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TECHNOLOGY REPORT

Conditional Gene Deletion in Primary Nociceptive Neurons of Trigeminal Ganglia and Dorsal Root Ganglia

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Summary: The use of Cre-loxP technology for conditional mutagenesis in pain pathways had been restricted by the unavailability of mice expressing Cre recombinase selectively in functionally distinct components of the nociceptive system. Here we describe the generation of transgenic mouse lines which express Cre recombinase selectively in sensory ganglia using promoter elements of the $Na_v1.8$ gene (Scn10a). Cre-mediated recombination was greatly evident in all nociceptive and thermoreceptive neurons of the dorsal root ganglia and trigeminal ganglia, but only in a small proportion of proprioceptive neurons. Cre-mediated recombination was not detectable in the brain, spinal cord, or any nonneural tissues and began perinatally after invasion of primary afferents into the developing spinal cord. Thus, these mice enable selective deletion of genes in subsets of sensory neurons and offer a wide scope for studying potential functions of genes in pain perception, independent of secondary effects arising from developmental defects or global gene ablation. genesis 38:122–129, 2004. © 2004 Wiley-Liss, Inc.

Key words: sensory-specific, Scn10a, Nav 1.8, chronic pain, Cre-loxP system

The precise molecular events underlying physiological nociception and pathological changes thereof in chronic pain states are still very poorly understood (Woolf and Costigan, 1999; Koltzenberg and Scadding, 2001). Recently, the application of gene targeting technology has delivered valuable insights into the pathophysiology of human pain disorders (Malmberg *et al.*, 1997; Caterina, 2001). However, due to the multiple limitations and liabilities of global ablation in classical mouse knockouts, systems enabling a tight spatial and temporal regulation of gene expression are required.

The Cre-loxP recombinase system is the most prominent of the binary systems available for inducible, tissue-specific gene expression (Nagy, 2000). The bottleneck for using this powerful technology in pain research is the availability of mouse lines expressing Cre recombinase selectively in anatomically and functionally distinct components of pain pathways. Fortunately, the recent cloning of genes that are expressed in discrete neuronal populations now makes their promoter elements available for driving Cre expression in specific peripheral or central neurons in pain pathways (Caterina and Julius, 1999; Julius and Basbaum, 2001). Proteins such as the sodium channel $Na_v1.8$ (synonymous with SNS or PN3) (Akopian *et al.*, 1996) and acid sensing ion channel-β (ASIC-β) (Chen *et al.*, 1998) are selectively expressed in the sensory ganglia, preferentially in neurons involved in nociception.

Here we report the generation of mice expressing Cre recombinase under the regulatory elements of the mouse Scn10a gene (Kozak and Sangameswaram, 1996; Souslova *et al.*, 1997), which encodes for the tetrodotoxin-resistant $Na_v1.8$ sodium channel. These mice provide a novel tool for conditionally manipulating genes in specific subsets of primary sensory neurons of the dorsal root ganglia and trigeminal ganglia in mouse models of acute and chronic pain.

Two of the eight identified founders carrying the targeted Scn10a BAC were characterized in detail and were found to express Cre recombinase in an identical pattern (henceforth referred to as SNS-Cre mice). LacZ staining performed on multiple organs of adult double-positive progeny of SNS-Cre transgenic founders and ROSA26-LacZ mice revealed a functional expression of Cre recombinase in the dorsal root ganglia (Fig. 1C) and trigeminal ganglia of both founders. In contrast, dorsal root ganglia of control mice carrying the SNS-Cre transgene or the ROSA26-LacZ allele alone did not demonstrate any staining for LacZ (Fig. 1C). Sections of the spinal cord, brain, and brainstem as well as all visceral and skeletal organs tested, such as the liver, lung, heart, intestine, and skeletal muscles of SNS-Cre;ROSA26-LacZ transgenics were completely devoid of staining, even upon 48-h incubation in the staining solution (Fig. 2A–C; micro-

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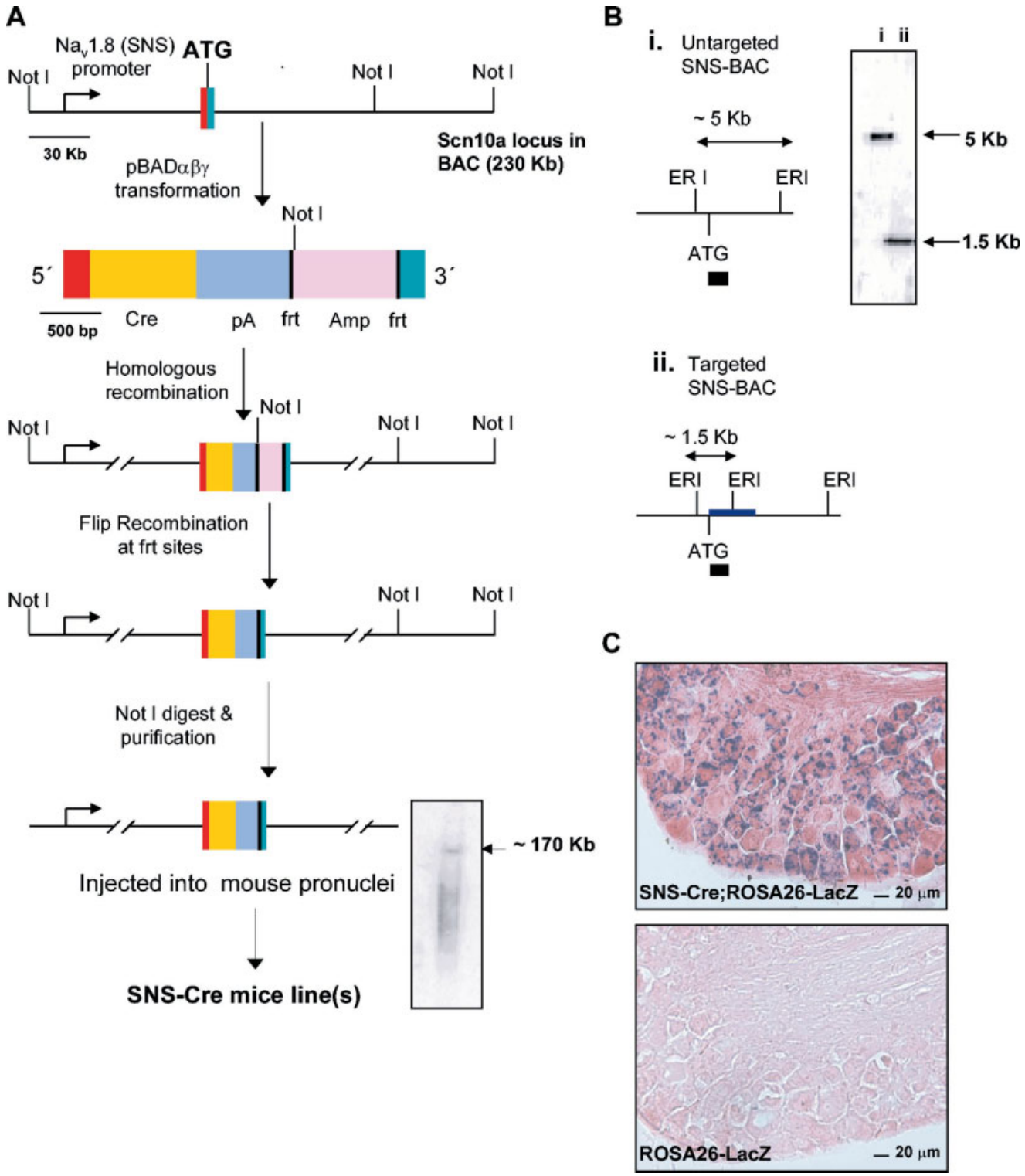

FIG. 1. Generation of SNS-Cre transgenic mice. **A:** Schematic representation of ET-recombination-based insertion of Cre recombinase into the coding region of a BAC containing the mouse Scn10a gene locus and preparation of 170 Kb fragment for pronucleus injection. **B:** Southern blot analysis for verification of correct insertion of Cre recombinase into coding ATG of the Scn10a gene in BAC. **C:** Dorsal root ganglia of adult SNS-Cre;ROSA26-LacZ double transgenic mice demonstrate expression of LacZ protein, in contrast to ROSA26-LacZ single transgenic (control) mice.

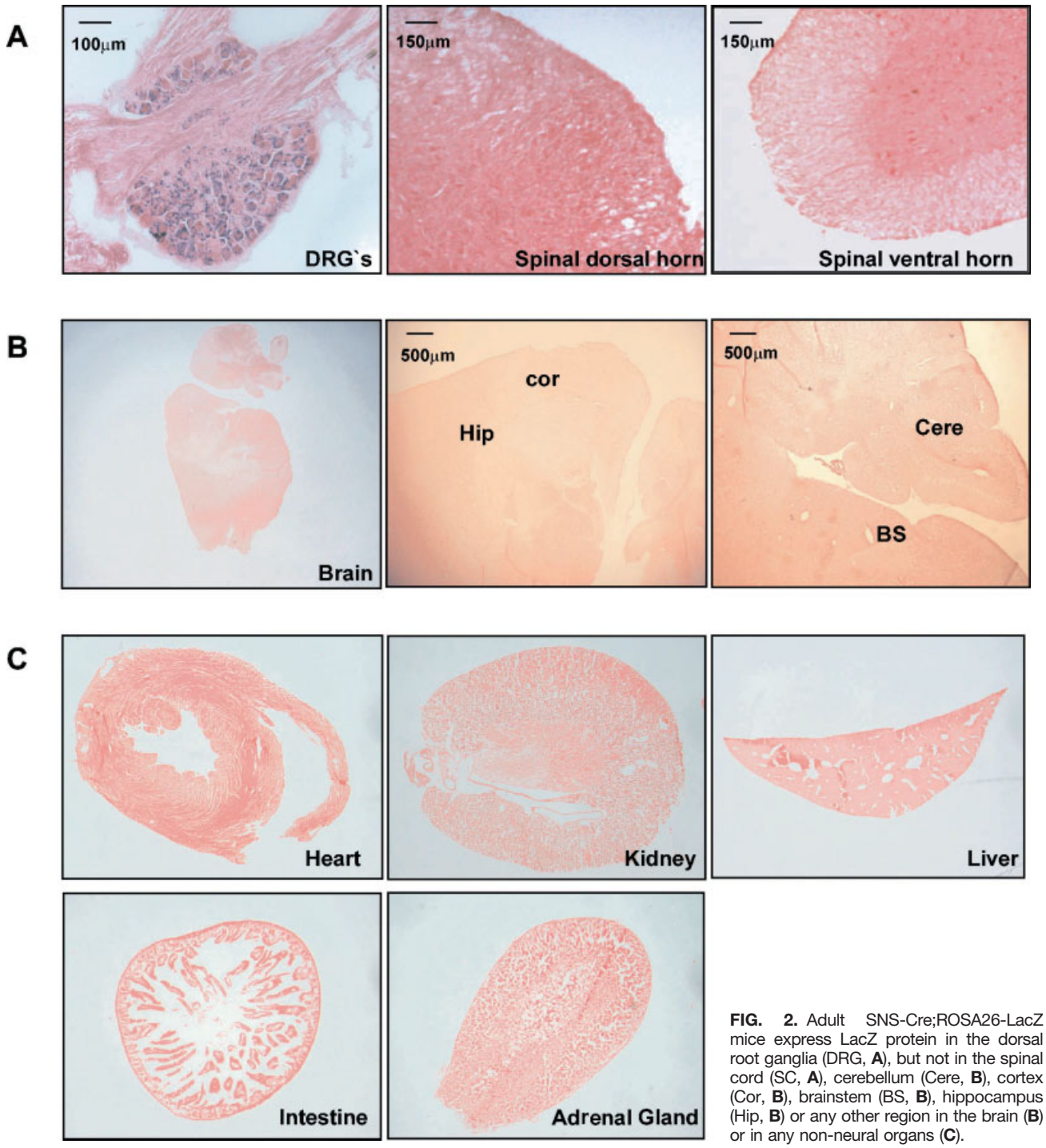

FIG. 2. Adult SNS-Cre;ROSA26-LacZ mice express LacZ protein in the dorsal root ganglia (DRG, **A**), but not in the spinal cord (SC, **A**), cerebellum (Cere, **B**), cortex (Cor, **B**), brainstem (BS, **B**), hippocampus (Hip, **B**) or any other region in the brain (**B**) or in any non-neural organs (**C**).

scopic analysis at higher magnification also yielded a complete lack of staining).

In order to characterize types of sensory neurons expressing Cre recombinase, we performed a systematic size analysis of stained adult DRG sections from SNS-Cre; ROSA26-LacZ double-positive progeny (Fig. 3A,B). A total of 93% of neurons with a cell diameter of $\leq$28 μm (small-diameter neurons, average size 16.0 $\pm$ 0.2) were positive for LacZ staining (see arrowheads in Fig. 3A, for example, Fig. 3B for average distribution). In contrast, 72% of large-diameter neurons ($\geq$28μm, average size 32 $\pm$ 1.2) failed to show LacZ staining (see arrows in Fig. 3A, for example, Fig. 3B for average distribution). We then sought to characterize the DRG neurons expressing Cre into specific subtypes based on staining with selected reagents, such as IB_4 and anti-substance P and

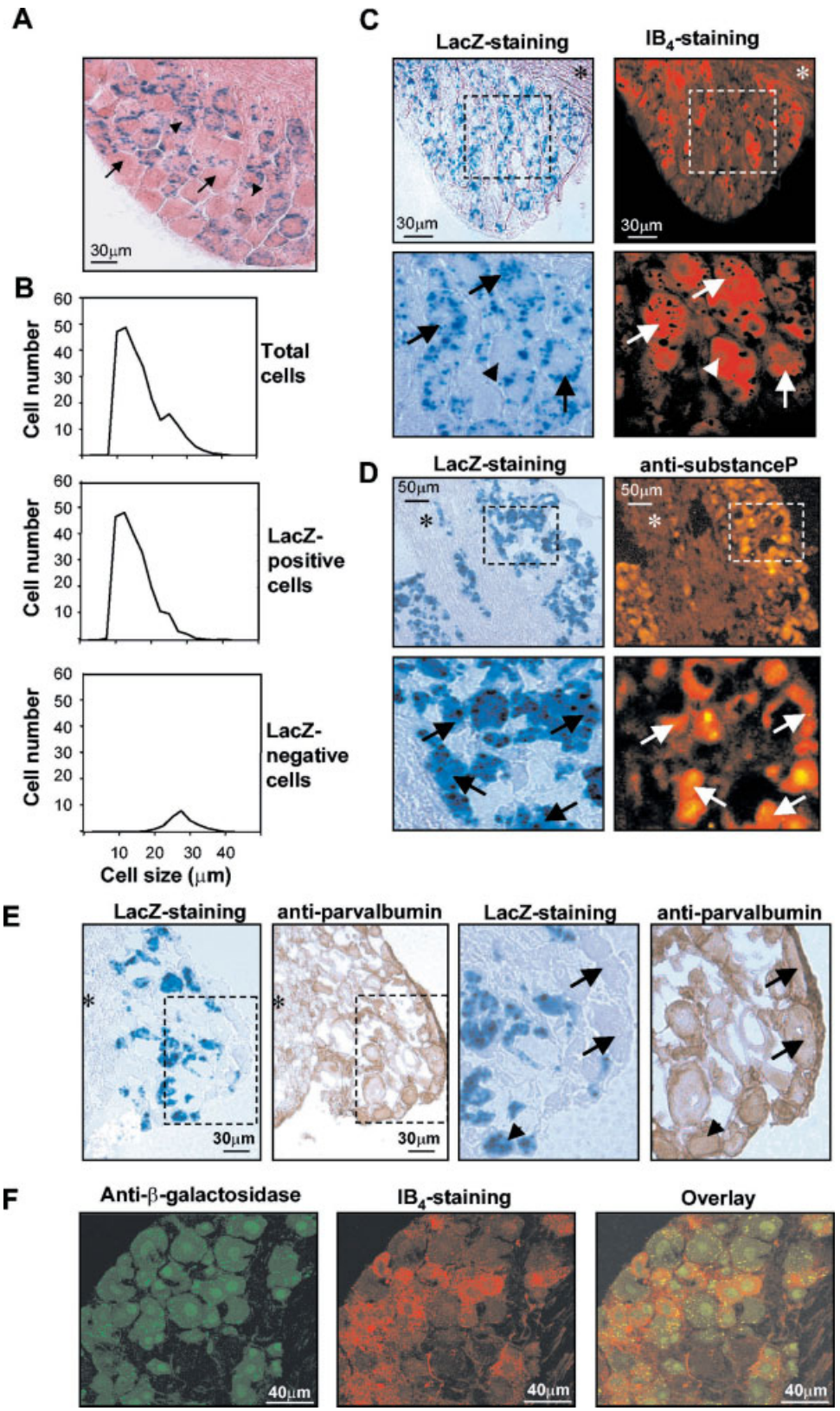

FIG. 3. Characterization of subtypes of DRG neurons expressing LacZ protein in adult SNS-Cre; ROSA26-LacZ double transgenic mice. **A:** Nearly all small-diameter neurons are LacZ-positive (arrowheads), but a majority of large-diameter neurons do not express LacZ (arrows). **B:** Size-distribution profile of Lac-positive and LacZ-negative DRG neurons in SNS-Cre;ROSA26-LacZ mice. A majority of cells lacking LacZ expression have a large diameter. Boxed area in **C, D, E** are magnified in adjoining panels. Coexpression in DRG sections was judged by double-labeling of sections (**C**) or by labeling of adjacent sections (**D,E**). Asterisks indicate the position of nerve fibers. Several LacZ-positive neurons bind isolectin-B_4 (IB_4, arrows in **C**) or express substance P (arrows in **D**). Very few IB_4-positive neurons are devoid of lacZ expression (arrowheads in **C**). In contrast, a majority of parvalbumin-positive large-diameter cells are devoid of LacZ expression (arrows in **E**), with a minority demonstrating coexpression (arrowhead in **E**). **F:** Confocal analysis of colabeling with anti-β-galactosidase antibody (FITC-labeled) and IB_4 (TRITC-labeled) showed that nearly all IB_4-positive cells expressed LacZ (overlay).

anti-parvalbumin antibodies. Approximately 36% and 34% of adult DRG neurons from SNS-Cre;ROSA26-LacZ mice demonstrated staining with IB_4 (Fig. 3C) and anti-substance P (Fig. 3D), respectively, consistent with a normal distribution of nociceptive neurons (Benn *et al.*, 2001) in SNS-Cre mice. About 95% of IB_4-positive neurons in DRGs from SNS-Cre, ROSA26-LacZ mice demonstrated LacZ expression (arrows in Fig. 3C). Similarly, 92% of substance P-positive neurons showed LacZ staining (arrows in Fig. 3D). Thus, nearly all IB_4-positive or substance P-expressing small-diameter DRG neurons of SNS-Cre mice functionally express the Cre recombinase. In contrast, a majority of parvalbumin-positive neurons (arrows in Fig. 3E) did not express LacZ in SNS-Cre; ROSA26-LacZ mice. Only 7% of parvalbumin-positive expressed LacZ (arrowhead in Fig. 3E). To unequivocally demonstrate coexpression of LacZ in nociceptors, DRG sections were colabeled with a monoclonal β-galactosidase antibody and TRITC-labeled IB_4 (Fig. 3F). Thus, nearly all small-diameter neurons, but only a minor fraction of large-diameter, parvalbumin-positive neurons express Cre recombinase in SNS-Cre mice.

Our next goal was to determine the developmental course of Cre expression in the DRG neurons of SNS-Cre mice. Whole-mount LacZ staining on SNS-Cre;ROSA26-LacZ mice at embryonic day 10 (E10, Fig. 4A) as well as sections derived from E17 embryos (cross-section of trunk and head shown in Fig. 4B) failed to demonstrate any functional expression of Cre recombinase, in contrast to positive controls that were processed in parallel (not shown). LacZ staining performed on SNS-Cre;ROSA26-LacZ mice on the day of birth (P0) revealed a functional expression of Cre recombinase in the dorsal root ganglia (Fig. 4C, arrow), but not in the spinal cord (Fig. 4C), brain (not shown), or brainstem (Fig. 4C) in a manner closely resembling the adult expression pattern. The trigeminal ganglia also demonstrated LacZ (arrowhead in Fig. 4C) staining in a pattern similar to dorsal root ganglia. A few neurons in the geniculate ganglion and the nodose ganglion were also weakly positive for LacZ staining (not shown). LacZ was not significantly detectable in sympathetic and parasympathetic ganglia and in all nonneural organs (for example, see Fig. 4C).

To rule out the possibility that expression of Cre in subsets of primary sensory neurons from birth onwards would affect their responses to somatic sensory stimuli, we recorded the latency and threshold of paw withdrawal following application of noxious heat and graded pressure, respectively, to the hindpaws of SNS-Cre mice (Hargreaves *et al.*, 1988; Walker *et al.*, 1999; Kolhekar *et al.*, 1997). Responses to thermal (Fig. 5A) and mechanical (Fig. 5B) stimuli were similar in SNS-Cre mice and their wildtype littermates.

Gene deletion studies in mice have provided significant insights into molecular mechanisms underlying reception, transduction, and processing of nociceptive and nonnociceptive sensory stimuli (Malmberg *et al.*, 1997; Caterina, 2000; Caterina and Julius, 1999; Julius and Basbaum, 2001). A common pitfall in constitutive and global mouse KOs is the lack of specificity of the site and onset of gene deletion (Nagy, 2000). A majority of candidate mediators for nociception or pathological pain are widely expressed in the peripheral and central nervous system (Woolf and Costigan, 1999; Caterina and Julius, 1999), which not only modulate the reception, gating, and processing of noxious and nonnoxious sensory inputs, but also emotion, anxiety, motivation, and mood. Furthermore, several behavioral pain studies involve the measurement of a motor response to a noxious sensory stimulus applied to a particular dermatome or viscerotome (Hargreaves *et al.*, 1988; Walker *et al.*, 1999; Kolhekar *et al.*, 1997), thereby raising the possibility that motor defects caused by gene deletion in motor neurons are erroneously read as changes in nociception and analgesia. Finally, many candidate genes proposed to be important in pathological pain are expressed in astrocytes and cells of the immune system, which can strongly modulate the course and analysis of pathological pain conditions such as in postinflammatory states. Thus, site-specific deletion of genes in individual avenues in pain pathways is a prerequisite for a rigorous analysis of pain and nociception in knockout mice. Here, ET recombination was used to drive the expression of Cre recombinase in a BAC harboring the native regulatory elements of the mouse Scn10a gene, a sensory neuron-specific sodium channel (Akopian *et al.*, 1996; Kozak and Sangameswaram 1996). Mice derived such enable Cre-loxP-mediated gene deletion selectively at the first avenue of the pain pathway, namely, primary sensory neurons of the DRG and TG, which are the most prominent sensory ganglia of the somatosensory nervous system. Sensory, motor, and emotional processing centers in the spinal cord and brain, as well as all nonneural tissues, were devoid of Cre expression.

Another distinguishing property of the SNS-Cre mice is that within the sensory ganglia expression of Cre is predominantly, but not exclusively, observed in subsets of neurons believed to play a crucial role in nociception and thermoreception. Double-labeling studies showed that nearly all peptidergic (substance P-positive) and nonpeptidergic (IB_4-positive) small-diameter neurons, which convey nociceptive and thermal inputs (Nagy and Hunt, 1982; Silverman and Kruger, 1990; Stucky and Lewin, 1999), expressed Cre recombinase in SNS-Cre mice. In contrast, less than a third of large-diameter DRG neurons, which mostly convey tactile and proprioceptive inputs (parvalbumin-positive, Patel *et al.*, 2003) express Cre in SNS-Cre mice. Thus, the SNS-Cre mice enable gene deletion in nearly all primary nociceptive neurons, likely without largely influencing gene expression in mechanoreceptive and proprioceptive neurons.

Another major disadvantage of knocking genes out constitutively stems from the potential developmental defects in nociceptive pathways or in other areas, which can occlude an objective analysis of pain responses. In rodents, the invasion and initial patterning of sensory axons into the spinal dorsal horn takes place over early embryonic stages between days 10–14 (Silos *et al.*, 1995;

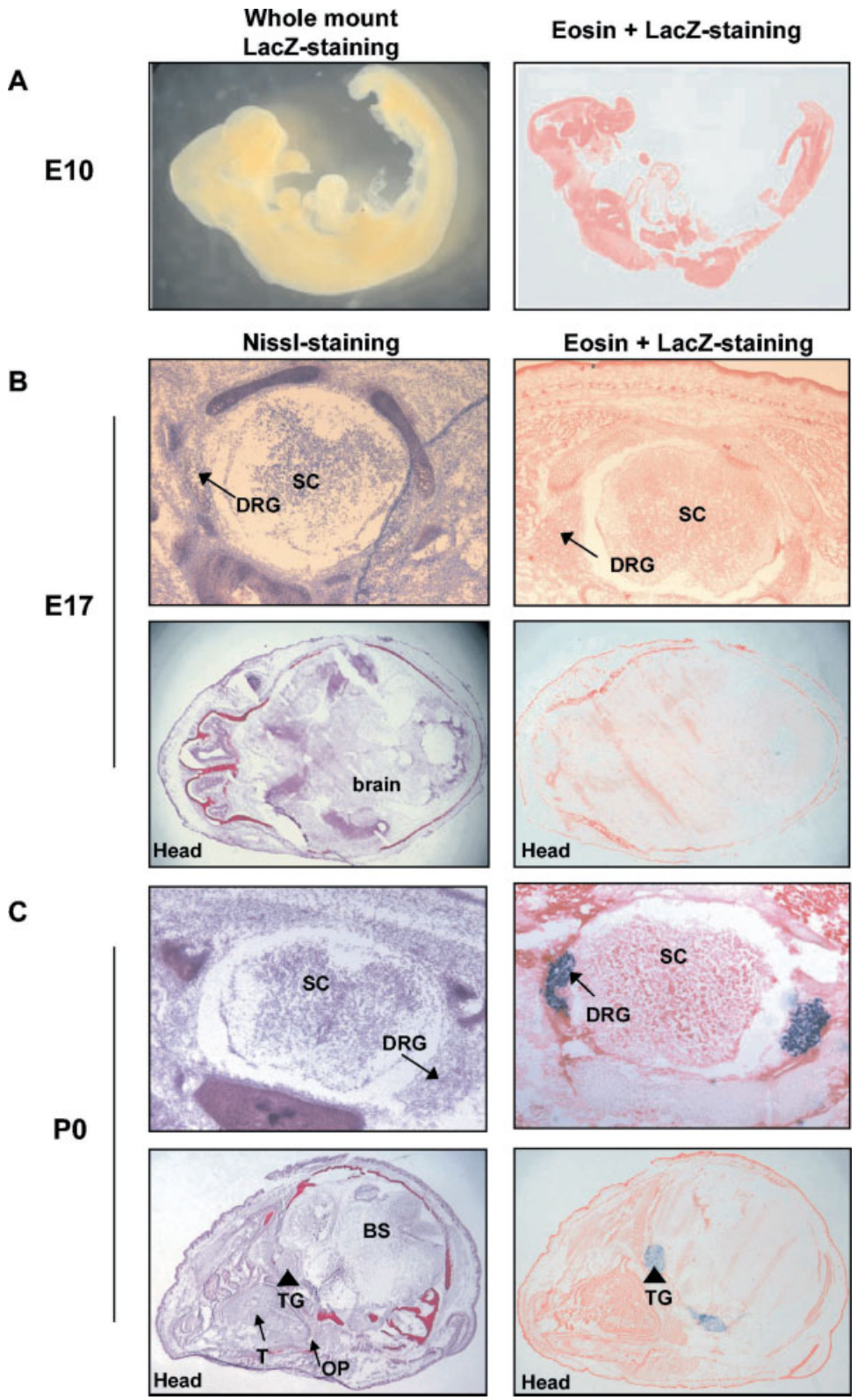

FIG. 4. Developmental course of Cre expression in SNS-Cre mice. LacZ staining was not evident in SNS-Cre;ROSA26-LacZ double-positive embryos at day 10 (E10, **A**) or 17 (E17, **B**) in the dorsal root ganglia (DRG), spinal cord (SC), brain, or other tissues. The right panel in **A** shows a eosin-counterstained section of the LacZ-stained whole-mount from the left panel. LacZ staining was first seen in SNS-Cre;ROSA26-LacZ double-positive mice on the day of birth (P0) in the DRGs (arrows in **C**) and trigeminal ganglia (TG, arrowheads in **C**), but not in the brainstem (BS) or other tissues (for example, OP, oropharynx, and T, tongue).

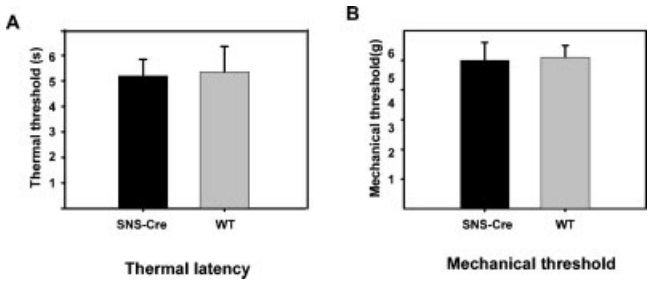

FIG. 5. Responses to nociceptive stimuli are normal in SNS-Cre mice. SNS-Cre mice are undistinguishable from wildtype littermates (WT) in terms of latency of paw withdrawal upon hindpaw application of noxious heat (**A**) or the mechanical threshold for eliciting paw withdrawal following hindpaw application of graded pressure (**B**).

Ozaki and Snider, 1997). In SNS-Cre mice, Cre-mediated gene deletion commences perinatally, thereby minimizing the risk of potential aberrations in spinal and peripheral trajectories of developing sensory neurons. However, some studies suggest that mechanoreceptive Aβ fibers reorganize in the spinal dorsal horn during postnatal periods (Fitzgerald *et al.*, 1994; Park *et al.*, 1999). Although the proportion of Aβ fibers expressing Cre recombinase in SNS-Cre mice is low, postnatal aberrations in the maturation of Aβ sensory afferents cannot be entirely ruled out when developmentally important genes are knocked out in SNS-Cre mice.

An important prerequisite for the usage of SNS-Cre mice in pain studies is that they themselves should not show any abnormalities in the processing of sensory inputs. Here, SNS-Cre mice failed to display any overt phenotype and were indistinguishable from wildtype littermates in terms of reception and processing of thermal and mechanical inputs. Thus, the SNS-Cre mice are ideally suited for achieving selective conditional gene expression in primary nociceptive neurons and provide a tool to explore the functions of proteins, which impart specific properties to nociceptors and spinal neurons in mouse models of physiological and pathological pain.

MATERIALS AND METHODS

Generation of Transgenic Construct

A 230-kb-large bacterial artificial chromosome (BAC) containing the mouse Scn10a locus flanked by NotI sites in pBeloBAC vector was identified by PCR from a 129-strain BAC library (BioCat, Heidelberg, Germany) and characterized. A cassette consisting of the Cre recombinase followed by a β-actin polyA sequence and a module containing the β-lactamase gene (ampicillin resistance) and a NotI restriction site flanked by frt sites was cloned into a cloning vector (pEGFP-N1, ClonTech, Palo Alto, CA) containing a marker for kanamycin resistance (Fig. 1A). The 200 bp genomic regions 5′ and 3′ to the coding ATG of the mouse Scn10a gene were cloned to flank the Cre cassette (Fig. 1A). The fragment consisting of the Cre cassette and the flanking homology arms were released by restriction digestion and purified as described previously (Zhang *et al.*, 1998; Muyrers *et al.*, 1999; Casanova *et al.*, 2001).

ET Recombination and Generation of Transgenic Mice

ET recombination was performed as described in former publications from the laboratory of Francis Stewart (Zhang *et al.*, 1998; Muyrers *et al.*, 1999; Casanova *et al.*, 2001). Recombinants were selected and screened for homologous recombination by PCR and Southern blotting (Fig. 1B). Furthermore, Southern blotting following Not1 digestion revealed that the coding ATG was 90 kb away from the 5′ end of the BAC (see Casanova *et al.*, 2002, fig. 1). The ampicillin gene with NotI site was excised from the positive clones by transient transfection of flp recombinase and correct recombinants were verified by Southern blotting (Zhang *et al.*, 1998; Muyrers *et al.*, 1999; Casanova *et al.*, 2001). The recombined BAC was released from the pBeloBAC vector by restriction digestion with NotI, isolated by pulse-field gel electrophoresis and agarase digestion, and purified on regenerated cellulose filters (MICROCON YM-30, Amicon, Germany). BAC DNA was dialyzed against microinjection buffer (10 mM Tris-HCl, 0.1 mM EDTA, and 100 mM NaCl) using nitrocellulose filters (Millipore, Germany) and injected into pronuclei derived from the hybrid B6D2 mouse strain (ZMBH, Heidelberg, Germany). Mice progeny thus obtained were analyzed for BAC insertion by genomic PCR amplification and backcrossed into the C57BL6 mouse genomic background.

LacZ Staining and Immunohistochemistry

To verify recombinase activity of the expressed Cre protein, two of the eight identified founders were mated with mice from the reporter transgenic line, GL(ROSA)26Sor^{tm1sor} (ROSA26-LacZ hereafter), which carry the gene encoding the β-galactosidase enzyme preceded by a stop codon, which is flanked by loxP sites (Soriano, 1999) (Jackson Laboratories, Bar Harbor, ME). Thus, in double-transgenic progeny, β-galactosidase protein (LacZ hereafter) is expressed selectively in tissues where Cre recombinase is active (Soriano, 1999). Cotransgenic progeny from SNS-Cre and ROSA26-LacZ mice were histologically analyzed for β-galactosidase activity (LacZ staining henceforth) in the DRGs, spinal cord, and brain in cryostat-sections (16 μm, Yamaguchi *et al.*, 1999). LacZ staining was done on whole-mount embryos at embryonic day 10 (E10, Yamaguchi *et al.*, 1999) or on 16 μm cryosections of E17 embryos, followed by Eosin counterstaining. Some adjacent sections were Nissl-stained as anatomical references.

To facilitate the identification of the DRG cell types, LacZ staining was performed on 16 μm sections followed by incubation with biotinylated *Griffonia simplicifolia* Isolectin-B4 (IB_4, 3.3 μg/ml, Vector Laboratories, Burlingame, CA) and streptavidin-TRITC (Dianova, Hamburg, Germany). Alternatively, double-immunofluorescence with a monoclonal β-galactosidase antibody

(ICN Pharmaceuticals, Frankfurt, Germany) and IB4 was performed on 16 μm DRG sections followed by confocal microscopy using a TCS-AOBS system (Leica, Bensheim, Germany). Furthermore, 7 μm adjacent sections were processed for LacZ staining or immunohistochemistry using monoclonal anti-substance P or anti-parvalbumin antibodies (Chemicon International, Temecula, CA) and TRITC-conjugated secondary antibodies (Dianova). The numbers of LacZ-stained cells were microscopically counted in four sections per DRG and costaining with IB_4 (in the same section), anti-substance P or parvalbumin antibodies (in adjacent sections). Then 30–40 DRGs were evaluated in total per transgenic line.

Testing of Paw Withdrawal Reflexes

All behavioral measurements were done on awake, unrestrained, and acclimatized mice. The mouse plantar test apparatus (Ugo Basile, Italy) was used to determine paw withdrawal latencies (in seconds, with a sensitivity of 0.1 sec) in response to noxious heat, which was applied via an infrared light source, as described in detail previously (Hargreaves *et al.*, 1988; Walker *et al.*, 1999; Kolhekar *et al.*, 1997). Latency measurements were done five times per paw. Similarly, von Frey filaments were utilized to apply graded pressure to the plantar surface of the hindpaws and mechanical stimulus thresholds were recorded in grams as described in detail previously (Hargreaves *et al.*, 1988; Walker *et al.*, 1999; Kolhekar *et al.*, 1997) (n = 4–5 per group).

Data and Statistics

All data are presented as mean ± SEM. Analysis of variance (ANOVA) for random measures was performed followed by post-hoc Scheffe's test. $P < 0.05$ was considered statistically significant.

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