Cellular/Molecular

Presynaptic α2-GABA$_A$ Receptors in Primary Afferent Depolarization and Spinal Pain Control

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Spinal dorsal horn GABA$_A$ receptors are found both postsynaptically on central neurons and presynaptically on axons and/or terminals of primary sensory neurons, where they mediate primary afferent depolarization (PAD) and presynaptic inhibition. Both phenomena have been studied extensively on a cellular level, but their role in sensory processing *in vivo* has remained elusive, due to inherent difficulties to selectively interfere with presynaptic receptors. Here, we address the contribution of a major subpopulation of GABA$_A$ receptors (those containing the α2 subunit) to spinal pain control in mice lacking α2-GABA$_A$ receptors specifically in primary nociceptors (*sns*-α2$^{-/-}$ mice). *sns*-α2$^{-/-}$ mice exhibited GABA$_A$ receptor currents and dorsal root potentials of normal amplitude *in vitro*, and normal response thresholds to thermal and mechanical stimulation *in vivo*, and developed normal inflammatory and neuropathic pain sensitization. However, the positive allosteric GABA$_A$ receptor modulator diazepam (DZP) had almost completely lost its potentiating effect on PAD and presynaptic inhibition *in vitro* and a major part of its spinal antihyperalgesic action against inflammatory hyperalgesia *in vivo*. Our results thus show that part of the antihyperalgesic action of spinally applied DZP occurs through facilitated activation of GABA$_A$ receptors residing on primary nociceptors.

Introduction

GABA$_A$ receptors mediate fast synaptic inhibition throughout the adult mammalian CNS. They are also densely expressed in the spinal dorsal horn (Bohlhalter et al., 1996), where they control the propagation of nociceptive signals (Roberts et al., 1986; Ishikawa et al., 2000). Diminished GABAergic and glycinergic inhibition at this site is a major factor in chronic pain syndromes (for a review, see Zeilhofer, 2008). Conversely, hyperalgesia originating from inflammatory and neuropathic diseases can be reversed by local spinal or systemic administration of benzodiazepines (BDZs) such as diazepam (DZP) (Knabl et al., 2008) and midazolam (Kontinen and Dickenson, 2000), which enhance GABA$_A$ receptor activation. GABA$_A$ receptors are heteropentameric ligand-gated ion channels, most of which are composed of α, β, and γ subunits (Olsen and Sieghart, 2008). BDZ-sensitive subtypes contain one γ2 subunit, which together with an α1, α2, α3, or α5 subunit forms the BDZ-binding site (Pritchett et al., 1989; Wieland et al., 1992). For each of these subunits, point-mutated mice have been generated that carry a histidine to arginine (H/R) substitution that destroys the DZP-sensitivity of the mutated α subunit without changing its responses to GABA (Möhler et al., 2002). Using these mice it has become possible to attribute to α2-GABA$_A$ receptors most of the antihyperalgesic effect of spinal DZP (Knabl et al., 2008).

In the spinal cord, α2-GABA$_A$ receptors are densely expressed in the superficial layers of the dorsal horn, the main termination area of primary nociceptors (Bohlhalter et al., 1996). At this site, α2-GABA$_A$ receptors are found not only postsynaptically on central neurons, where they cause classical hyperpolarization, but most likely also presynaptically on the terminals of primary sensory neurons [discussed in Persohn et al. (1991) and Bohlhalter et al. (1996)]. These terminals are depolarized by GABA$_A$ receptors (Labrakakis et al., 2003), because their intracellular chloride concentration is kept above electrochemical equilibrium by the chloride importer NKCC1 (Alvarez-Leefmans, 2009). This primary afferent depolarization (PAD) causes presynaptic inhibition, i.e., a reduction in synaptic glutamate release, possibly through inactivation of presynaptic sodium channels and/or through activation of a shunting conductance, both of which can result in inhibition of action potential propagation into presynaptic terminals (Kullmann et al., 2005). Both processes will result in reduction of nociceptive input to the spinal cord. However, if PAD

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Table 1. Genotypes and cell type-specific phenotypes of the mouse lines analyzed

Genotype			*Gabra2* phenotype		
Gabra2 allele 1	*Gabra2* allele 2	*sns*-cre transgene	Primary nociceptors	All other cell types	Designation (short)
fl	fl	−	wt/wt	wt/wt	$\alpha2^{fl/fl}$_*sns*−cre^{tg-} ($\alpha2^{fl/fl}$)
fl	fl	+	−/−	wt/wt	$\alpha2^{fl/fl}$_*sns*−cre^{tg+} (sns−$\alpha2^{-/-}$)
fl	H	−	wt/wt	wt/wt	$\alpha2^{fl/H}$_*sns*−cre^{tg-} ($\alpha2^{fl/H}$)
fl	H	+	−/wt	wt/wt	$\alpha2^{fl/H}$_*sns*−cre^{tg+} (sns−$\alpha2^{-/H}$)
fl	R	−	wt/R	wt/R	$\alpha2^{fl/R}$_*sns*−cre^{tg-} ($\alpha2^{fl/R}$)
fl	R	+	−/R	wt/R	$\alpha2^{fl/R}$_*sns*−cre^{tg+} (*sns*−$\alpha2^{-/R}$)
R	R	−	R/R	R/R	($\alpha2^{R/R}$)
−	−	−	−/−	−/−	($\alpha2^{-/-}$)

Phenotypically, floxed (fl) alleles are regarded as wild type (wt) in the absence of *cre*.

Table 2. qRT-PCR (Taqman) assays used to quantify $GABA_A$ receptor α subunit expression

Assay ID	Context sequence (including the probe sequence)	Gene
Mm00607939_s1	CTGTTACTGAGCTGCGTTTTACACC	*Actb*
Mm00439046_m1	TTCCAGAAAAGCCAAAGAAAGTAAA	*Gabra1*
Mm00433435_m1[a]	TATATACCATGAGGCTTACAGTCCA	*Gabra2*
Mm00433440_m1	AGTGACTGTGACACTCGATCTCACA	*Gabra3*
Mm00802631_m1	GAAACATCCCTTCAGAATACACATG	*Gabra4*
Mm00621092_m1	ACACCATGCGTCTGACAATCTCTGC	*Gabra5*
Mm01227754_m1	CCAGGATTTGGGGGTGCTGTAACAG	*Gabra6*
Mm01266203_g1	TCTCAGAGGCAAACATGGAATACAC	*Gabrd*
Mm00489932_m1	CCAGACATGGAATATTCCATTGACA	*Gabre*
Mm01193033_m1	GTAACATGGACTACACAGCCACTAT	*Gabrp*
Mm00445057_m1	AGCAAATGTGCAGGATGGCCTGATT	*Gabrq*
Mm00433499_m1	GCAAGGCAGCCCAATCCTGAGACGA	*Gabrr1*
Mm00433507_m1	TCCAAGCCAAGCCATTTGTATAAAA	*Gabrr2*
Mm01344096_m1	GTTTCCCTGGGGATCACGACGGTGC	*Gabrr3*

[a]This assay amplifies a gene segment including the floxed *gabra2* exon 5 and, therefore, does not yield a PCR product from chromosomes that have undergone *cre*-mediated excision of exon 5.

becomes sufficiently strong to trigger action potentials, it may also elicit so-called dorsal root reflexes and exaggerate pain and neurogenic inflammation (Willis, 1999).

The contribution of PAD to the processing of nociceptive signals and to the antihyperalgesic effect $GABA_A$ receptor modulators is unknown, mainly due to the lack of suitable tools for the specific targeting of presynaptic $GABA_A$ receptors. Here, we used a genetic approach and investigated conditional nociceptor-specific α2-$GABA_A$ receptor-deficient and point-mutated mice in morphological, electrophysiological, and behavioral experiments. Deletion of the α2-$GABA_A$ receptor in nociceptive primary afferents reduced DZP sensitivity of GABAergic membrane currents in nociceptive dorsal root ganglion (DRG) neurons and $GABA_A$ receptor-mediated presynaptic inhibition, and led to a reduction in the antihyperalgesic effect of spinal DZP.

Materials and Methods

Mice. To generate a floxed *Gabra2* allele, a 6.3 kb PstI-NcoI genomic fragment containing exons 5 (221 bp) and 6 (83 bp), together with 2 SphI sites, was isolated. The 1 kb SphI-SphI fragment was removed from the 6.3 kb PstI-NcoI fragment and replaced by an oligo hybrid containing a *lox*P site with adjacent KpnI and SalI sites, recreating a single SphI site, into which the 1 kb SphI-SphI fragment containing exon 5 was reinserted. A neomycine resistance cassette (FRT-Pol2-neo-bpA-FRT-*lox*P) was then subcloned into the SalI site. The vector was linearized at the 5′ end of the genomic homology at a NotI site and electroporated into embryonic stem (ES) cells (C57BL/6N, Eurogentec). Clones harboring a single targeting event (see Fig. 1*A*, "targeted allele") were injected into blastocysts (Polygene). The neomycine resistance cassette was bred out using ACTFlpe mice (Jackson Laboratories) to obtain the floxed allele (Gabra2$^{tm2.1Uru}$). Floxed mice were crossed with *EIIa*-cre mice (Jackson Laboratories) to obtain *Gabra2* global knock-out mice (allele designated Gabra2$^{tm2.2Uru}$). Nociceptor-specific *sns*-$\alpha2^{-/-}$ mice and *sns*-$\alpha2^{-/R}$ point-mutated mice were generated from *sns*-cre transgenic mice (Agarwal et al., 2004) crossed with $\alpha2^{fl/fl}$ and/or $\alpha2^{R/R}$ mice (Löw et al., 2000) (for the designations of the different genotypes, see Table 1). All mice were maintained on a C57BL/6J background.

mRNA quantification. Four to six lumbar DRGs, lumbar spinal cords, and cerebral cortices were rapidly removed from killed adult *sns*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates, as well as from global $\alpha2^{-/-}$ mice. mRNA expression of $GABA_A$ receptor subunits was quantified with quantitative reverse transcriptase PCR (qRT-PCR) using β-actin as reference gene (for Taqman assays, see Table 2).

Morphology. Lumbar spinal cords prepared from 6–8-week-old *sns*-$\alpha2^{-/-}$ mice and $\alpha2^{fl/fl}$ littermates were cut into 300-μm-thick parasagittal slices, fixed in 4% paraformaldehyde for 10 min, and subsequently cut into 14-μm-thick sections using a cryostat. Immunofluorescence stainings were made to study the colocalization of $GABA_A$ receptor α2 and α3 subunits using guinea pig affinity purified antisera [guinea pig affinity purified antisera (Knabl et al., 2008)] with markers of primary afferent nociceptive fibers (CGRP and IB4). A polyclonal rabbit antiserum against CGRP (Millipore Bioscience Research Reagents, cat. no. AB 15360) and an IB4-Alexa 488 conjugate (Invitrogen, cat. no. 121411) were used to label spinal axons and terminals of peptidergic and nonpeptidergic nociceptors, respectively. Thick myelinated (non-nociceptive) fiber terminals were labeled with a rabbit antiserum against VGluT1 (Synaptic Systems). High-resolution confocal images were processed and analyzed with Imaris (Bitplane) software. Double-immunofluorescence staining was visualized by confocal microscopy (Zeiss LSM-710 Meta) using a 63× Plan-Apochromat objective (NA 1.4). The pinhole was set to 1 Airy unit for each channel and separate color channels were acquired sequentially. The acquisition settings were adjusted to cover the entire dynamic range of the photomultipliers. High-resolution confocal images were processed and analyzed with Imaris (Bitplane) with minimal adjustments of contrast and brightness. Images from both channels were overlaid (maximal intensity projection) and background was subtracted, when necessary. A low-pass "edge-preserving" filter was used for images displaying α2 or α3 staining. Colocalization of α2-subunit immunoreactivity with primary afferent terminals was quantified from single confocal sections (1024 × 1024 pixels) at a magnification of 78 nm/pixel in 8-bit grayscale images, using a threshold segmentation algorithm (minimal intensity, 90–130; size 0.08–0.8 μm^2). Colocalizations were counted in six fields per slide each from a different mouse. Three mice per genotype were analyzed. Colocalizations were considered to be true only if (1) the α-subunit staining appeared completely inside the primary afferent staining, (2) covered an area > 0.057 μm^2, and (3) the colocalization was visible in the previous and next images of the Z-stack.

Electrophysiology. Whole-cell patch-clamp recordings were made at room temperature from acutely isolated nociceptive DRG neurons and from superficial dorsal horn neurons. DRG neurons were prepared from 3–4-week-old mice (Knabl et al., 2008). Nociceptive DRG neurons were identified by the presence of Na^+ currents resistant to tetrodotoxin (TTX) (0.3 μM) and exhibiting pronounced reduction in amplitudes during repetitive (5 Hz) depolarizations for 30 ms to 0 mV (Blair and Bean, 2003). Transverse spinal cord slices with short dorsal roots attached were prepared from 2–3-week-old mice (Ahmadi et al., 2002). Dorsal roots were stimulated electrically (duration ≥100 μs; 17–70 V) at a frequency of 0.07 Hz to elicit primary afferent-evoked EPSCs. Dorsal root potential (DRP) recordings were made from isolated spinal cords of 18–27-d-old mice at 28.5°C (Martinez-Gomez and Lopez-Garcia, 2005). Dorsal roots S2 or S3 were stimulated and the cranially adjacent root was recorded. Suction electrodes were used for both stimulation and recording.

Behavior. Experiments were done in 7–10-week-old mice. Care was taken to ensure equal numbers of male and female mice in all experiments. Inflammatory and neuropathic pain induction, thermal and mechanical testing, and intrathecal injections, i.e., injections into the subarachnoid space of the spinal canal, of DZP and vehicle were done as

described previously (Knabl et al., 2008). Capsaicin was dissolved in Tween 80 (10%), ethanol (10%), and saline (80%). Permission for the animal experiments was obtained from the Veterinäramt des Kantons Zürich (ref. no. 121/2006 and 34/2007).

Results

Nociceptor-specific α2-$GABA_A$ receptor-deficient mice

Conditional nociceptor-specific α2-$GABA_A$ receptor-deficient mice ($\alpha2^{fl/fl}$_*sns*-cre^{tg+}; short *sns*-$\alpha2^{-/-}$ mice) were generated by crossing mice carrying a floxed α2-$GABA_A$ receptor (*Gabra2*) gene (Fig. 1*A*) to transgenic mice expressing the *cre* recombinase under the transcriptional control of the sensory neuron-specific sodium channel (*sns*) gene (Agarwal et al., 2004). To quantify changes in $GABA_A$ receptor α2 subunit expression and to test for possible compensatory upregulations or downregulations of other BDZ-sensitive $GABA_A$ receptor subunits, we used qRT-PCR in lumbar dorsal root ganglia and spinal cords and in cerebral cortices. Compared with $\alpha2^{fl/fl}$ mice, *sns*-$\alpha2^{-/-}$ mice showed pronounced reduction in $GABA_A$ receptor α2 mRNA subunit copy numbers with no significant changes in the spinal cord or cerebral cortex (Fig. 1*B*). The expression of the other BDZ-sensitive $GABA_A$ receptor subunits was not significantly changed in DRGs of *sns*-$\alpha2^{-/-}$ mice (Fig. 1*C*). We also analyzed possible changes in the expression of the BDZ-insensitive $GABA_A$ receptor subunits α4, α6, δ, ε, π, θ, and ρ1-ρ3 (Table 3). Transcripts encoding for six of these subunits (α4, δ, ε, θ, ρ1, and ρ3) were reliably detected in DRGs of both $\alpha2^{fl/fl}$ and *sns*-$\alpha2^{-/-}$ mice. mRNA encoding for the α4 subunit was significantly upregulated in *sns*-$\alpha2^{-/-}$ mice by 44.5 ± 9.5% (mean ± SEM). Upregulations by between 20 and 40% were also found for δ, θ, and ρ1, but these did not reach statistical significance.

No detectable levels of α2 subunit mRNA were found in global $\alpha2^{-/-}$ mice [generated from $\alpha2^{fl/fl}$ mice crossed to *EIIa*-cre mice (Lakso et al., 1996)], verifying the specificity of the assay and suggesting that the α2 mRNA remaining in DRGs of *sns*-$\alpha2^{-/-}$ mice derived most likely from non-nociceptive (*sns*-cre negative) DRG neurons.

α2-$GABA_A$ receptors expressed in spinal terminals of primary afferent sensory fibers

High-resolution confocal microscopy was used in parasagittal sections of the lumbar spinal cord to quantify the expression of α2-$GABA_A$ receptors in the three major subpopulations of primary afferent fibers. Peptidergic and nonpeptidergic nociceptive fiber axons and terminals were labeled with antiserum against calcitonin gene-related peptide (CGRP) and with a fluorescent isolectin B4 (IB4) conjugate, respectively, while non-nociceptive fiber terminals were labeled with an antiserum against the vesicular glutamate transporter 1 (VGluT1), which is in the dorsal horn selectively expressed by thick myelinated (non-nociceptive) primary afferent fiber terminals (Todd et al., 2003). All sections were counterstained with an antiserum against the $GABA_A$ receptor α2 subunit (Fig. 2*A*,*B*). In the major termination area of nociceptive fibers (laminae I and II of the spinal dorsal horn),

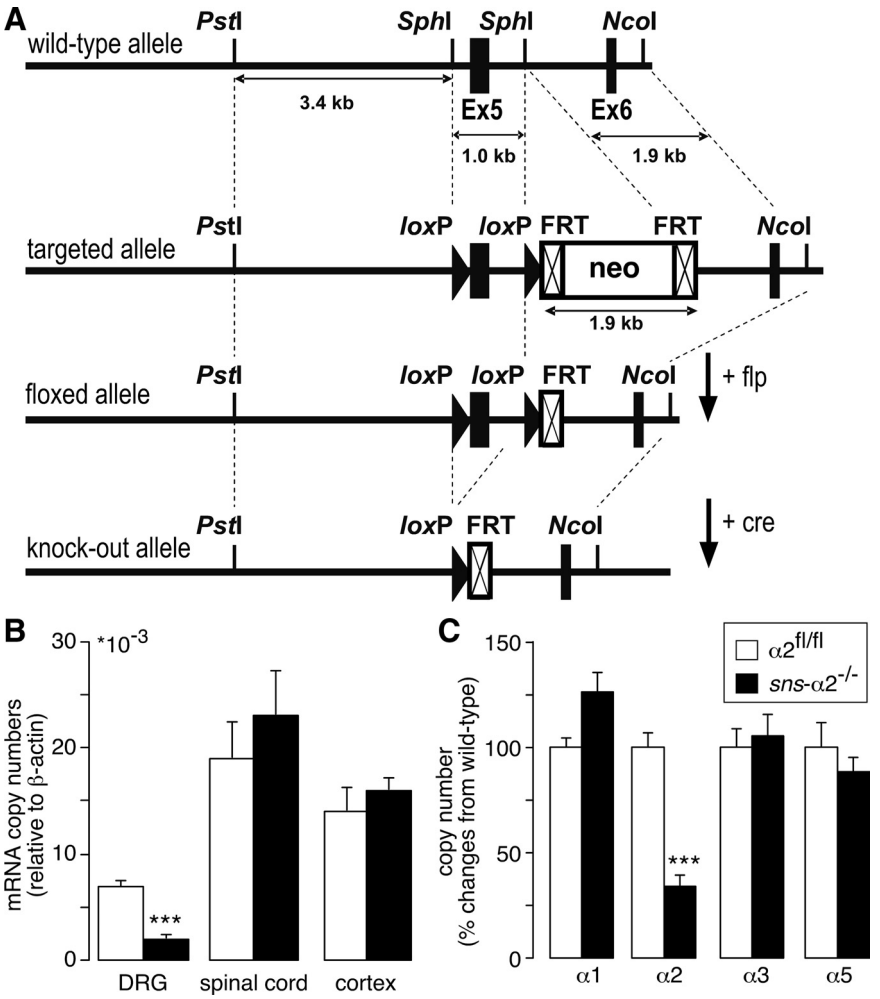

Figure 1. Generation of $GABA_A$ receptor $\alpha2^{fl/fl}$ mice and qRT-PCR analyses. ***A***, Generation of mice carrying a floxed *Gabra2* allele. For details, see Materials and Methods. ***B***, Quantification (mean ± SEM) of *Gabra2* transcript numbers (relative to β-actin) in lumbar DRGs, spinal cords, and cerebral cortices of *sns*-$\alpha2^{-/-}$ mice ($n = 7$) and wild-type ($\alpha2^{fl/fl}$) littermates ($n = 9$) with qRT-PCR. ***C***, Quantification of *gabra1*, *Gabra2*, *Gabra3*, and *Gabra5* gene transcripts (encoding for the BDZ-sensitive subunits α1, α2, α3, and α5) in the DRGs of *sns*-$\alpha2^{-/-}$ mice and wild-type ($\alpha2^{fl/fl}$) littermates. ***$p \leq 0.001$. Statistical comparisons between wild-type and *sns*-$\alpha2^{-/-}$ were made with unpaired *t* tests followed by Bonferroni corrections for three (***B***) and four (***C***) independent comparisons.

Table 3. Changes in gene expression in *sns*-$\alpha2^{-/-}$ mice compared to $\alpha2^{fl/fl}$ mice

$GABA_A$ receptor subunit (gene)	Expression relative to β-actin in $\alpha2^{fl/fl}$ mice (mean ± SEM)	Expression ratio (*sns*-$\alpha2^{-/-}/\alpha2^{fl/fl}$) (mean ± SEM)	*P*
α4 (*Gabra4*)	$7.6 \pm 0.34 \times 10^{-5}$	1.45 ± 0.25	0.004
α6 (*Gabra6*)	n.d.		
δ (*Gabrd*)	$2.9 \pm 0.45 \times 10^{-4}$	1.41 ± 0.45	n.s.
ε (*Gabre*)	$2.2 \pm 0.21 \times 10^{-4}$	1.10 ± 0.26	n.s.
π (*Gabrp*)	n.d.		
θ (*Gabrq*)	$2.5 \pm 0.41 \times 10^{-4}$	1.37 ± 0.29	n.s.
ρ1 (*Gabrr1*)	$2.6 \pm 0.39 \times 10^{-4}$	1.23 ± 0.14	n.s.
ρ2 (*Gabrr2*)	n.d.		
ρ3 (*Gabrr3*)	$1.8 \pm 0.33 \times 10^{-4}$	0.90 ± 0.11	n.s.

Gene expression for both genotypes was first calculated relative to β-actin expression, and then compared between *sns*-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice. n.d., Not detectable. α6 transcripts were not detectable in any of the samples, and transcripts for π and ρ2 were only found in two out of 16 samples. *P*, Significance calculated by ANOVA followed by Bonferroni correction for six independent samples. n.s., Not significant after Bonferroni correction. Number of mice, $n = 8$ and 7, for $\alpha2^{fl/fl}$ and *sns*-$\alpha2^{-/-}$, respectively.

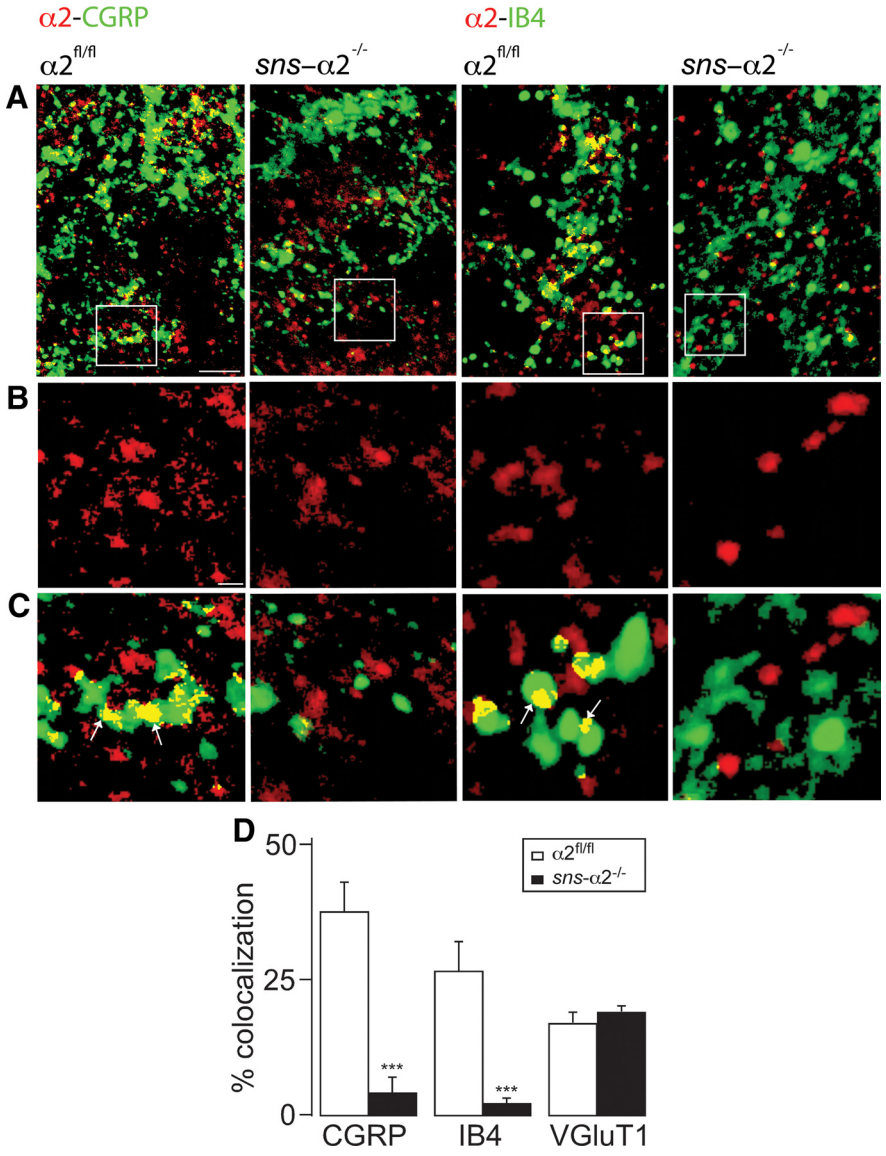

Figure 2. $\alpha 2$-$GABA_A$ receptors in the spinal dorsal horn. ***A***, Colocalization of $\alpha 2$-$GABA_A$ receptors (red) with peptidergic (CGRP-positive, lamina II outer) and nonpeptidergic (IB4-positive, lamina II inner) axons and terminals (green) in parasagittal sections of lumbar spinal cord of adult wild-type ($\alpha 2^{fl/fl}$) and *sns*-$\alpha 2^{-/-}$ mice. ***B***, ***C***, Higher magnification of the areas indicated in ***A*** showing the $\alpha 2$-subunit immunoreactivity alone (***B***) or superimposed with colocalized pixels (yellow, ***C***). Arrows in ***C*** point to the terminals containing the an $\alpha 2$-$GABA_A$ receptor. ***B***, $\alpha 2$-$GABA_A$ receptor immunoreactivity. ***C***, Colocalization (indicated by arrows). ***D***, Statistical analysis. Percentage colocalization (mean ± SD) of CGRP- (lamina IIo), IB4- (lamina IIi), and VGluT1- (lamina III) positive axons and terminals with $\alpha 2$-$GABA_A$ receptors. Colocalizations (for criteria, see Materials and Methods) were counted in six fields per slide each from a different mouse. Three mice per genotype were analyzed. ANOVA followed by Bonferroni *post hoc* test $F_{(5,12)} = 47.0$; $^{***}p \leq 0.001$. Scale bars: ***A***, 5 μm; ***B***, ***C***, 0.5 μm (scale bar only shown in ***B***).

approximately one third and one fourth of CGRP- and IB4-positive structures stained also positive for $\alpha 2$-$GABA_A$ receptors in wild-type ($\alpha 2^{fl/fl}$) mice. These colocalizations were virtually absent in *sns*-$\alpha 2^{-/-}$ mice (Fig. 2*C*,*D*). As expected, VGluT1-positive structures were mainly located in the deep dorsal horn (lamina III and deeper). They also showed a considerable but lower degree of colocalization with $\alpha 2$-$GABA_A$ receptors, which was unchanged in *sns*-$\alpha 2^{-/-}$ mice. We also found a significant expression of $\alpha 3$-$GABA_A$ receptors in all three types of primary afferent fibers (52 ± 12%, 41 ± 16%, 27 ± 4% (mean ± SD) with CGRP, IB4, and VGluT1, respectively). The distribution of $\alpha 3$-$GABA_A$ receptors was not altered in *sns*-$\alpha 2^{-/-}$ mice.

Electrophysiological analysis of *sns*-$\alpha 2^{-/-}$ mice

To analyze functional consequences of *sns*-$\alpha 2$ gene deletion at the cellular level, we first made whole-cell recordings from acutely isolated nociceptive DRG neurons identified by the presence of TTX-resistant Na^+ currents with pronounced use-dependent inactivation upon repetitive stimulation (Pearce and Duchen, 1994; Arbuckle and Docherty, 1995; Blair and Bean, 2003). Amplitudes of GABAergic membrane currents evoked by exogenous application of muscimol remained unchanged in *sns*-$\alpha 2^{-/-}$ mice, but their facilitation by DZP (1 μM) was significantly reduced (Fig. 3*A*).

We next analyzed the modulation of primary afferent-evoked synaptic transmission by presynaptic $GABA_A$ receptors in transverse spinal cord slices. AMPA receptor-mediated EPSCs were evoked by electrical stimulation of attached dorsal rootlets and recorded from visually identified superficial (laminae I/II) dorsal horn neurons. Electrical stimulation thresholds of AMPA-EPSCs were virtually identical in wild-type and *sns*-$\alpha 2^{-/-}$ mice [32.4 ± 2.9 V ($n = 17$) and 34.1 ± 2.8 V ($n = 10$), means ± SEM]. In the absence of muscimol or DZP, the vast majority of AMPA-EPSCs were reliably triggered by dorsal root stimulation and occurred with constant latencies. They, therefore, most likely represented monosynaptic events. After a stable AMPA-EPSC was established, slices were superfused with different concentrations of muscimol to activate $GABA_A$ receptors. To avoid confounding effects arising from activation of postsynaptic $GABA_A$ receptors, we replaced in the intracellular recording solution Cl^- with F^- (Turecek and Trussell, 2001), which does not permeate $GABA_A$ receptor channels (Bormann et al., 1987). AMPA-EPSC amplitudes were not significantly different between *sns*-$\alpha 2^{-/-}$ mice and $\alpha 2^{fl/fl}$ littermates, and were similarly decreased with the $GABA_A$ receptor agonist muscimol in both genotypes (Fig. 3*B*). However, when DZP (1 μM) was applied in addition to a low concentration (0.1 μM) of muscimol, the rate of successful transmissions (i.e., of presynaptic stimulations eliciting EPSCs) dropped significantly in $\alpha 2^{fl/fl}$ mice as expected for a presynaptic site of action. This increased inhibition was not observed in *sns*-$\alpha 2^{-/-}$ mice (Fig. 3*C*).

The functioning of $GABA_A$ receptors on the presynaptic terminals of primary nociceptors was also assessed through the analysis of DRPs. These are local field potentials generated by GABAergic interneurons and occurring in one dorsal root after electrical stimulation of another dorsal root in a neighboring segment. We compared DRPs of *sns*-$\alpha 2^{-/-}$ and $\alpha 2^{fl/fl}$ mice in terms of amplitude, sensitivity to the $GABA_A$ receptor blocker

bicuculline, and DZP sensitivity. DRPs of *sns*-$\alpha2^{-/-}$ mice were of similar size and similarly sensitive to bicuculline (1 and 3 μM), but their potentiation by DZP was strongly reduced (0.3–3 μM) (Fig. 4).

Acute nociception and inflammatory and neuropathic hyperalgesia in *sns*-$\alpha2^{-/-}$ mice

Before analyzing conditional $\alpha2$-$GABA_A$ receptor mutant mice, we verified that the presence of the *sns*-cre transgene alone did not affect the development of hyperalgesia or the responsiveness of mice to DZP. *sns*-cre mice with no mutations in the *Gabra2* gene developed normal hyperalgesia and responded normally to spinal DZP (Table 4). We then continue with the analysis of *sns*-$\alpha2^{-/-}$ mice. These mice responded normally to acute noxious heat and to mechanical stimulation with von Frey filaments, and exhibited normal nociceptive responses (flinches) after chemical activation of nociceptors through subcutaneous capsaicin injection into one hindpaw (Table 5). When tested in an inflammatory pain model (subcutaneous injection of the yeast extract zymosan A into one hindpaw), *sns*-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice developed virtually identical thermal and mechanical hyperalgesia and similar paw swelling (Fig. 5*A–C*). Likewise, *sns*-$\alpha2^{-/-}$ and $\alpha2^{fl/fl}$ mice responded with nearly identical thermal and mechanical hyperalgesia after chronic constriction injury (CCI) of the left sciatic nerve (Fig. 5*D*,*E*), and developed unchanged mechanical hyperalgesia after subcutaneous capsaicin injection (Fig. 5*F*).

In separate experiments, we assessed the consequences of *sns*-$\alpha2$ gene deletion for the antihyperalgesic effects of spinal DZP in inflammatory and neuropathic pain. DZP [0.09 mg/kg body weight, compare with Knabl et al. (2008)] was injected intrathecally at the level of the lower lumbar spine. Injections were made 2 d after zymosan A injection and 7 d after CCI surgery, when inflammatory or neuropathic hyperalgesia had reached a maximum [for the time course of sensitization, compare with Reinold et al. (2005) and Hösl et al. (2006)]. DZP reversibly reduced thermal and mechanical hyperalgesia to similar degrees in both pain models. This antihyperalgesia was profoundly reduced in global $\alpha2$-$GABA_A$ point-mutated mice ($\alpha2^{R/R}$ mice), confirming the dominant contribution of $\alpha2$-$GABA_A$ receptors (Fig. 6). In the inflammatory pain model, the antihyperalgesic effect of intrathecal DZP in *sns*-$\alpha2^{-/-}$ mice fell between those of wild-type ($\alpha2^{fl/fl}$) and $\alpha2^{R/R}$ mice for thermal and mechanical hyperalgesia, indicating that presynaptic $\alpha2$-$GABA_A$ receptors contributed significantly to $\alpha2$-dependent antihyperalgesia (Fig. 6*A*,*B*).

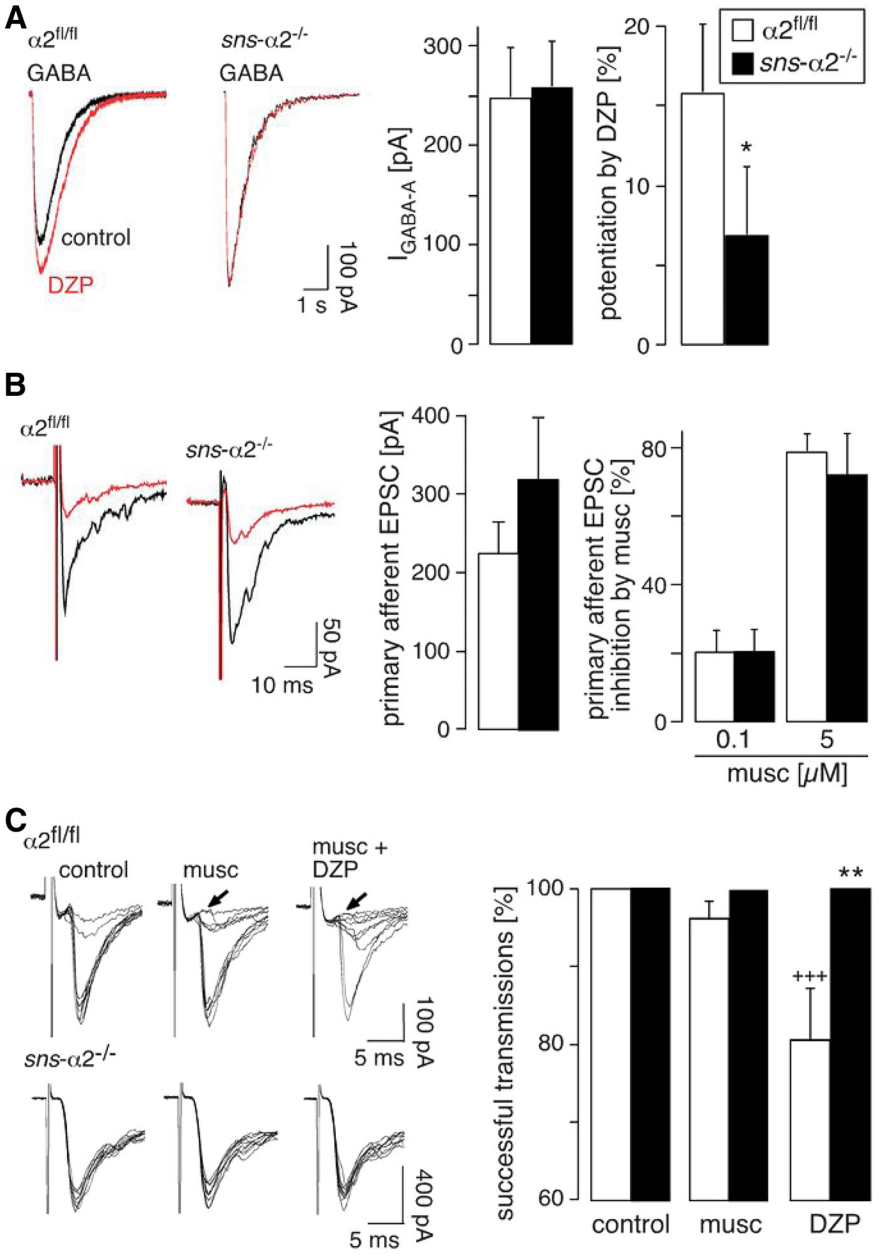

Figure 3. GABAergic membrane currents and primary afferent-evoked synaptic transmission in wild-type ($\alpha2^{fl/fl}$) and *sns*-$\alpha2^{-/-}$ mice. ***A***, GABAergic membrane currents recorded from nociceptive DRG neurons. Left, Individual current traces evoked through puffer application of GABA (1 mM) to the soma of the recorded DRG neuron in $\alpha2^{fl/fl}$ and *sns*-$\alpha2^{-/-}$ mice in the absence (black) or presence (red) of DZP (1 μM). Right, Statistical analysis (mean ± SEM). $n = 26$ ($\alpha2^{fl/fl}$) and 14 (*sns*-$\alpha2^{-/-}$). *$p < 0.05$ (unpaired *t* test). ***B***, ***C***, Primary afferent-evoked EPSCs recorded from lamina I/II neurons in transverse spinal cord slices. ***B***, Left, Current traces under control conditions (black) and in the presence of muscimol (musc, 5 μM, red). Right, Statistical analysis (mean ± SEM). EPSC amplitudes: unpaired *t* test, $n = 19$ ($\alpha2^{fl/fl}$), $n = 18$ (*sns*-$\alpha2^{-/-}$); inhibition by muscimol, $n = 6–17$. ***C***, Analyses of synaptic failure rates. Left, Superposition of 10 consecutive primary afferent-evoked EPSCs under control conditions, in the presence of muscimol (0.1 μM) and in the additional presence of DZP (1 μM). Right, Statistics (mean ± SEM). $n = 17$ ($\alpha2^{fl/fl}$) and 10 (*sns*-$\alpha2^{-/-}$). ANOVA (genotype × treatment); $F_{(3,81)} = 3.96$; *$p = 0.03$; **$p < 0.01$ significant against $\alpha2^{fl/fl}$; $^{+++}p < 0.001$ significant against control.

Although intrathecal DZP was similarly effective against neuropathic hyperalgesia, and although this antihyperalgesia was also mainly mediated by $\alpha2$-$GABA_A$ receptors, neuropathic *sns*-$\alpha2^{-/-}$ mice responded normally to intrathecal DZP (Fig. 6*C*,*D*).

Because compensatory processes are of major concern in gene deletion studies (Rudolph and Möhler, 2004), we included nociceptor-specific $\alpha2$ point-mutated mice (*sns*-$\alpha2^{-/R}$) in addition

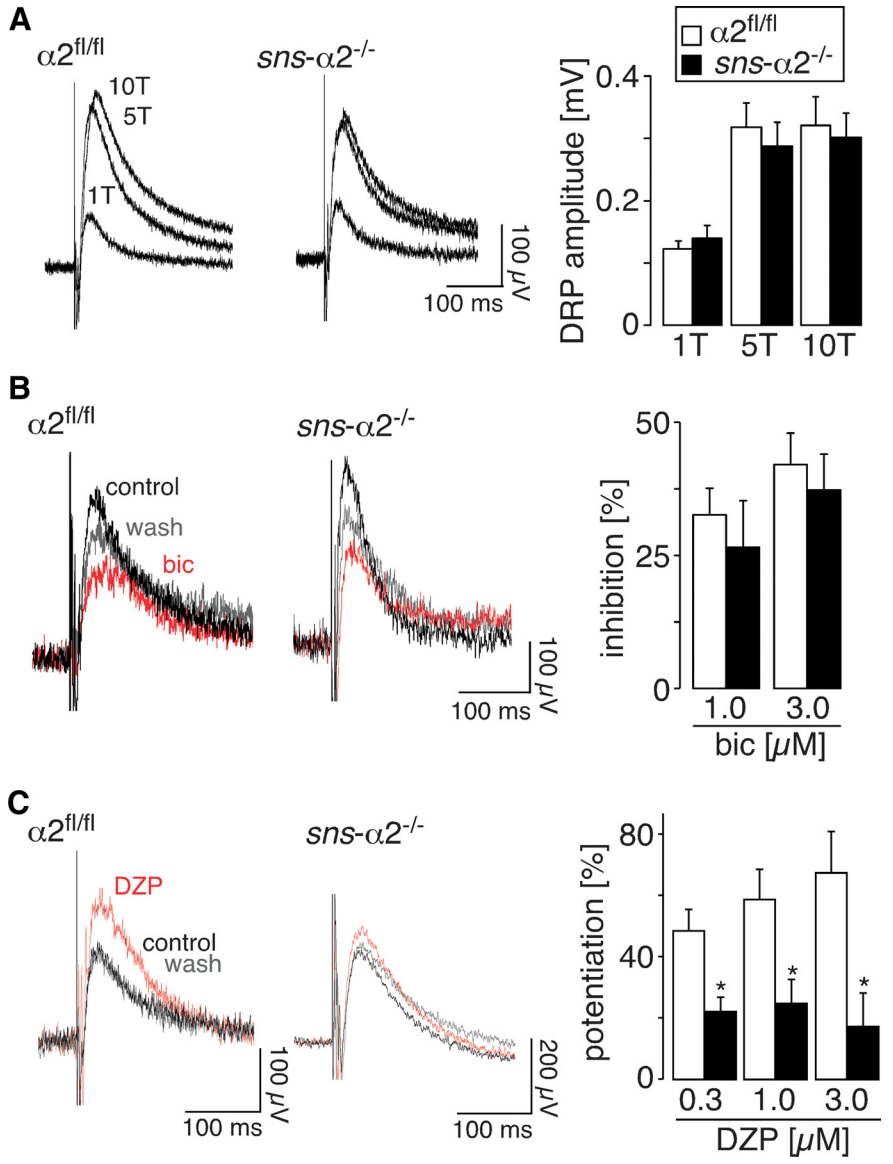

Figure 4. Dorsal root potentials. ***A***, Left, Average traces of DRPs recorded at threshold stimulation (1T) and at fivefold (5T) and tenfold (10T) higher stimulation intensities in wild-type ($\alpha2^{fl/fl}$) and *sns*-$\alpha2^{-/-}$ mice. Right, Statistical analysis (mean ± SEM). $n = 18$ ($\alpha2^{fl/fl}$) and 14 (*sns*-$\alpha2^{-/-}$). ***B***, Same as ***A***, but inhibition by bicuculline (bic, 1.0 μM, red) of DRPs elicited at 5T. $n = 9$ ($\alpha2^{fl/fl}$) and 7 (*sns*-$\alpha2^{-/-}$). ***C***, Same as ***B*** but potentiation by DZP (1 μM, red). $n = 9$ ($\alpha2^{fl/fl}$) and 5 (*sns*-$\alpha2^{-/-}$). *$p < 0.05$ (unpaired *t* test) significant against $\alpha2^{fl/fl}$.

Discussion

Although presynaptic $GABA_A$ receptors have been extensively studied in various CNS areas (Kullmann et al., 2005), their roles in integrative CNS functions and as targets for GABAergic drugs have remained difficult to assess. Here, we have used a genetic approach to selectively interfere with presynaptic $GABA_A$ receptors on spinal nociceptor terminals and to investigate their contribution to spinal pain control. We used confocal double labeling experiments to study the expression pattern of α2-$GABA_A$ receptors in the spinal dorsal horn, electrophysiological recordings in spinal cord slices, and isolated spinal cords to assess their contribution to the modulation of primary afferent-evoked synaptic transmission, and finally behavioral experiments to study their role in pain control.

Previous *in situ* hybridization (Persohn et al., 1991; Ma et al., 1993), immunofluorescence (Bohlhalter et al., 1996; Knabl et al., 2008), and electrophysiological (Knabl et al., 2008) experiments have suggested that $GABA_A$ receptors on primary sensory neurons are mainly, if not exclusively, of the α2 subtype. Our confocal double labeling experiments confirm the presence of α2-$GABA_A$ receptors on peptidergic and nonpeptidergic nociceptors as well as on non-nociceptive fibers. The additional presence of α3 subunits found in all three fiber types is consistent with our electrophysiological results, which demonstrate that GABAergic membrane currents in nociceptive DRG neurons and DRPs were still potentiated by DZP in *sns*-$\alpha2^{-/-}$ mice, albeit to a lesser extent than in wild-type mice.

GABAergic axo-axonic synapses onto the presynaptic terminals of primary afferent nerve fibers have been extensively investigated in monkey (Alvarez et al., 1993) and cat (Alvarez et al., 1992), but data in mice is sparse. In monkey and cat electron microscopy studies, GABAergic terminals were found presynaptic to Aδ fiber terminals but not to C fiber terminals. Our study, however, provides clear evidence for the presence of $GABA_A$ receptors on the intraspinal segments of peptidergic and nonpeptidergic C fibers in mice, and also for their functionality, as ablation of α2-$GABA_A$ receptors in the *sns*-$\alpha2^{-/-}$ mice almost completely abolished the potentiating effect of DZP on DRPs. Although the *sns*-cre is active not only in C fiber nociceptors but also in Aδ nociceptors (Gangadharan et al., 2009), these actions cannot be ascribed to α2-$GABA_A$ receptors on Aδ fibers alone, because recent evidence indicates that in particular heat hyperalgesia is largely, if not exclusively, mediated by peptidergic C fibers (Abrahamsen et al., 2008; Cavanaugh et al., 2009). Provided that the absence in monkey and cat of GABAergic terminals presynaptic to C fiber endings translates to

to *sns*-$\alpha2^{-/-}$ and $\alpha2^{R/R}$ mice in a subset of experiments (those on mechanical hyperalgesia) (Fig. 6*B*,*D*). These "tissue-specific point-mutated" mice carry a point-mutated and a floxed (wild type) allele in all cells of the body, with the exception of primary nociceptors that only express the mutated allele after cre-mediated deletion of the wild-type allele. In all tests performed, the phenotypes of these *sns*-$\alpha2^{-/R}$ mice closely resembled those of *sns*-$\alpha2^{-/-}$ mice. Because heterozygous nociceptor-specific α2-deficient (*sns*-$\alpha2^{-/+}$) mice and heterozygous α2-point-mutated ($\alpha2^{H/R}$) mice showed no behavioral changes compared with wild-type ($\alpha2^{fl/fl}$) mice (Table 6), the phenotype of *sns*-$\alpha2^{-/R}$ mice clearly originated from the presence of the point mutation in primary nociceptors. These experiments therefore render compensatory upregulations of other DZP-sensitive $GABA_A$ receptors in the *sns*-$\alpha2^{-/-}$ mice unlikely.

Table 4. Baseline nociceptive sensitivity, inflammatory hyperalgesia (48 h after subcutaneous zymosan A injection), and antihyperalgesic effect of diazepam (0.09 mg/kg, i.t.) in wild-type and sns-cre transgenic mice

	Acute nociception		Inflamed		Antihyperalgesia by diazepam	
	Thermal (PWL, s)	Mechanical (PWT, g)	Thermal (PWL, s)	Mechanical (PWT, g)	Thermal (AUC, s×h)	Mechanical (AUC, g×h)
Wild type	14.8 ± 0.8 ($n = 7$)	3.5 ± 0.06 ($n = 9$)	5.65 ± 0.19 ($n = 7$)	0.94 ± 0.10 ($n = 9$)	19.9 ± 2.9 ($n = 7$)	6.55 ± 1.0 ($n = 9$)
sns-cre $^{tg+}$	14.4 ± 2.6 ($n = 8$)	3.6 ± 0.09 ($n = 7$)	5.73 ± 0.91 ($n = 8$)	1.02 ± 0.06 ($n = 7$)	20.4 ± 3.0 ($n = 8$)	6.34 ± 0.3 ($n = 7$)
P (unpaired *t* test)	0.71	0.63	0.95	0.58	0.91	0.83

Paw withdrawal latencies (PWL; s) in response to stimulation with defined radiant heat, mechanical thresholds (PWT; g) in response to stimulation with electronic von Frey filaments in wild-type and *sns*-cre $^{+}$ littermates. Antihyperalgesia was quantified as the area under the curve (AUC) of the change from before-drug baseline plotted versus time. All values mean ± SEM.

Table 5. Baseline nociceptive sensitivity and inflammatory and neuropathic hyperalgesia in wild-type and *sns*-α2 $^{-/-}$ mice

	Acute nociception			Inflammatory hyperalgesia/paw swelling			Neuropathic hyperalgesia		Capsaicin-induced sensitization
	Thermal (PWL, s)	Mechanical (PWT, g)	Chemical (flinches/5 min)	Thermal (AUC, s×d)	Mechanical (AUC, g×d)	Paw swelling (AUC, ml×h)	Thermal (AUC, s×d)	Mechanical (AUC, g×d)	Mechanical (AUC, g×h)
α2 $^{fl/fl}$	15.0 ± 0.6 ($n = 6$)	3.0 ± 0.1 ($n = 6$)	49.5 ± 6.6 ($n = 6$)	33.3 ± 4.6 ($n = 6$)	9.1 ± 0.3 ($n = 6$)	3.62 ± 0.42 ($n = 6$)	224 ± 9 ($n = 6$)	47.3 ± 1.5 ($n = 6$)	4.6 ± 0.24 ($n = 5$)
sns-α2 $^{-/-}$	14.5 ± 0.8 ($n = 10$)	3.1 ± 0.1 ($n = 10$)	49.0 ± 7.1 ($n = 6$)	33.0 ± 4.6 ($n = 10$)	10.6 ± 0.7 ($n = 10$)	3.25 ± 0.31 ($n = 10$)	230 ± 8 ($n = 6$)	44.8 ± 1.4 ($n = 6$)	4.5 ± 0.22 ($n = 6$)

Paw withdrawal latencies (PWL; s) in response to stimulation with defined radiant heat, mechanical thresholds (PWT; g) in response to stimulation with electronic von Frey filaments, and numbers of flinches within 5 min after subcutaneous injection of capsaicin (1.6 μg in 10 μl) in *sns*-α2 $^{-/-}$ mice and in wild-type (α2 $^{fl/fl}$) littermates. Hyperalgesia was quantified as the area under the curve (AUC) of the change from baseline sensitivity plotted versus time. $p > 0.1$ (unpaired *t* test) for all comparisons between genotypes. All values mean ± SEM.

mice, our findings may prompt for structural arrangements of the GABAergic input different from classical axo-axonic synapses. In such an alternative scenario, GABAergic inhibition of C fiber nociceptors might not originate from $GABA_A$ receptors located at the presynaptic terminal itself, but from axonal receptors located farther away from the terminals. Such an arrangement would impair action potential propagation rather than directly interfere with transmitter release, and would be similar to what has been described for muscle spindle afferents in the rat brainstem (Verdier et al., 2003). These axonal receptors might become activated through ambient GABA rather than through GABA released directly onto these receptors.

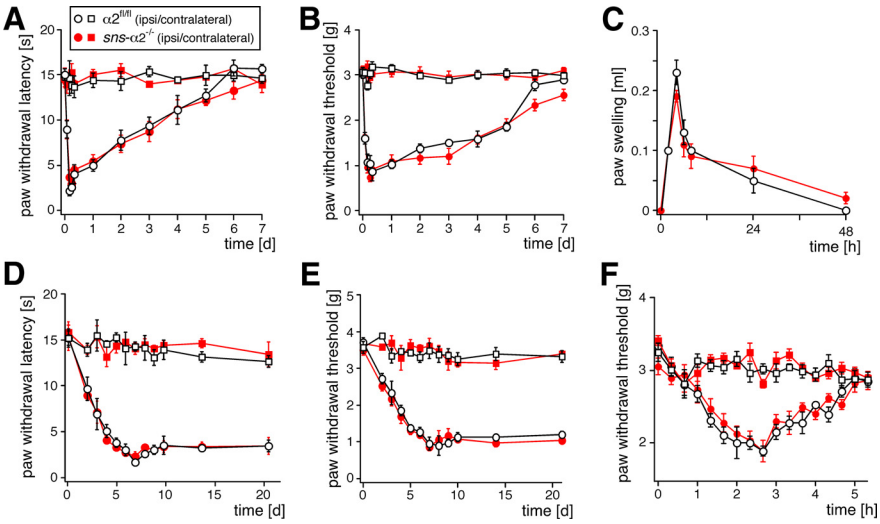

Figure 5. Nociceptive behavior in *sns*-α2 $^{-/-}$ mice. Inflammation induced by subcutaneous zymosan A injection (0.06 mg/10 μl) into the plantar side of the left hindpaw. Thermal hyperalgesia (paw withdrawal latencies, s) (***A***), mechanical sensitization (paw withdrawal thresholds, g) (***B***), and paw swelling (***C***) in *sns*-α2 $^{-/-}$ and wild-type (α2 $^{fl/fl}$) littermates. $n = 6$–10 mice/group. ***D***, ***E***, Same as ***B***, ***C***, but neuropathic pain induced through CCI surgery of the left sciatic nerve. $n = 6$ mice/group. ***F***, Secondary hyperalgesia induced through subcutaneous injection of capsaicin (30 μg in 10 μl) into the plantar left hindpaw. Mechanical withdrawal thresholds (g); $n = 5$–6 mice/group. For statistics, see Table 5.

The most obvious behavioral phenotype observed in *sns*-α2 $^{-/-}$ mice was a reduction in the antihyperalgesic effect of spinal DZP against inflammatory hyperalgesia. At least for inflammatory hyperalgesia, this phenotype unambiguously indicates that the antihyperalgesic action of spinal benzodiazepines is largely due to a direct action on the sensory pain pathway and not to indirect effects, such as a reduction in anxiety-induced hyperalgesia. It also indicates that the enhancement on primary afferent depolarization by spinally applied BDZs increases presynaptic inhibition in primary nociceptors and, thereby, reduces nociceptive input to the spinal dorsal horn. Diminished DZP-induced antihyperalgesia in *sns*-α2 $^{-/-}$ mice correlates well with the decreased ability of DZP to facilitate $GABA_A$ receptor-mediated inhibition of synaptic transmission between primary nociceptors and second order neurons, and with the diminished DZP-sensitivity of GABAergic DRPs in these mice. Approximately one third of the α2-$GABA_A$ receptor-mediated antihyperalgesia was maintained in *sns*-α2 $^{-/-}$ mice. This part may originate from α2-$GABA_A$ receptors expressed by intrinsic dorsal horn neurons. Expression of α2-$GABA_A$ receptors on intrinsic dorsal horn neurons has not been generally accepted previously, because *in situ* hybridization studies had revealed significant amounts of α2 mRNA in the ventral but not in the dorsal horn (Ma et al., 1993). Our experiments demonstrate that much of the α2 immunofluorescence is retained in *sns*-α2 $^{-/-}$ mice, consistent with our previous electrophysiological data showing reduced DZP-sensitivity in dorsal horn neurons of α2 $^{R/R}$ mice (Knabl et al., 2008). Alternatively, the remaining α2-$GABA_A$ receptor-mediated antihyperalgesia could come from α2-$GABA_A$ receptors residing on primary sensory neurons that do not express the *sns*-cre.

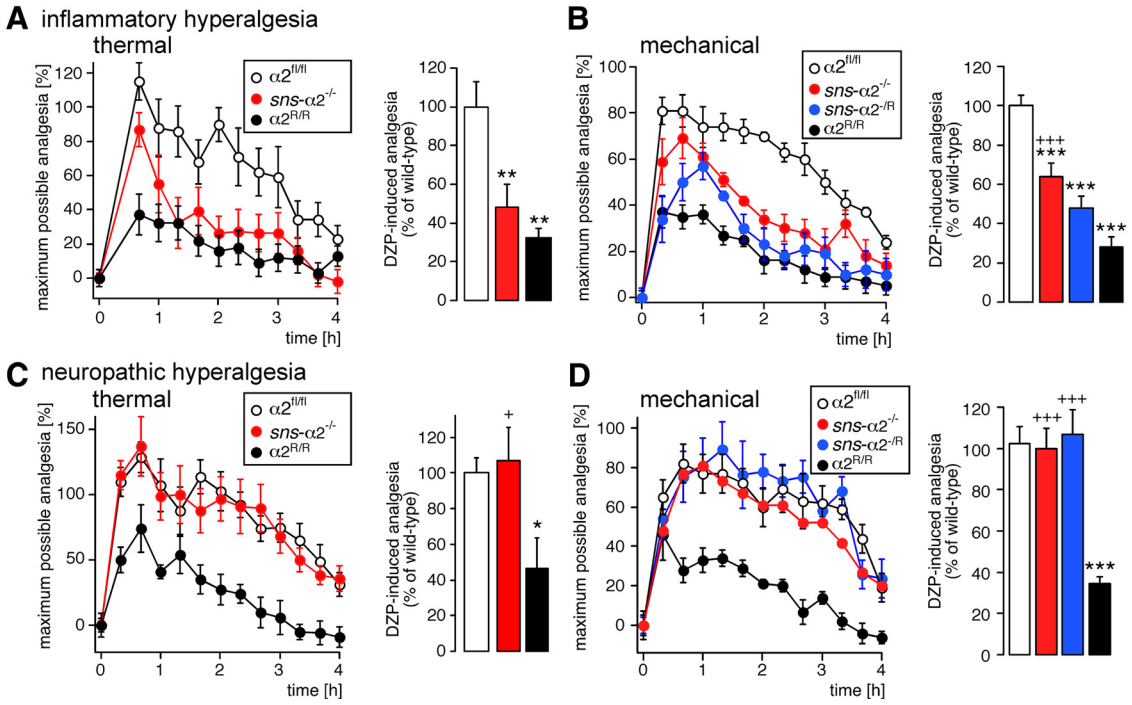

Figure 6. Antihyperalgesic effects of DZP. ***A*—*D***, Antihyperalgesic effects of intrathecally injected DZP (0.09 mg/kg body weight) on thermal (***A***, ***C***) and mechanical (***B***, ***D***) hyperalgesia expressed as percentage maximum possible analgesia (mean ± SEM). Area under the curve (AUC), 0–4 h after DZP injection. ***A***, ***B***, Inflammatory hyperalgesia induced by subcutaneous zymosan A injection (0.06 mg in 10 μl) into the left hindpaw. DZP was given 48 h after zymosan A injection. Left, Time course; right, Statistics. AUC expressed as percentage of wild-type littermates ($\alpha2^{fl/fl}$ mice). ANOVA $F_{(2,25)} = 8.71$ followed by Bonferroni *post hoc* text, $n = 8–10$ mice/group (thermal hyperalgesia); ANOVA $F_{(3,33)} = 36.82$, $n = 7–12$ mice/group (mechanical hyperalgesia). ***C***, ***D***, Same as ***A***, ***B*** but neuropathic pain 7 d after CCI surgery of the left sciatic nerve. ANOVA followed by Bonferroni *post hoc* test $F_{(2,21)} = 5.18$, $n = 7–9$ mice/group (thermal hyperalgesia); $F_{(3,23)} = 11.16$, $n = 5–10$ mice/group (mechanical hyperalgesia). $^*p \le 0.05$; $^{**}p \le 0.01$; $^{***}p \le 0.001$, significant against $\alpha2^{fl/fl}$, $^+p \le 0.05$; $^{+++}p \le 0.001$, against $\alpha2^{R/R}$.

Table 6. Baseline mechanical sensitivity, inflammatory hyperalgesia, and antihyperalgesic effect of DZP (0.09 mg/kg, i.t.) in heterozygous nociceptor-specific α2-deficient (*sns*-$\alpha2^{-/H}$) mice, heterozygous point-mutated ($\alpha2^{H/R}$) mice, and heterozygous α2-floxed (wild-type) mice

	Acute nociception (PWT, g)	Inflammatory hyperalgesia (PWT, g)	Antihyperalgesia by DZP (AUC, g×h)
$\alpha2^{fl/H}$ ($n = 7$)	3.59 ± 0.06	0.82 ± 0.11	7.80 ± 2.95
sns-$\alpha2^{-/H}$ ($n = 6$)	3.55 ± 0.13	1.02 ± 0.08	7.59 ± 0.35
$\alpha2^{R/H}$ ($n = 6$)	3.50 ± 0.11	0.99 ± 0.08	8.12 ± 0.63
P (ANOVA)	0.56	0.90	0.76

Antihyperalgesia was quantified as the area under the curve (AUC) of the change from before-drug baseline sensitivity plotted versus time. All three genotypes had virtually identical baseline mechanical sensitivities, developed similar mechanical hyperalgesia, and responded normally to DZP (0.09 mg/kg, i.t.). Because the floxed *Gabra2* allele behaves as a wild-type allele in the absence of cre expression, $\alpha2^{fl/H}$ mice can be considered as wild-type mice. Hence, these experiments demonstrate that the histidine to arginine point mutation of only one *Gabra2* allele ($\alpha2^{H/R}$ mice) has no apparent consequences for pain control or for the anti-hyperalgesic effect of intrathecal DZP. Similarly, the nociceptor-specific deletion of one gabra2 allele (in sns-$\alpha2^{-/H}$ mice) affected neither baseline mechanical sensitivity nor the development of mechanical hyperalgesia or its reversal by intrathecal DZP. The phenotype described for the nociceptor specific α2 point-mutated (sns-$\alpha2^{-/R}$) mice can thus be specifically attributed to the lack of DZP-sensitive α2-GABA$_A$ receptors in primary nociceptors. All values mean ± SEM.

In contrast to the antihyperalgesic activity of spinal DZP against inflammatory pain, its activity against neuropathic pain was not changed in *sns*-$\alpha2^{-/-}$ or *sns*-$\alpha2^{-/R}$ mice. It is tempting to speculate that presynaptic inhibition by α2-GABA$_A$ receptors might be less important under neuropathic conditions. However, Abrahamsen et al. (2008) demonstrated that different types of primary afferent sensory fibers mediate inflammatory and neuropathic hyperalgesia. In fact, neuropathic hyperalgesia developed normally in mice lacking *sns*-positive primary nociceptors, whereas inflammatory hyperalgesia was largely abolished (Abrahamsen et al., 2008). It is therefore possible that the antihyperalgesic action of intrathecal DZP against neuropathic pain also occurred through presynaptic α2-GABA$_A$ receptors, but residing on primary afferent sensory fibers that did not express *sns*-cre.

GABA$_A$ receptors on spinal nociceptor terminals have been suggested to inhibit the transmission of nociceptive signals through PAD and subsequent presynaptic inhibition (Willis, 1999). The *sns*-$\alpha2^{-/-}$ mice studied here had normal baseline nociceptive sensitivities and developed normal inflammatory or neuropathic hyperalgesia. Very intense nociceptor stimulation and inflammation may, however, enhance PAD to levels sufficient to trigger action potentials and to elicit so-called dorsal root reflexes (Cervero and Laird, 1996; Willis, 1999). Input from primary afferent nerve fibers could then, via interconnected GABAergic interneurons, elicit action potentials in other primary afferent fiber terminals, from which excitation could spread both anterogradely and retrogradely, to exaggerate pain and inflammation. Again, *sns*-$\alpha2^{-/-}$ mice exhibited unaltered hyperalgesia after capsaicin injection and unchanged hyperalgesia or paw swelling after zymosan A injection. Nevertheless, our findings do not exclude a contribution of GABAergic PAD to presynaptic inhibition or dorsal root reflexes, because the GABA$_A$ receptors remaining in nociceptors of *sns*-$\alpha2^{-/-}$ mice were apparently sufficient to sustain GABAergic membrane currents and DRPs of nearly normal amplitude. Reduced BDZ sensitivity of GABA$_A$ receptor currents in nociceptive DRG neurons and of dorsal root potentials but nearly unchanged amplitudes and unaffected bicuculline sensitivity may be explained by the upregulation of BDZ-insensitive GABA$_A$ receptor subunits. A significant upregulation was found for the α4 subunit. In addition, other BDZ-insensitive but bicuculline-sensitive subunits (δ and θ) showed a trend toward increased expression in *sns*-$\alpha2^{-/-}$ mice. One might speculate that a facilitation of GABA$_A$ receptor-

mediated dorsal root reflexes by BDZs could also counteract antihyperalgesia by spinal BDZs. However, although DRPs in *sns*-$\alpha 2^{-/-}$ mice were less sensitive to DZP, these mice did not show increased antihyperalgesia.

In summary, the generation of mice with a genetic ablation of a specific $GABA_A$ receptor subtype in primary nociceptors allowed us to attribute to presynaptic $GABA_A$ receptors residing on the axons or terminals of primary nociceptors a significant role in spinal pain control, namely a contribution to antihyperalgesia mediated by spinal DZP.

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