synaptic inhibition by GABA. We therefore compared the amplitudes and charge tansfer of the total (mixed GABAergic/glycinergic) IPSC in superficial dorsal horn neurons of hoxb8-α2$^{-/-}$ and α2$^{fl/fl}$ mice (Fig. 3A,B). In these experiments, we relied again on the analysis of light-evoked IPSCs in presumed excitatory (photocurrent-negative) neurons. After recording of the total IPSCs for 1 min, we added strychnine (500 nM) to the bath perfusion to determine (via subtraction of the remaining amplitude) the amplitude of the glycinergic component. In case of the amplitudes of light-evoked IPSCs, we found a trend towards reduced IPSC amplitudes in hoxb8-α2$^{-/-}$ mice both for the total IPSC (-1054 ± 200 pA, n = 8, versus −558 ± 154 pA, n = 10, for α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, respectively) and for its glycinergic component (-633 ± 155 pA, n = 8, versus −374 ± 133 pA, n = 10). When the total charge transfer was analyzed, a significant reduction was observed both of the total IPSC (-100 ± 24 pC, n = 8, versus -28.0 ± 3.4 pC, n = 8; *P* = 0.004, unpaired *t*-test) and for its glycinergic component (-49.6 ± 13.8 pC, n = 8, versus -14.0 ± 4.0 pC, n = 8, *P* = 0.027) clearly ruling out an increase in glycinergic synaptic currents as a compensatory process.

Although α2$GABA_A$Rs are primarily located at synapses (Sassoe-Pognetto et al., 2000), they are also found at extrasynaptic sites, where they may form a receptor reserve (Gouzer et al., 2014). $GABA_A$Rs located at extrasynaptic sites also underlie tonic membrane currents that are found in a subpopulation of dorsal horn neurons, where they are activated by ambient GABA (for review, see (Farrant and Nusser, 2005). These tonic GABAergic currents are thought to be carried by high-affinity α5$GABA_A$R or δ subunit-containing $GABA_A$Rs (Delgado-Lezama et al., 2013; Fritschy and Panzanelli, 2014). However, a possible contribution of α2$GABA_A$R to tonic currents, for example in situations of increased ambient GABA concentrations, cannot be excluded. To examine tonic membrane currents in hoxb8-α2$^{-/-}$ mice, we performed again whole-cell patch-clamp recordings from presumed excitatory



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neurons. After 5 min of baseline holding current recording, we added bicuculline (20 μM) to the bath perfusion to determine the GABAergic contribution (Fig. 3C). Neurons recorded in slices obtained from hoxb8-$\alpha2^{-/-}$ mice had on average slightly larger holding currents (-41.2 ± 4 pA, n = 12 cells) relative to neurons in slices from $\alpha2^{fl/fl}$ mice (-27 ± 2.0 pA, n = 9). This increase was likely due to larger GABAergic tonic currents as the bicuculline-sensitive component was also larger in hoxb8-$\alpha2^{-/-}$ mice (-5.8 ± 2.8 pA, n = 12) than in $\alpha2^{fl/}$

Fig. 3. No compensatory increase in glycinergic IPSCs or tonic GABAergic currents in hoxb8α2$^{-/-}$ mice. A, Amplitudes (left) and charge transfer (right) of total (mixed GABAergic/glycinergic) light-evoked IPSCs in α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice (n = 8 cells from 6 mice and n = 10 cells from 9 mice, for α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, respectively, unpaired *t*-test). B, Same as A, but for the glycinergic IPSC component (n = 8 cells from 6 mice and n = 10 cells from 9 mice, for α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, respectively). For 2 cells from the hoxb8-α2$^{-/-}$ group, no meaningful charge transfer value was obtained for the glycinergic IPSC component. C-E, tonic GABAergic currents. C, Example traces of tonic currents in α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice before and during application of bicuculline (20 μM). Grey and red bars indicate the areas that were analyzed for the all-point histograms of control and bicuculline conditions, respectively. The all-point histograms follow a Gaussian distribution and depict the amplitude scattering in a 30 s range analyzed from the current recorded in control and bicuculline conditions, respectively. D, Plot showing holding currents in baseline conditions (before application of bicuculline) in hoxb8-α2$^{-/-}$ mice (n = 7 cells) compared to α2$^{fl/fl}$ mice (n = 9 cells), unpaired *t*-test. E, same as D, but GABAergic component (after application of bicuculline) (n = 12 cells from 7 mice and n = 7 cells from 4 mice, for α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, respectively, unpaired *t*-test. F,G, Quantitative expression of mRNAs encoding for certain GABAA (F) and glycine receptor subunit genes in the spinal cord (top) and DRGs (bottom) of α2$^{fl/fl}$ mice and hoxb8-α2$^{-/-}$ mice. Expression *gabra2* (encoding for the α2 $GABA_A$ receptor subunit) in α2$^{fl/fl}$ mice is shown for comparison (grey column). Data on *gabra2* are taken from Paul et al., 2012. Expression of *glra2*, *glra3* and *glra4* in DRGs was below the detection threshold (*N.D.*, not detectable). *P* values were obtained using unpaired two-tailed unpaired *t*-test. n = 6–7 mice per group.

^{fl} mice (α2$^{fl/fl}$: −0.3 ± 1.1 pA, n = 9) (Fig. 3D,E).

To complement these functional data with data on gene expression, we performed quantitative RT-PCR of $GABA_A$ and glycine receptor subunits (Fig. 3F, G). In a previous study (Paul et al., 2012), we already reported that the expression of $GABA_A$R α1 and α3 - α6 subunits was unchanged in spinal cords and DRGs of hoxb8-α2$^{-/-}$. In the present study, we have complemented these previous results and investigated in addition the expression of the $GABA_A$R δ subunit, which together with the α4 subunit is a part of many extrasynaptic $GABA_A$Rs (Delgado-Lezama et al., 2013), of the three $GABA_A$R ρ subunits, which give rise to atypical bicuculline-insensitive $GABA_A$Rs previously also called $GABA_C$Rs (Bormann and Feigenspan, 1995), and of all five glycine receptor subunits (*glra1* - *glra4* and *glrb*). Relative to the $GABA_A$R α2 subunit, all four $GABA_A$R subunits analyzed here were expressed at much lower levels both in the spinal cord and the DRGs. No significant differences were found between α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice. In the spinal cord, all glycine receptor subunits, with the exception of the α4 subunit (*glra4*), were expressed at higher levels than the $GABA_A$R α2 subunit (*gabra2*). A minor, yet statistically significant, down-regulation in hoxb8-α2$^{-/-}$ mice was obtained for *glra2* and *glrb*. In DRGs, expression of glycine receptor subunits was below the detection limit (*glra2* – *glra4*) or very low (*glra1*). In both α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice, a relatively high expression was found for *glrb*, which is however in many tissues not translated into protein despite high mRNA levels (Weltzien et al., 2012).

In addition to mediating synaptic (phasic) and extrasynaptic (tonic) inhibition via dendritic and somatic $GABA_A$Rs, $GABA_A$Rs contribute also to presynaptic inhibition via receptors located on spinal axons and terminals of peripheral sensory neurons, including nociceptors (Rudomin and Schmidt, 1999; Willis, 1999). We therefore also investigated a possible impact of $GABA_A$R α2 subunit deletion on excitatory neurotransmission between primary sensory fibers and dorsal horn second order neurons using optogenetics for presynaptic primary afferent stimulation. Bilateral sciatic nerve injections of AAV1 ChR2-mCherry virus were made into the sciatic nerve of α2$^{fl/fl}$ and hoxb8-α2$^{-/-}$ mice. These injections led to the infection of dorsal root ganglion neurons of the lumbar segment L4 (Fig. 4A). Soma diameter analysis of the infected neurons indicated that the great majority of infected cells had a soma diameter of approximately 20 μm (Fig. 4B, n = 72 cells from 3 mice), in line with a preferred infection of nociceptive (small diameter) neurons. Seven days after virus injection, we prepared transverse spinal cord slices of the L4 segment for electrophysiological recordings from neurons in the superficial dorsal horn (laminae I and II). After establishing a whole-cell recording, we stimulated the dorsal horn with blue light (λ = 473 nm, 4 ms, power density: 20.4 mW/mm^2) and recorded light-evoked EPSCs (Fig. 4C,D). No statistically significant difference was observed between genotypes for EPSC amplitude (α2$^{fl/fl}$ mice: −108.2 ± 34.7 pA, n = 11; hoxb8-α2$^{-/-}$: −117.8 ± 41.3 pA, n = 10), decay kinetics (α2$^{fl/fl}$: 7.6 ± 1.6 ms; hoxb8-α2$^{-/-}$: 8.5 ± 0.9 ms) or success rate (α2$^{fl/fl}$: 94.2 ± 3.4%, hoxb8-α2$^{-/-}$: 99 ± 1.0%) (Fig. 4E).

2.4. Other potential compensatory mechanisms preventing a pronociceptive phenotype in hoxb8-α2$^{-/-}$ mice

Other potential mechanisms possibly counteracting a loss in GABAergic inhibition include a compensatory weakening of glutamatergic transmission within the spinal dorsal horn or a strengthening of descending inhibitory pain control. Vesicular glutamate transporters of which three isoforms (vGluT1-3) exist are marker genes of glutamatergic neurons (El Mestikawy et al., 2011). They are localized to axon terminals of glutamatergic neurons where they mediate glutamate uptake into presynaptic storage vesicles. In the spinal cord, vGluT1 expression originates almost exclusively from peripheral non-nociceptive touch-sensitive and proprioceptive sensory neurons (Oliveira et al., 2003). vGluT2 is expressed by nociceptive sensory neurons, but most vGluT2 protein in the spinal cord is found in interneurons. vGluT3 is only weakly expressed in the dorsal horn but it is present in a small band at the border between laminae II and III where it originates from vGluT3 expression in the terminals of tyrosine hydroxylase positive peripheral sensory neurons (Seal et al., 2009) and neurons descending from the brainstem (Oliveira et al., 2003). To assess potential changes in vGluT1-expression in the dorsal horn of hoxb8-α2$^{-/-}$ mice and α2$^{fl/fl}$ mice, we performed quantitative (densitometric) immunohistochemical analyses (Fig. 5). Because of the differential expression of vGluTs in the superficial and deep dorsal horn, we analyzed expression in the superficial and deep dorsal horn separately. While we did not detect differences for vGluT1 and vGluT2, there was a small but statistically significant difference in vGluT3 signal intensity in the superficial dorsal horn. Because many of the descending neurons in this area are serotonergic and co-express vGluT3 with markers of serotonergic transmission (Oliveira et al., 2003), we analyzed potential changes in the serotonergic system. We found a pronounced and highly significant increase in immunoreactivity against both serotonin and tryptophan hydroxylase 2 (TPH2) in hoxb8-α2$^{-/-}$ mice compared to α2$^{fl/fl}$ mice (Fig. 5). As serotonergic input to the dorsal horn is generally believed to contribute to endogenous pain control (Fields et al., 2006), we hypothesized that this up-regulation may constitute a compensatory mechanism possibly explaining the absence of a hyperalgesic phenotype in hoxb8-α2$^{-/-}$ mice.

2.5. Ablation of serotonergic axon terminals from the dorsal horn of hoxb8-α2$^{-/-}$ mice fails to unmask a pro-nociceptive phenotype

To examine, whether the observed up-regulation of markers of the dorsal horn serotonergic innervation underlies the absence of a pronociceptive phenotype in hoxb8-α2$^{-/-}$ mice, we pharmacologically ablated serotonergic axon terminals in the spinal cord through three intraspinal injections of 5,7 dihydroxytryptamine (DHT; 2.5 μg in 500 nl) covering the left spinal segments L4-L6 (Fig. 6). Mice were pretreated with desipramine (25 mg/kg, i.p.) to avoid ablation of noradrenergic terminals, which are also present in the spinal dorsal horn. Efficient ablation of serotonergic terminals was verified with serotonin immunostaining for each mouse analyzed in the sensory tests (Fig. 6A).

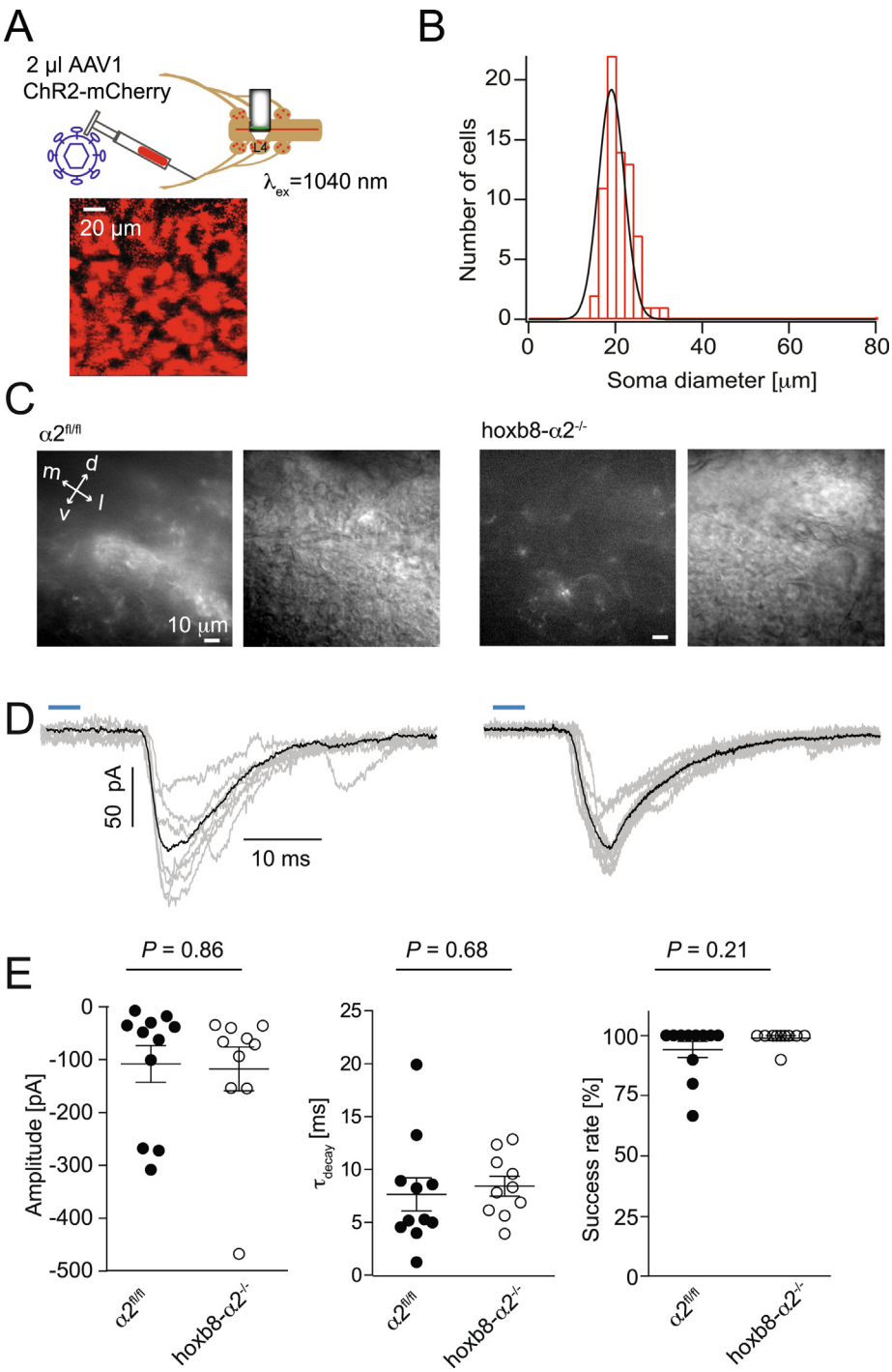

Fig. 4. Excitatory input from primary nociceptive fibers onto dorsal horn second order neurons. A, Scheme illustrating the AAV1 ChR2-mCherry injection into the sciatic nerve of $\alpha2^{fl/fl}$ or hoxb8-$\alpha2^{-/-}$ mice. Pseudo-coloured z-projection two-photon image of virus-infected ganglion cells in DRGs of lumbar segment L4. B, Histogram showing the distribution of infected dorsal root ganglion cell soma diameters which could be fitted with a Gaussian function (average diameter: 20.9 ± 0.4 µm; n = 72 cells from 3 mice). C, Epifluorescence (left) and infra-red contrast images (right) of the superficial dorsal horn in acute spinal cord slices prepared from nerve-injected mice. Epifluorescence images show punctate mCherry expression on the spinal terminals of infected dorsal root ganglion neurons. D, Example traces from the two genotypes showing the average (black) of 7 ($\alpha2^{fl/fl}$) and 10 (hoxb8-$\alpha2^{-/-}$) EPSCs (grey) after 4 ms blue light exposure (horizontal blue bars) of primary afferent terminals. E, Statistical comparisons of amplitude, success rate and decay time constants (n = 11 cells and n = 10 cells, for $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice, respectively, unpaired *t*-test).

We then tested the sensitivity of the ipsilateral hindpaw to stimulation with von Frey filaments, in the pin-prick test, the Hargreaves test and the cold plantar test, to respectively assess sensitivity to innocuous and noxious punctate mechanical stimulation, noxious heat and noxious cold stimuli (Fig. 6B). In agreement with previous work, ablation of serotonergic terminals did not sensitize naïve wild-type mice (Carr et al., 2014). However, it also failed to induce sensitization in hoxb8-$\alpha2^{-/-}$ mice.

3. Discussion

α2GABA$_A$Rs serve critical functions in the control of spinal nociceptive circuits (Zeilhofer et al., 2012b; Zeilhofer et al., 2012a). Several studies have shown that specific activation (via positive allosteric modulation) of α2GABA$_A$Rs reduces pain sensitivity in several rodent pain models (Knabl et al., 2009; Reichl et al., 2012; Ralvenius et al., 2015), and in human experimental pain models (van Amerongen et al., 2019). Based on these findings one might expect that ablation of α2GABA$_A$Rs from the spinal cord would lead to an hyperalgesic phenotype or spontaneous pain behaviors as observed after ablation or silencing of inhibitory dorsal horn neurons (Foster et al., 2015) or after spinal blockade of GABA$_A$Rs (Roberts et al., 1986; Sivilotti and Woolf, 1994). However, in a previous study, hoxb8-$\alpha2^{-/-}$ mice, which lack α2GABA$_A$Rs from the spinal cord, showed no increased sensitivity to acute noxious mechanical and thermal stimuli and no signs of spontaneous pain (Paul et al., 2014). This discrepancy may indicate the

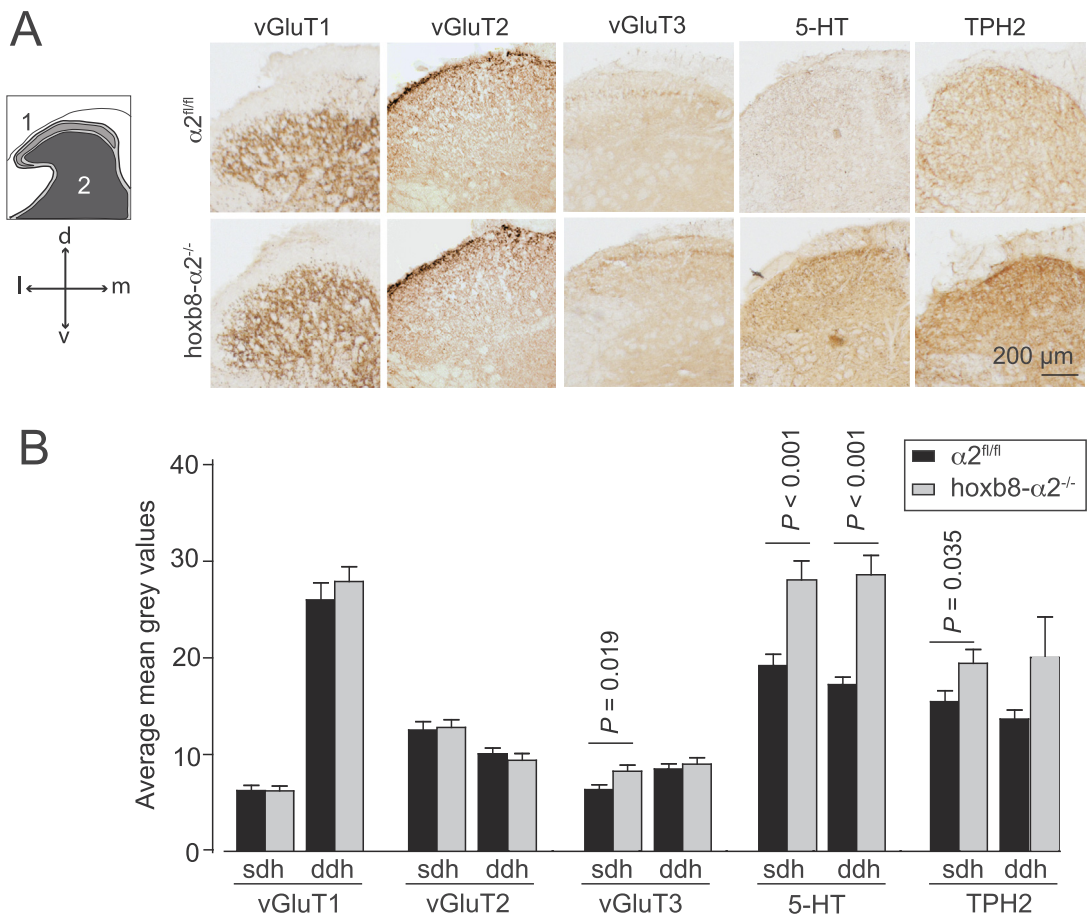

Fig. 5. vGluT1-3 and serotonergic marker expression. A, Transverse sections of lumbar spinal cord dorsal horns of $\alpha2^{fl/fl}$ and hoxb-8$\alpha2^{-/-}$ mice stained for vesicular glutamate transporter vGlT1 – vGluT3 and markers of serotonergic expression (5-HT and TPH2). 1, sdh; 2, ddh. B, Quantification of immunoperoxidase intensities in stainings for vGluT1, vGluT2, vGluT3, 5-HT, and TPH2 (in $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice, n = 4, each). Separate analyses for the superficial and deep dorsal horn (sdh, superficial dorsal horn; ddh, deep dorsal horn).

presence of compensatory mechanisms at the cellular and/or molecular level, which may counteract the reduction in GABAergic inhibition. Revealing such mechanisms might benefit the discovery of novel anti-hyperalgesic drug targets.

In the present study, we have examined the functional consequences of $GABA_AR$ α2 subunit deletion from the spinal cord on the cellular and behavioral level. We confirmed that hoxb8-$\alpha2^{-/-}$ mice show reduced synaptic inhibition in the superficial dorsal horn but nevertheless exhibit normal acute pain sensitivities. We then continued with an in-depth analysis of GABAergic neurotransmission in hoxb8-$\alpha2^{-/-}$ and corresponding wild-type ($\alpha2^{fl/fl}$) mice. Our electrophysiological recordings revealed a reduction in the amplitudes of light-evoked GABAergic IPSCs without a compensatory increase in glycinergic inhibition. In fact, the total IPSC amplitude was reduced by a similar percentage as the GABAergic IPSC component, suggesting that the loss of α2$GABA_ARs$ also reduced the glycinergic IPSC component. This might suggest the presence of presynaptic α2$GABA_ARs$ that facilitate transmitter release (Kawaguchi and Sakaba, 2017). Alternatively, the loss of $GABA_ARs$ might impair the clustering of glycine receptors at postsynaptic sites. Direct evidence for either of the two processes is however missing.

In subsequent analyses we found a trend towards *increased* amplitudes of tonic GABAergic membrane currents in hoxb8-$\alpha2^{-/-}$ mice. The absence of a pronociceptive phenotype in hoxb8-$\alpha2^{-/-}$ mice might hence be due to preserved *tonic* GABAergic inhibition mediated by extrasynaptic $GABA_ARs$. Although this explanation cannot be fully ruled out, it appears unlikely. Tonic GABAergic currents originate from the activation of extrasynaptic $GABA_ARs$ by ambient GABA. Since GABA concentrations are low outside synaptic clefts, $GABA_ARs$ mediating tonic GABAergic currents should have high affinities for GABA. Previous work has shown that α4/δ and α5$GABA_ARs$ fulfill these criteria (Delgado-Lezama et al., 2013; Fritschy and Panzanelli, 2014). It has been shown that α5$GABA_ARs$ contribute to tonic currents in the spinal dorsal horn, but α5 subunit-deficient mice exhibit normal acute nociceptive pain behavior (Perez-Sanchez et al., 2016). Similar findings have been made in case of α4$GABA_ARs$, which typically assemble with δ $GABA_AR$ subunits to form benzodiazepine-insensitive extrasynaptic $GABA_ARs$ (Storustovu and Ebert, 2006). Mice lacking these δ subunits exhibit reduced tonic GABAergic membrane currents but also show no changes in acute nociceptive sensitivity (Bonin et al., 2011). Furthermore, tonic GABAergic currents in superficial dorsal horn neurons are small and are present only in a subset of neurons (Mitchell et al., 2007) consistent with the low abundance of $GABA_AR$ α4 subunits in the spinal dorsal horn (Paul et al., 2012). These results argue against a major role of extrasynaptic $GABA_AR$ and of tonic GABAergic currents in the regulation of nociception. On the other hand, gaboxadol (also known as THIP), which enhances currents through α4/δ containing $GABA_AR$ (Adkins et al., 2001), reduced acute nociception in wild-type but not in δ subunit-deficient mice (Bonin et al., 2011). These results on acute nociception are hence reminiscent of what was observed in the present study with HZ-166 in chronic pain models.

An alternative and in our opinion more likely explanation for the absence of a pronociceptive phenotype in hoxb8-$\alpha2^{-/-}$ mice is the presence of compensatory mechanisms that counteract diminished GABAergic inhibition. In this context, it is interesting to note that hyperalgesia induced by ablation or silencing of inhibitory dorsal horn

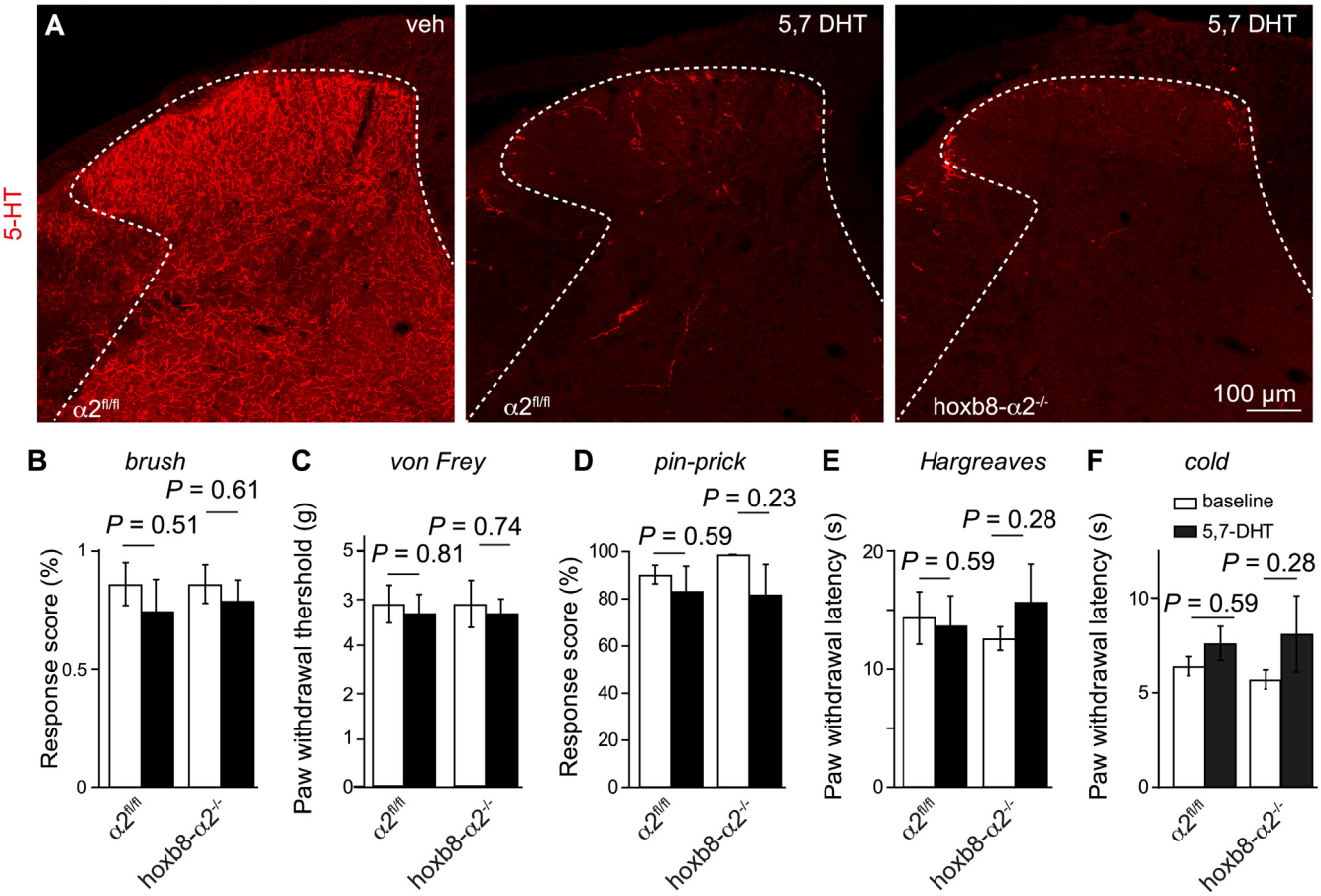

Fig. 6. Ablation of serotonergic axon terminals in the lumbar dorsal horn. A, Transverse spinal cord sections of $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice prepared on day 6 after local spinal injection of 5,7-DHT (3 injections à 500 nl of 5 µg/µl) and stained for serotonin. B-F, Somatosensory and nociceptive responses in $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice five days after 5,7-DHT injection (same doses as above; n = 7 mice per group, paired *t*-test).

neurons also shows some recovery over the course of a few weeks (Foster et al., 2015). As the reduction in GABAergic IPSC amplitudes was not compensated by an increase in glycinergic inhibition, we speculated whether the loss in inhibition might be compensated by a reduction in excitatory drive to the dorsal horn or an increased inhibitory control via descending antinociceptive fiber tracts. We did not detect a reduction in nociceptive input strength from peripheral sensory fibers onto dorsal horn second-order neurons, which is in line with unaltered expression of vGluT1 and vGluT2 protein in the dorsal horn of hoxb8-$\alpha2^{-/-}$ mice. We found however a significant up-regulation of the vGluT3 isoform which is found not only in low-threshold mechanoreceptive C fibers (Seal et al., 2009) but also serotonergic neurons of the brainstem (Domonkos et al., 2016). This drew our attention to possible changes in descending serotonergic inhibition. Serotonin is synthesized in neurons of the nucleus raphe magnus, located in the hindbrain, which is the main source of spinal 5-HT release (Hornung, 2003). Although spinally released serotonin can mediate both hyperalgesia and antinociception, depending on which subtype of serotonin receptors is activated (Yaksh and Wilson, 1979; Furst, 1999; Diniz et al., 2015), most studies support an analgesic effect of descending serotonergic input to the spinal dorsal horn. We demonstrated increased levels of serotonin and TPH2 in both the superficial and deep dorsal horn of the spinal cord in hoxb8-$\alpha2^{-/-}$ mice. These results might suggest an increased serotonergic tone in the spinal cord of hoxb8-$\alpha2^{-/-}$ mice as a possible compensatory mechanism. However, local and nearly complete ablation of serotonergic terminals from one side of the lumbar spinal cord segments L3-L5 failed to unmask a hyperalgesic phenotype in hoxb8-$\alpha2^{-/-}$ mice.

In summary, the results obtained with HZ-166 support an important contribution of α2GABA$_A$Rs to spinal nociceptive control. Their ablation early in development appears to induce an up-regulation of dorsal horn serotonergic innervation and a yet-to-be identified compensatory mechanism that protects hoxb8-$\alpha2^{-/-}$ mice from a hyperalgesic phenotype.

4. Methods and materials

4.1. Animals

All experimental procedures were approved by the Cantonal Veterinary Office (licenses 126/2012, 74/2013, 86/2013, 031/2016). Behavioral experiments were performed in $\alpha2^{fl/fl}$; hoxb8::cre double transgenic mice (short hoxb8-$\alpha2^{-/-}$ mice) and in cre-negative $\alpha2^{fl/fl}$ mice. HoxB8-cre and GABA$_A$R $\alpha2^{fl/fl}$ mice have been described previously (Witschi et al., 2010; Witschi et al., 2011). For combined electrophysiological/optogenetic experiments these two strains of mice were crossed with vGAT::ChR2 BAC transgenic mice (Zhao et al., 2011) to obtain $\alpha2^{fl/fl}$; vGAT::ChR2-YFP and $\alpha2^{fl/fl}$; hoxb8cre; vGAT::ChR2 double and triple transgenic mice. Electrophysiological/optogenetic experiments were performed in 3–4 week old mice of either sex. In immunohistochemical experiments global GABA$_A$R α2 subunit-deficient mice ($\alpha2^{-/-}$ mice) were used in addition to hoxb8-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice. We observed occasional hoxb8-cre-mediated gene recombination in the germ line. In order to avoid confounding results originating from the presence of non-conditional knock-out alleles, we performed post-hoc PCR genotyping of brain and spinal cord tissue for all mice included in our experiments. Mice were included in our analyses only when PCR genotyping of their brain tissue confirmed the

presence of a non-recombined floxed *Gabra2* allele and the absence of a recombined ("knock-out") *Gabra2* allele.

4.2. Drugs and chemicals

For electrophysiological experiments, (-)-bicuculline methochloride, strychnine hydrochloride, and HZ-166 were dissolved in extracellular solution. NBQX was dissolved in DMSO and diluted with extracellular solution to a final concentration of 20 μM in 0.1% DMSO. All chemicals except for HZ-166 were purchased from Tocris (Germany). HZ-166 was synthesized as described earlier (Rivas et al., 2009). Desipramine hydrochloride was provided by Bio-Techne AG and 5,7-dihydroxytryptamine creatinine sulphate was provided by ANAWA Trading SA.

4.3. Spinal cord slice preparation

Acute transverse 400 μm thick slices of the lumbar spinal cord were prepared from 20 to 30 day old mice of either sex. Slices were cut in ice-cold solution of the following composition in mM (Dugue et al., 2009): 130 K-gluconate, 15 KCl, 0.05 EGTA, 20 HEPES and 25 glucose titrated to pH 7.4 with KOH and supplemented with 50 μM D-APV to prevent glutamate excitotoxicity. Slices were then allowed to recover for 30 min in a solution containing (in mM) 225 D-mannitol, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.8 $CaCl_2$ and 8 $MgCl_2$ and 25 glucose (37 °C, gassed with 95% O_2, 5% CO_2). In a final step, slices were transferred to an artificial cerebrospinal fluid (aCSF), of the following composition (in mM): 120 NaCl, 2.5 KCl, 1.25 NaH_2PO4, 26 $NaHCO_3$, 5 HEPES, 1 $MgCl_2$, 2 $CaCl_2$ and 14.6 glucose. Slices were transferred to the recording chamber and continuously perfused with aCSF equilibrated with 95% O_2, 5% CO_2 at a flow rate of 1 ml/min.

4.4. Electrophysiological recordings in spinal cord slices

Patch pipettes were prepared from borosilicate glass capillaries and had an open tip resistance of 3–5 MΩ. Recording pipettes were filled with an internal solution containing (in mM): 120 CsCl, 2 $MgCl_2$, 10 HEPES, 0.05 EGTA, 2 MgATP, 0.1 NaGTP. CsCl was used to block $GABA_B$ receptor-mediated K^+ currents. QX-314 (5 mM) was added to block voltage-activated Na^+ channels in the recorded cell. Excitatory postsynaptic currents (EPSCs) were recorded using a K-gluconate-based internal solution containing (in mM): 130 K-gluconate, 20 KCl, 0.05 EGTA, 2 $MgCl_2$, 2 MgATP, 0.1 NaGTP, 10Na-Hepes, 5 QX-314. Neurons located less than 150 μm from the dorsal margin of the spinal cord (laminae I and II) were visually identified with an iXON Ultra camera (Andor Technology, Belfast, UK) equipped with infrared gradient contrast equipment (Zeiss Examiner A1, Göttingen, Germany). Whole-cell voltage-clamp recordings were performed at room temperature at a holding potential of −60 mV using a double patch-clamp EPC 9 amplifier controlled with Patchmaster acquisition software (both HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany).

All experiments on $GABA_A$R-mediated and glycine receptor-mediated currents were performed in $\alpha2^{fl/fl}$;vGAT::ChR2-YFP and $\alpha2^{fl/fl}$;hoxb8cre;vGAT::ChR2 double and triple transgenic mice. Recordings were exclusively made from presumed excitatory neurons, characterized by the absence of a blue light-induced photocurrent evoked by blue light directly applied to the cell soma using a UGA-40 GEO laser system (473 nm, 1 s, spot size 10 μm, 715.9 mW/mm^2, RAPP OptoElectronic GmbH, Hamburg, Germany). IPSCs were evoked by brief 4 ms blue light stimuli applied in wide-field mode (473 nm, field of illumination ± 200–300 μm, 20.4 mW/mm^2) with a monochromator (Polychrome V, Thermo Fisher Scientific Munich GmbH, Germany) to evoke IPSCs. IPSCs were evoked at a frequency of 4 stimulations per min using wide-field illumination of the dorsal spinal cord. The GABAergic component of the light-evoked IPSCs was isolated by bath-applied strychnine (0.5 μM). A steady-state block by strychnine was usually reached after 2–3 min of continuous application. At the end of the experiment, the $GABA_A$R antagonist bicuculline was bath-applied to verify that the recorded IPSC was exclusively mediated by $GABA_A$Rs.

During whole-cell recording, access resistance was continuously monitored using short hyperpolarizing voltage steps (-5 mV) applied at regular intervals. Recordings were discarded if the access resistance changed > 20% or if recovery to baseline currents before GABA application was less than 85–90% in the case of tonic currents. Electrical signals were sampled either at 20 kHz (light-induced IPSCs) or 5 kHz (tonic currents) and filtered at 2.9 kHz. Data was analyzed using IgorPro (WaveMetrics, Inc.,USA).

Primary afferent-evoked EPSCs were recorded from unidentified (excitatory and inhibitory) neurons in slices prepared from hoxb8-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice one week after these mice had been injected into both sciatic nerves with a non-cre dependent AAV1 expressing a ChR2-mCherry fusion protein under the chicken β-actin (CAG) promoter. Intraneural AAV injections (titer 2.96x10^{13} GC/ml, approximately 2 μl each side) were performed in 15–28 day-old mice. mCherry-expressing infected dorsal root ganglion (DRG) cells at lumbar segment L4 were visualized using a two-photon microscope for a gross morphological characterization in situ (excitation wavelength λ = 1040 nm). The soma diameter of the cells (72 cells from 3 animals) was determined from a series of projected z-stacks using the 2-axis average diameter method (Scroggs and Fox, 1992). In a few recordings, NBQX (20 μl of 20 mM stock solution) was added to the recording chamber at the end of the experiment to verify that the recorded EPSCs were mediated by AMPA receptors.

4.5. Electrophysiological recordings in HEK293 cells

The effects of HZ-166 on currents through recombinant $GABA_A$Rs were studied in HEK293 cells transiently expressing $GABA_A$Rs. HEK293 cells were transfected using lipofectamine LTX28. To ensure expression of the γ2 subunit (required for modulation of $GABA_A$Rs by benzodiazepines) in all recorded cells, we transfected cells with a plasmid expressing the γ2 subunit plus eGFP from an IRES, and only selected eGFP-positive cells for recordings. The transfection mixture contained (in μg): 1 αx, 1 β2, 3 γ2/eGFP (in case of α1 and α5) or 1 αx, 1 β3, 3 γ2/eGFP (in case of α2 and α3). Recordings were made 18–36 hrs after transfection. Whole-cell patch-clamp recordings of GABA-evoked currents were made at room temperature (20–24 °C) at a holding potential of −60 mV. Recording electrodes were filled with solution containing (in mM): 120 CsCl, 10 EGTA, 10 HEPES (pH 7.40), 4 MgCl2, 0.5 GTP and 2 ATP. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. GABA was applied to the recorded cell using a manually controlled pulse (4–6 s) of a low sub-saturating GABA concentration (EC_{10}). EC_{10} values of GABA were determined individually for all subunit combinations analyzed. EC_{50} values of HZ-166 and Hill coefficients (nH) were obtained from fits of normalized concentration–response curves to the equation $I_{GABA} = I_{max}\,[GABA]^{nH}/([GABA]^{nH} + [EC_{50}]^{nH})$. I_{max} was determined as the average maximal current elicited by a concentration of 1 mM GABA. HZ-166 was dissolved in DMSO, subsequently diluted with recording solution, and was co-applied together with GABA without preincubation.

4.6. Immunohistochemistry

Adult $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice were injected i.p with 0.25 ml pentobarbital and perfused through the ascending aorta with 50 ml ice-cold ACSF at room temperature for 2 min (Paul et al., 2012). Spinal cord and brain were then removed and fixed in cold 4% PFA for 90–120 min before being transferred to 30% sucrose in phosphate buffer saline (PBS) at 4 °C overnight for cryoprotection (Notter et al., 2014). Twenty to thirty μm thick coronal sections were made from frozen blocks and mounted onto Superfrost Plus microscope slides (Thermo Scientific, Zurich, Switzerland).

The distribution of the $GABA_AR$ α2 subunit, vesicular glutamate transporter 1–3 (vGluT1 - vGluT3), serotonin (5-HT) and tryptophan hydroxylase 2 (TPH2) was visualized on 20 μm thick lumbar spinal cord cryosections using diaminobenzidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) staining. In brief, sections from $\alpha2^{fl/fl}$ and hoxb8-$\alpha2^{-/-}$ mice were mounted on the same slide and incubated overnight at 4 °C in primary antibodies diluted in Tris triton (pH 7.4) containing 4% NGS and 0.2% Triton X-100. Sections were washed three times with PBS and incubated with the avidin–biotin complex (ABC) immunoperoxidase method according to specifications of the manufacturer. DAB hydrochloride, diluted 0.05% in Tris saline (pH 7.7) with 0.01% hydrogen peroxide was used as a chromogen. The staining reaction was carried out for 2–5 min at room temperature and stopped by transferring the sections to ice-cold buffer. Sections were air-dried, dehydrated with an ascending series of ethanol and xylene and coverslipped with Eukitt (Erne Chemie, Dallikon, Switzerland) (Paul et al., 2012). The dilutions of antibodies were: guinea-pig anti-$GABA_AR$ α2 subunit, 1:1000 (Paul et al., 2012); rabbit anti-vGluT1, 1:12′000 (Synaptic systems; RRID: AB_2336884); rabbit anti-vGluT2, 1:2000 (Synaptic systems; RRID: AB_2336885); guinea-pig anti-vGluT3, 1:3000 (Chemicon; RRID: AB_2336888); rabbit anti-5-HT antibody (ImmunoStar; RRID: AB_572263), 1:500, rabbit anti-TPH2, 1:500 (Novus Biologicals; vertraulich). ImageJ was used for quantifying the intensity of the staining. For this, ROIs were drawn in the superficial and deep spinal dorsal horn and the mean intensity was measured. Mean background intensity was subtracted from the ROI mean intensity values. Immunofluorescence staining for the detection of serotonergic axon terminals was performed on 30 μm thick spinal cord sections on glass slides using a 5-HT rabbit primary antibody (ImmunoStar; RRID: AB_572263), 1:500, and Cy3 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories; RRID: AB_2307443), 1:800.

4.7. Quantitative RT-PCR

Lumbar spinal cords and lumbar DRGs were rapidly removed from euthanized adult hoxb8-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates (n = 6–7 mice per genotype). mRNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen no.205311). Expression of $GABA_A$and glycine receptor subunits was assessed using β-actin as reference gene (for details of the assays see Witschi et al. (2011)).

4.8. Behavioral testing

All behavioral experiments were performed in 8–10 week mice of either sex during the light phase (ZT 2–9). Experiments were conducted by an experimenter blinded either to the genotype of the mice or to their treatment with drug or vehicle. Mice were randomly assigned to treatment groups. Mechanical withdrawal thresholds and thermal withdrawal latencies were assessed using an electronic von Frey anesthesiometer and Hargreaves test apparatus with a temperature controlled glass platform (30 °C) (both from IITC, Woodland Hills, CA). Responses to noxious cold were determined following the protocol by (Brenner et al., 2012) using a 5 mm thick borosilicate glass platform and applying dry ice below the paw of the animal with a 5 ml syringe. Pin-prick tests were performed using a blunt syringe that did not perforate the skin, as described in (Foster et al., 2015). Six to ten measurements were made for each time point per animal for both mechanical and heat tests. Sensitivity of the mice to chemically evoked pain was assessed in the formalin test. Formalin (4%, 20 μl) was injected subcutaneously into the dorsal surface of the left hind paw. Licking bouts of the injected paw were counted for 60 min in 5 min intervals starting immediately after formalin injection (Hösl et al., 2006). HZ-166 was suspended in 0.5% methyl cellulose and injected at a dose of 16 mg/kg body weight (Paul et al., 2014) intraperitoneally (i.p.) one hour before formalin injection. Nocifensive responses were quantified as licking bouts of the injected paw.

4.9. Serotonergic terminal ablation

Desipramine (25 mg/kg, i.p.) was administered 45 min prior to intraspinal injection of 5,7-dihydroxytryptamine (5,7-DHT; 5 μg/μl, 3 × 500 nl). 5,7- DHT was dissolved in saline, sonicated and filtered before the use. Intraspinal injections were performed in isoflurane-anesthetized mice on a motorized stereotaxic frame to target lumbar spinal cord segments L3 - L5 as previously described (Haenraets et al., 2018). von Frey, pin-prick, Hargreaves and noxious cold tests were performed on day 4 and 5 after 5,7-DHT administration (Yesilyurt et al., 2015).

4.10. Data analysis

Average amplitudes and decay time constants of light-induced IPSCs were calculated from 10 consecutive current traces in control, strychnine, or strychnine with HZ-166 conditions. Total charge transfer per IPSC (Q) was calculated by integrating an IPSC current averaged over 10 consecutive traces (I) over time (from the peak of the IPSC until the end of the recording). The resulting trace ($\int$ I(t) dt) was fitted to the double-exponential function $Q = y_0 + A_1*\exp(-k_1*t) + A_2*\exp(-k_2*t)$. The value of Q(t) for $t \rightarrow \infty$ (i.e., y_0) was used as a measure of the total charge transfer. To analyze decay kinetics of the light-evoked IPSCs, a weighted τ obtained from double exponential fits (Labrakakis et al., 2014) was determined. For tonic current measurement, an all-points histogram was plotted for a 30 s period immediately preceding drug application (i.e., baseline condition) and at the end of a 5–7 min drug application. Gaussian function was fitted to the side of the distribution not skewed by synaptic events, and the peak was used to determine the mean baseline holding current required to maintain the cell's membrane voltage at −60 mV. Tonic currents in the presence of bicuculline were determined by repeating the fitting procedure after drug application and measuring the difference in mean baseline holding currents before and after application of bicuculline. All *P* values indicate α errors obtained from paired or unpaired t-tests.

Author contributions

LT, EN and HCJ performed and analyzed the electrophysiological experiments in slices and the morphological experiments, LS performed the RT-PCR experiments. WTR performed and analyzed the behavioral experiments on the baseline nociceptive sensitivities, MAA performed and analyzed the electrophysiological experiments in HEK 293 cells, GWA did the experiments involving serotonergic terminal ablation, MP and JCC provided reagents and suggested experiments, LT, HCJ and HUZ designed research and wrote the manuscript, all authors commented on the manuscript.

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