Published in final edited form as:
Genesis. 2010 October 1; 48(10): 596–602. doi:10.1002/dvg.20656.

Hoxb8-Cre Mice: a Tool for Brain-Sparing Conditional Gene Deletion

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SUMMARY

The spinal cord is the first site of temporal and spatial integration of nociceptive signals in the pain pathway. Neuroplastic changes occurring at this site contribute critically to various chronic pain syndromes. Gene targeting in mice has generated important insights into these processes. However, the analysis of constitutive (global) gene-deficient mice is often hampered by confounding effects arising from supraspinal sites. Here, we describe a novel *Cre* mouse line which expresses the Cre recombinase under the transcriptional control of the *Hoxb8* gene. Within the neural axis of these mice, *Hoxb8-Cre* expression is found in spinal cord neurons and glial cells, and in virtually all neurons of the dorsal root ganglia, but spares the brain apart from a few cells in the spinal trigeminal nucleus. The *Hoxb8-Cre* mouse line should be a valuable new tool for the *in vivo* analysis of peripheral and spinal gene functions in pain pathways.

Keywords

spinal cord; dorsal root ganglia; astrocytes; pain; Cre-*lox*P system; *Hox* genes; brain-sparing gene-deletion; glycine transporter type 1

Noxious (i.e. painful or potentially tissue damaging) stimuli are sensed by specialized nerve cells, called peripheral or primary nociceptors, which connect the peripheral tissues with the spinal cord dorsal horn, the first site of synaptic processing in the pain pathway. From there, nociceptive signals are relayed to higher central nervous system areas where pain finally becomes conscious. It is generally accepted that chronic/pathological pain syndromes can originate from dysfunctions at all three levels. Persistent activity of peripheral nociceptors as well as plastic changes in the spinal and supra-spinal processing of nociceptive stimuli have been shown to contribute to these pathologies. In addition, these sites are also critically involved in the action of many analgesic drugs, in particular of opioids (Dickenson and Kieffer, 2006) but also of aspirin-like drugs (cyclooxygenase inhibitors) (Brune and Zeilhofer, 2006). Constitutive (global) gene targeting has yielded important insights into mechanisms of pain and analgesia. It does however not allow spatial discrimination of these mechanisms at the different sites, although this was highly desirable in many aspects of basic pain research and analgesic drug development. One strategy to address this issue relies on conditional gene deletion through the Cre-*lox*P system. Primary nociceptor-specific gene

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deletion can be achieved with mice expressing the Cre recombinase under the transcriptional control of the gene encoding the sensory neuron-specific sodium channel (*sns*) *Nav1.8* (Agarwal et al., 2004; Akopian et al., 1999). Other mouse lines, *Peripherin-Cre* (Zhou et al., 2002) and *HtPA-Cre* (Pietri et al., 2003), have been reported to express the Cre recombinase in primary sensory neurons of dorsal root ganglia. To further discriminate spinal (and peripheral) sites from supraspinal sites, we aimed at a *Cre* mouse line allowing brain-sparing gene deletion. To this end, we generated a novel mouse line expressing the Cre recombinase under the transcriptional control of the murine homeobox gene *Hoxb8* (previously called *Hox-2.4*). *Hox* genes are expressed in spatially and temporally restricted domains along the anterior-posterior axis of the body, where they usually show a sharp rostral expression boundary (McGinnis and Krumlauf, 1992). The expression of the *Hoxb8* gene extends to the cervical segment C2 (Charité et al., 1995; Deschamps and Wijgerde, 1993) and thereby above makes *Hoxb8* an appropriate gene to drive *Cre* expression for brain-sparing gene deletion.

Charité et al. (1995) characterized upstream cis-acting regulatory elements of the *Hoxb8* gene and found that a 11kb DNA segment upstream of the *Hoxb8* translational start was sufficient to closely mimic the endogenous *Hoxb8* expression pattern. To generate *Hoxb8-Cre* transgenic mice, we fused the 11 kb DNA segment (Charité *et al.*, 1995) to a *Cre* expression cassette and used this construct for pronuclear injections (figure 1A). Four transgenic founders were obtained each of which gave rise to a transgenic line. These mouse lines were back crossed continuously to the C57BL/6 background and maintained in a heterozygous state. Two lines (1403, 1404) showed the desired expression pattern on a gross scale depicted here at E9.5 and E11.5 (figure 1B). One of these lines (1403), which carries a single copy of the transgene (figure 1C), was characterized in detail and is described here.

To analyze the expression pattern of *Hoxb8-Cre*-mediated gene recombination along the neural axis, *Hoxb8-Cre* mice were first crossed with heterozygous floxed *Rosa26lacZ* mice (*R26R*) (Soriano, 1999). Cryostat sections from spinal cords, brains and spinal dorsal root ganglion (DRG) neurons (which harbour the somata of peripheral sensory neurons, including nociceptors) were prepared from 4 – 7 week old co-transgenic (*Hoxb8-Cre*$^{tg+}$*/R26R)* mice and stained with X-Gal followed by a counterstain with acidified hematoxylin. Coronal sections from the lumbar spinal cord of these mice revealed *lacZ* expression throughout the white and grey matter in a pattern reminiscent of Nissl staining, suggesting that *lacZ* expression occurred in neurons as well as in glial cells (figure 2A). Sections obtained from *Hoxb8-Cre*$^{tg-}$*/R26R* littermates did not show any visible *lacZ* activity. To determine the rostral *Cre* expression boundary in the *Hoxb8-Cre* mouse line, coronal and horizontal spinal cord sections representing different anterior-posterior spinal cord segments were analyzed. *Hoxb8-Cre*-induced *lacZ* expression was similar at the lumbar and thoracic segment, but gradually decreased in a caudo-rostral direction within the cervical segments (figure 2B). While full *lacZ* activity was still observed at cervical segment C7 in both the grey and the white matter, *lacZ* activity disappeared around cervical segment C4 and became restricted to a few cells scattered in the grey matter at cervical segment C2. The brain was largely devoid of *lacZ* activity (figure 2C) even after prolonged (24 hours) X-Gal exposure with the exception of a few cells in the spinal trigeminal nucleus (figure 2D). *Hoxb8-Cre*-mediated gene recombination was also analyzed in cryostat sections of lumbar DRGs from *Hoxb8-Cre*$^{tg+}$*/R26R* mice (figure 2E). *Cre*-induced *lacZ* activity and immunostaining of β-gal protein (not shown) was found in virtually all DRG neurons, indicating efficient *Cre*-mediated gene recombination in primary somatosensory neurons and in primary nociceptors. No *lacZ* activity or β-gal immunoreactivity was apparent in satellite cells of DRGs.

We next aimed at determining the types of cells that exhibited *Hoxb8-Cre*-induced *lacZ* activity in the spinal cord. To demonstrate the presence of *lacZ* in neurons, we performed co-immunostainings of coronal spinal cord sections with anti-sera against the bacterial β-galactosidase (β–gal) and the neuron-specific nuclear protein (NeuN) (figure 3A,B). For quantitative analyses, 508 NeuN positive neurons were identified in stacks of confocal images obtained from 3 independent sections. Virtually all of these neurons (490 / 508; 96.0 ± 0.8%, mean ± sd) contained β–gal immunoreactivity. *Hoxb8-Cre*-mediated recombination could also be verified in astrocytes. This was shown through *Hoxb8-Cre*-mediated (conditional) deletion of the glycine transporter type 1 (GlyT1; *Slc6a9*) gene, which is abundantly expressed in spinal glial cells (Zafra et al., 1995). *Hoxb8-Cre* mice were crossed with mice carrying floxed GlyT1 alleles (Yee et al, 2006) to generate *Hoxb8-Cre*$^{tg+}$/*GlytT1*$^{flox/flox}$ (*Hoxb8-GlyT1*$^{-/-}$) mice. Immunohistochemical analysis of postnatal day 10 (P10) mice revealed intense GlyT1 immunofluorescence throughout the spinal grey matter of wild-type mice, but not in *Hoxb8-GlyT1*$^{-/-}$ mice (figure 3C).

The mesodermic (vascular) expression pattern in the spinal cord was assessed with the use of the *RA/EG* reporter strain (Constien et al., 2001), which carries a *Cre*-inducible enhanced green fluorescence protein (EGFP) reporter gene in the locus of the receptor for advanced glycated end products (*RA/EG*). We analyzed coronal sections at different spinal cord segments of co-trangenic progeny from *Hoxb8-Cre* mice crossed with the *RA/EG* strain (figure 3D). At lumbar segments, intense *Hoxb8-Cre*-induced EGFP fluorescence was seen in cells around and along blood vessels. EGFP fluorescence extended rostrally to the upper thoracic segments (approximately T2). *RA/EG* reporter mice did not reveal neural *Hoxb8-Cre*-mediated EGFP fluorescence consistent with previous findings showing that the *RA/EG* promoter is inactive in most neurons (Brett et al., 1993; Constien et al., 2001).

We next analyzed *Hoxb8-Cre*-induced *lacZ* expression in non-neural tissues (figure 4). Transverse sections through the lower hindlimb showed that the epidermis was devoid of *Hoxb8-Cre*-induced *lacZ* activity, while *lacZ* activity was found in cells scattered in the reticular dermis and the subcutis (figure 4A). Striated muscle exhibited intense *lacZ* activity. Kidney sections showed strong *lacZ* activity in about half of the epithelial cells and in cells surrounding blood vessels (figure 4B), while liver and heart sections did not reveal any apparent lacZ staining (figure 4C,D).

Finally, the temporal onset of *Hoxb8*-induced *Cre* activity was analyzed in co-transgenic *Hoxb8-Cre*$^{tg+}$*/R26R* embryos recovered at embryonic states E9.5 to E15. The tissue distribution of *lacZ* expression at early stages of development like E9.5 already anticipated the expression pattern described above for adult mice (compare Fig. 1B). Because most dorsal horn neurons are born between E10 and E12 (Helms and Johnson, 2003), this early onset of *lacZ* expression suggests that Hoxb8-Cre is already active in neuronal precursor cells of the spinal cord.

Hoxb8-Cre-induced *lacZ* expression pattern described here closely resembles that of endogenous *Hoxb8* expression studied in wild-type embryos with *in situ* hybridization (Charité et al., 1995; Deschamps and Wijgerde, 1993). A subtle difference exists however in the exact rostral expression boundary, which in the case of the endogenous *Hoxb8* gene extends more rostrally up to C2 (Charité et al., 1995). The rostral expression boundary of our *Hoxb8-cre* transgenic mice is located approximately two segments more posterior than the rostral limit of endogenous *Hoxb8*, presumably because of the lack on our construct of a retinoic acid-responsive element located in the *Hox* cluster between *Hoxb4* and *Hoxb5* (Oosterveen *et al.*, 2003; Valarche *et al.*, 1997). Another difference became apparent when we compared the *lacZ* expression pattern of our *Hoxb8-cre*$^{tg+}$*/R26R* cotransgenic mice with published data on *Hoxb8lacZ* "knock-in" mice (Holstege et al., 2008), in which the

endogenous *Hoxb8* had been replaced by a *lacZ* expression cassette. In postnatal (P11) *Hoxb8lacZ* mice, *lacZ* expression was less intense in the ventral than in the dorsal spinal cord and was found only in a subpopulation of DRG neurons (Holstege et al., 2008). This difference reflects most likely the dynamic change in the expression of *Hoxb8* transcripts during development, which is detectable in the *Hoxb8lacZ* mice but occluded through the irreversible *lacZ* activation in our mice.

One prerequisite for the use of *Hoxb8-Cre* mice in pain studies is that they themselves do not show abnormalities in their responses to painful stimuli. Because insertion of *Cre* transgenes can potentially lead to a loss of function of genes or to copy number-dependent Cre-induced toxicity (Schmidt-Supprian and Rajewsky, 2007), we performed a gross characterization of nociceptive sensitivity in these mice. Exposure to noxious thermal or mechanical stimuli did not reveal any differences in the nociceptive thresholds of *Hoxb8-Cre* mice compared to *Hoxb8-Cre* negative littermates (figure 5A,B).

In summary, the *Hoxb8-Cre* transgenic mice described here exhibit the desired *Cre* expression pattern and should hence be suitable for brain-sparing gene deletion experiments. This will be very helpful in the site-specific analysis e.g. of pain-related genes which often exhibit a wide-spread expression along the neural axis including peripheral, spinal and supraspinal sites. In such studies, our *Hoxb8-Cre* mice will allow distinguishing supraspinal effects from those occurring at spinal and peripheral sites.

METHODS

Generation of Transgenic Construct and Mice

The *Hoxb8-Cre* transgene was built from the reporter construct no.1 used by Charité at al. (1995). This construct contains a *lacZ* reporter gene fused in-frame to the first exon of *Hoxb8,* and about 11 kb of *Hoxb8* upstream sequence including most of the *Hoxb9*, and the *Hoxb9-Hoxb8* intergenic region. The 11.4 kb DNA sequence including the first 1058 bp of the *Hoxb8* gene was fused to an additional 35 bp sequence (see figure 1A) containing a Kozak sequence and an ATG starting codon and subcloned into pBC SK (Stratagene). A *Cre* recombinase cassette and a bovine poly(A) sequence were inserted in-frame downstream of the ATG. The final construct (12,754 bp) was verified by sequencing, excised with *Sal I* and *Not I*, purified and injected into fertilized early stage oocytes from C57BL/6 × DBA2 mice. Four transgenic founders were obtained which were back crossed to C57BL/6 background for at least 2 generations before crossing these heterozygous *Hoxb8-Cre* mice with the *RA/EG* (Constien et al., 2001) and *ROSA26lacZ* reporter mouse lines (Soriano, 1999). The mice analyzed here had on average a C57BL/6 background of at least 93.75%.

Southern Blot Analysis

The number of *Hoxb8-Cre* transgene copies integrated into the genome of mouse line 1403 was determined using quantitative southern blot. A probe (749 bp) directed against the *Hoxb8* promoter was generated by PCR using the following primers: FWD: 5′-TTG TTG TGA GGC AAG AGA TA-3′ and REV: 5′-TTT ATT GAA TTT TGA GGC G-3′. Labelling with this probe of *EcoR V*-digested genomic liver DNA yielded bands of 4.4 kb and 1.3 kb for wild-type and *Hoxb8-Cre* transgenic mice, respectively.

LacZ Staining and Immunohistochemistry

To study the pattern of *Cre* activity the mouse lines were crossed with 2 reporter mouse lines, B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J (*ROSA26lacZ*) (Soriano, 1999) and B6.129P2-Ager ^{tm1Arnd} (*RA/EG*) (Constien et al., 2001), as well as with the use of mice carrying a floxed GlyT1 allele (GlyT1$^{fl/fl}$; Slc6a9$^{tm1.1Bois}$). Co-transgenic progeny (4 - 7 weeks old) of

Hoxb8-Cre and *ROSA26-lacZ* mice were histologically analyzed for β-galactosidase activity. Following decapitation of the mice, organs were rapidly removed, rinsed in ice cold phosphate buffered solution (PBS), embedded in dry ice and stored at −80°C. Fresh tissue was cut in 16 μm thick sections using a sliding microtome (HM 560; Micron, Heidelberg) at −20°C. Sections were placed on superfrost plus glass slides, dried at room temperature and fixed with 0.2% glutaraldehyde for 10 min on ice. Counterstaining was performed with acidified (4% acetic acid) hematoxylin. All X-Gal stainings were performed at 37°C for 1 - 24 hours. Whole-mount embryos (plug was considered as embryonic day E 0.5) were freed of their extra-embryonic membranes before being fixed in 0.2% glutaraldehyde between 15 and 30 min on ice (for X-Gal staining protocols see Hogan et al., 1994). Presence of EGFP was detected through its endogenous fluorescence.

For immunostaining experiments, 4 - 6 week old mice (10 day old mice for the GlyT1 stainings) were perfused transcardially through the ascending aorta with PBS followed by fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.15 M phosphate buffer, pH 7.4. After perfusion, the organs were dissected out rapidly and post-fixed over night in the same fixative solution followed by cryoprotection in PBS containing 30% sucrose over night. Skin, kidney, liver, and heart were cut at −40°C into 40 μm thick sections with a sliding microtome (HM400; Micron, Heidelberg). The free-floating sections were washed in Tris-Triton (Tris-buffer with 0.05% Triton X-100, pH 7.4) and then incubated in a mixture of primary antibodies diluted in Tris-Triton containing 2% normal goat serum (NGS) and 0.2% Triton X-100 in a moist chamber with continuous agitation (100 rpm) overnight at 4°C. Sections were then washed 3 times 10 min in Tris-Triton and incubated for 30 min at room temperature in a mixture of secondary antibodies coupled to the fluorochromes Alexa Fluor488 and cyanine dye Cy3 (Jackson Immunoresearch, West Grove, PA). Non-specific staining was blocked with 2% NGS. Sections were washed 3 times for 10 min in PBS and mounted on gelatine-coated slides, air dried and protected with coverslips in fluorescence mounting medium (Dako Cytomation). Antisera used: chicken bacterial anti-β-galactosidase 1:3,000 (Abcam, ab9361), NeuN 1:5,000 (Chemicon), rabbit anti-GlyT1a,b 1:1,000 (gift from Dr. D. Boison, Legacy Institute, Portland, USA). Co-expression of NeuN and β-gal was analyzed in 3 independent lumbar spinal cord sections obtained from *Hoxb8-Cre*$^{Ig+}$/*R26R* co-transgenic mice. Stacks of 15 confocal images at 0.3 μm distance were recorded from 4 areas (115 × 115 μm) per section located in the superficial dorsal horn. 508 NeuN-positive cells were identified, reconstructed in z-direction, and carefully screened for β-gal immunoreactivity.

Testing of Paw Withdrawal Reflexes

Behavioral measurements were done on awake, free-moving 9 week old mice. A mouse plantar test apparatus (Ugo Basile, Italy) was used to determine paw withdrawal latencies in response to noxious heat, which was applied to the plantar surface of the hind paws via an infrared light source. Similarly, dynamic von Frey filaments (IITC, Woodland Hills, USA) were used to apply increasing mechanical pressure to the plantar surface of one hindpaw and mechanical stimulus thresholds were recorded in grams. At least 5 measurements were taken per paw and animal (n = 4 - 6 mice per group). Permissions for all animal experiments were obtained from the Kanton of Zurich (licences 34/2007 and 35/2009).

Acknowledgments

The authors thank Dr. Pawel Pelczar for the pronuclear injections, Dr. Thomas Müller for helpful suggestions, Dres. Jean-Marc Fritschy, Irene Knüsel, and Venceslas Duveau for scientific advice, and Isabelle Camenisch for technical assistance. This work has been supported in part by the Swiss National Science Foundation (grant 31003A-116064 to HUZ).

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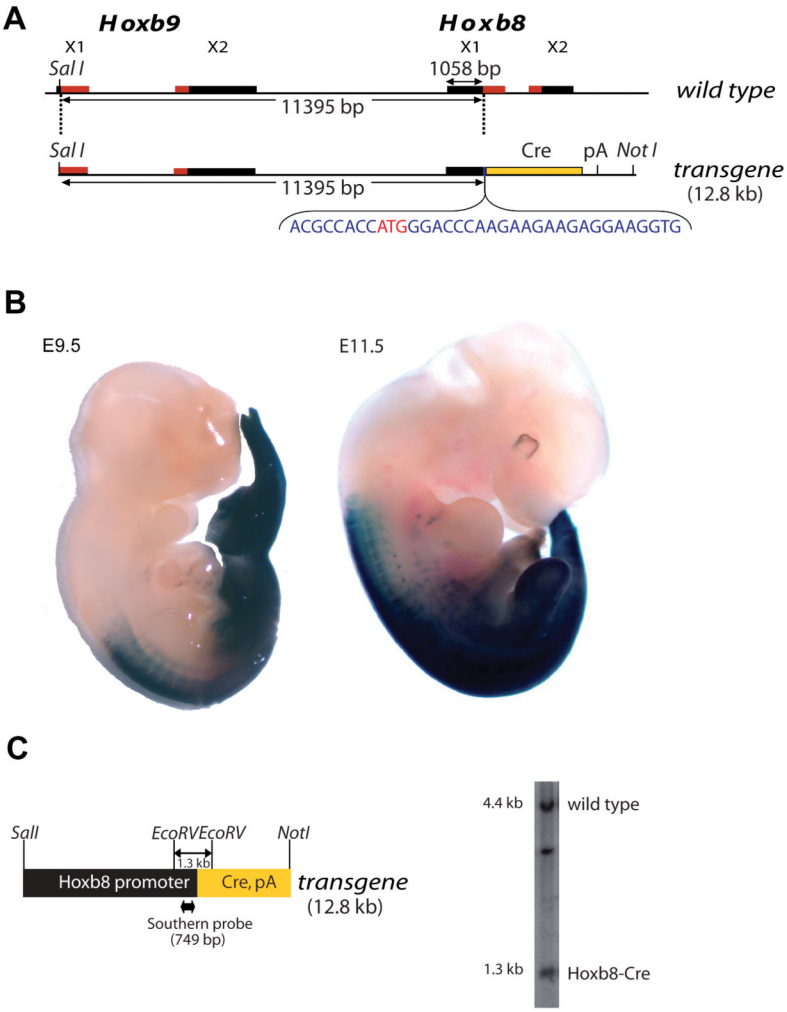

Fig. 1. Generation of *Hoxb8-Cre* mice

(A) Transgene construct and respective genomic context in the murine locus. Red bars indicate coding regions of exons (X). Between *Hoxb9* and *Hoxb8* genes an artificial sequence (blue letters) containing a starting ATG codon was inserted. (B) *Hoxb8-Cre*-induced *lacZ* activity in E9.5 and E11.5 embryos. (C) Southern blot of *EcoRV* digested genomic liver DNA from a *Hoxb8-Cre* transgenic mouse (line 1403) hybridized with a probe against the *Hoxb8* promoter. A hybridization intensity ratio of about 0.6 between *Hoxb8Cre* transgene (1.3 kb) and *Hoxb8* wild-type (4.4 kb) suggests the presence of a single copy of the transgene.

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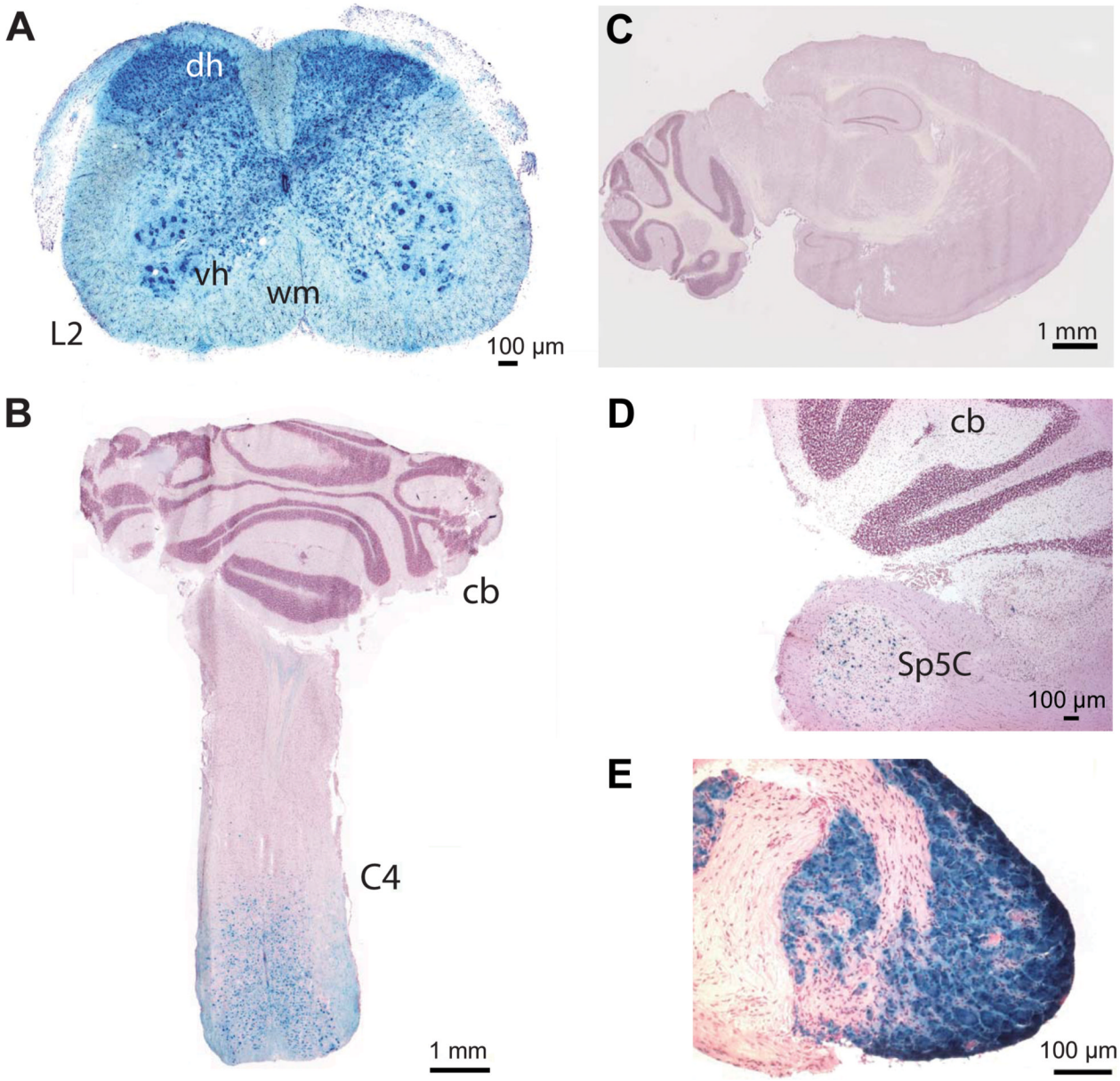

Fig. 2. lacZ activity in co-transgenic *Hoxb8-Cre*$^{tg+}$*/R26R* mice in neural tissue

(A) Coronal section of the spinal cord at lumbar segment L2. Dorsal horn (dh); ventral horn (vh); white matter (wm). (B) Horizontal section of the upper cervical spinal cord and cerebellum (cb) showing a gradual decrease of lacZ activity towards more anterior cervical segments. (C) Sagittal brain section with no visible *Hoxb8-Cre*-induced lacZ activity. (D) Sagittal section including brainstem, spinal trigeminal nucleus (Sp5C), and cerebellum (cb). (E) Lumbar DRG. All sections were obtained from 6 – 7 week old mice, incubated with X-Gal, and counterstained with acidified hematoxylin.

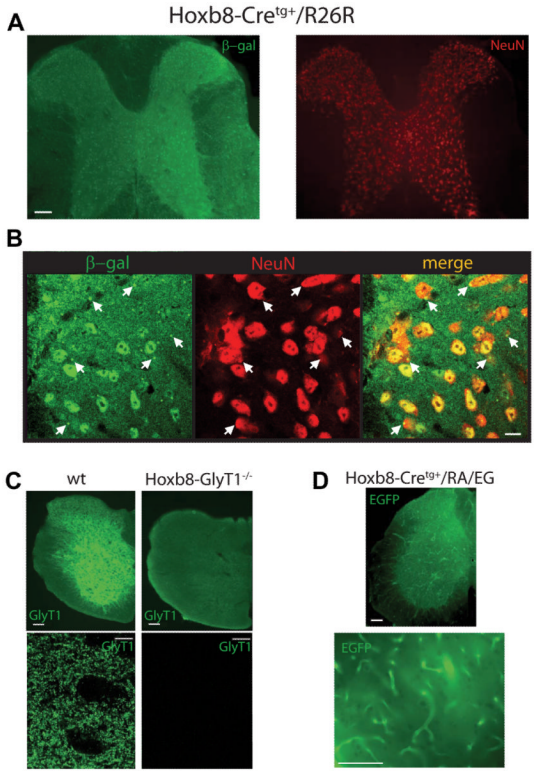

Fig. 3. Histochemical analysis of co-transgenic progeny of *Hoxb8-Cre* mice crossed with *R26R* and *RA/EG* reporter strains

(A, B) Neuronal expression. (A) Coronal thoracic spinal cord section from a co-transgenic *Hoxb8-Cre^{tg+}/R26R* mouse. β–gal (Alexa Fluor488; left panel) and NeuN (Cy3; right panel) immunofluorescence on the same section. Scale bar: 100 μm. (B) Confocal immunofluorescence analysis of a coronal section of the lumbar spinal dorsal horn of a co-transgenic *Hoxb8-Cre^{tg+}/R26R* mouse. Superposition of 15 images taken at 0.3 μm intervals. β-gal (Alexa Fluor488), NeuN (Cy3) and merged view. Arrows indicate β-gal immunoreactive granula in close association with NeuN positive structures. Scale bar, 20 μm. (C) Glial expression analysis. Coronal section from the lumbar spinal cord of *Hoxb8-GlyT1$^{-/-}$* mice and *Hoxb8-Cre* negative wild-type (GlyT1$^{fl/fl}$) littermates stained with GlyT1 antiserum. Scale bars, 100 μm (top panels) and 5 μm (bottom panels). (D) Mesodermic expression analysis. Endogenous EGFP fluorescence in coronal spinal cord sections (level L3) of a *Hoxb8-Cre^{tg+}/RA/EG* co-transgenic mouse. Top, overview; bottom, grey matter area. Scale bars, 100 μm. All sections were obtained from 5 – 6 week old mice.

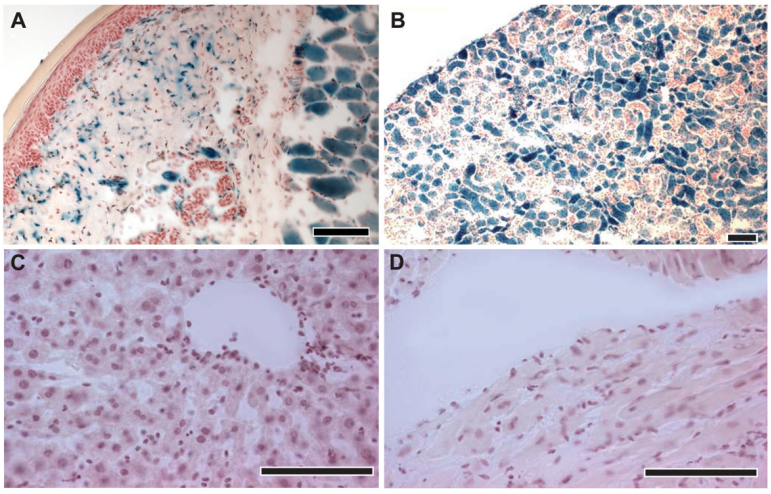

Fig. 4. β-gal activity in co-transgenic *Hoxb8-Cre^{tg+}/R26R* mice in non-neural tissue

Histological analysis of β–gal activity in 5 - 6 week old co-transgenic *Hoxb8-Cre^{tg+}/R26R* progeny. (A) Skin at the metatarsal region of the hindlimb, (B) kidney, (C) liver, and (D) heart. All sections were from 5 – 6 week old mice, incubated with X-Gal, and counterstained with acidified hematoxylin. Scale bars, 100 μm.

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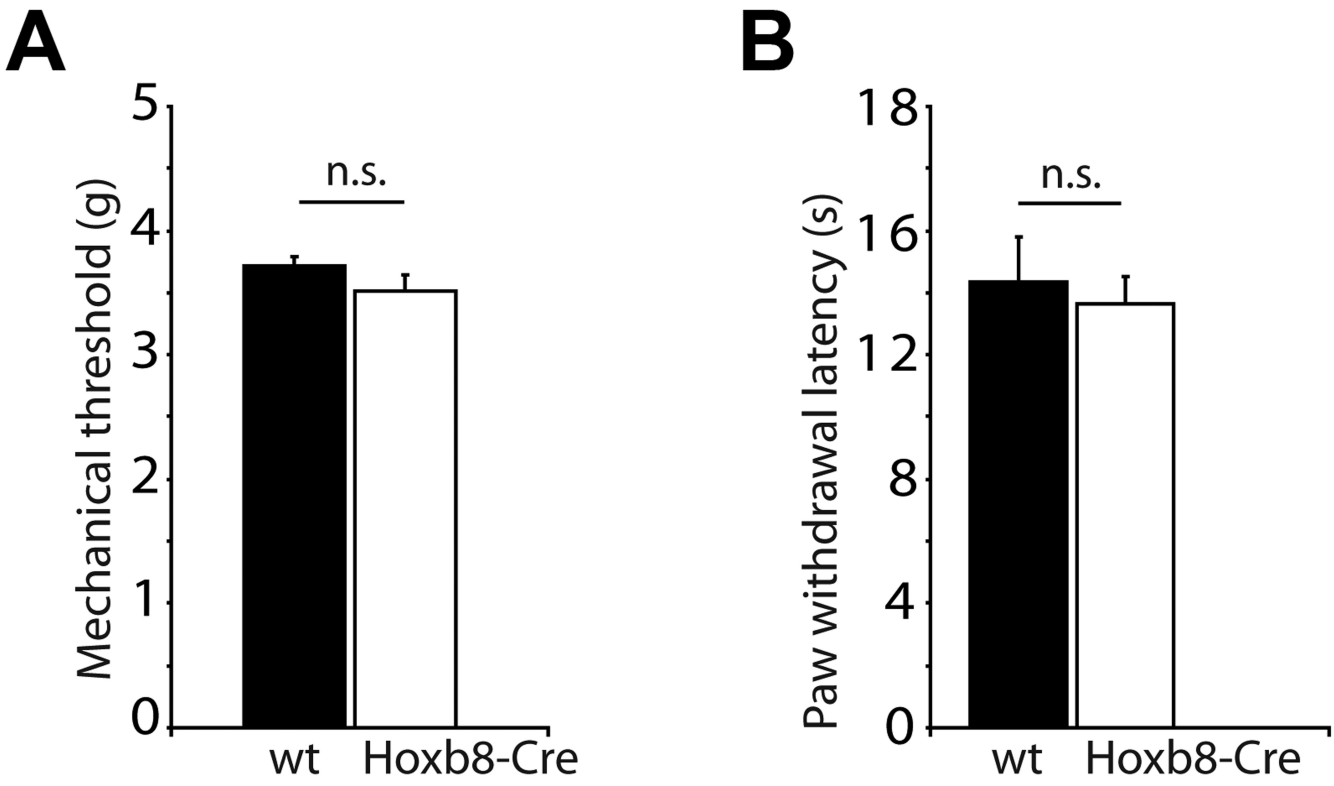

Fig. 5. Responses to noxious thermal and mechanical stimulation in *Hoxb8-Cre* mice

Hoxb8-Cre and wild-type littermates (wt) showed virtually identical mechanical thresholds (A) and paw withdrawal latencies upon exposure to noxious heat (B). Mean ± sem (n = 4 - 6 mice / group).

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