

# Estrogen receptor $\beta$ is essential for sprouting of nociceptive primary afferents and for morphogenesis and maintenance of the dorsal horn interneurons

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Estrogen is known to influence pain, but the specific roles of the two estrogen receptors (ERs) in the spinal cord are unknown. In the present study, we have examined the expression of ER $\alpha$  and ER $\beta$  in the spinal cord and have looked for defects in pain pathways in ER $\beta$  knockout (ER $\beta^{-/-}$ ) mice. In the spinal cords of 10-month-old WT mice, ER $\beta$ -positive cells were localized in lamina II, whereas ER $\alpha$ -positive cells were mainly localized in lamina I. In ER $\beta^{-/-}$  mice, there were higher levels of calcitonin gene-regulated peptide and substance P in spinal cord dorsal horn and isolectin B4 in the dorsal root ganglion. In the superficial layers of the spinal cord, there was a decrease in the number of calretinin (CR)-positive neurons, and in the outer layer II, there was a loss of calbindin-positive interneurons. During embryogenesis, ER $\beta$  was first detectable in the spinal cord at embryonic day 13.5 (E13.5), and ER $\alpha$  was first detectable at E15.5. During middle and later embryonic stages, ER $\beta$  was abundantly expressed in the superficial layers of the dorsal horn. ER $\alpha$  was also expressed in the dorsal horn but was limited to fewer neurons. Double staining for ER $\beta$  and CR showed that, in the superficial dorsal horn of WT neonates [postnatal day 0 (P0)], most CR neurons also expressed ER $\beta$ . At this stage, few CR-positive cells were detected in the dorsal horn of ER $\beta^{-/-}$  mice. Taken together, these findings suggest that, early in embryogenesis, ER $\beta$  is involved in dorsal horn morphogenesis and in sensory afferent fiber projections to the dorsal horn and that ER $\beta$  is essential for survival of dorsal horn interneurons throughout life.

spinal cord | embryo | development | calretinin | pain

Estrogen is known to influence multiple functions in brain tissue, including neuronal development, plasticity and survival, neurotransmitter and neuropeptide synthesis, and neurotransmitter receptors (1–4). There are recent studies on the effects of estradiol on the spinal cord and on the peripheral nervous system (5, 6). Animal experiments as well as observations in humans have shown that somatosensory perception and pain sensitivity are influenced by estrogen (7–10), but little is known about the underlying mechanisms. In the rat and mouse, both estrogen receptor  $\alpha$  (ER $\alpha$ ) and ER $\beta$  have been shown to be expressed in the dorsal horn of adult spinal cords, in laminae I and II, an area involved in receiving and processing nociceptive information (11–14). In an animal model of inflammatory pain, it has been demonstrated that estradiol-induced analgesia can be reversed by tamoxifen (a selective ER modulator) (15).

To better understand the ontogenic role of ER $\alpha$  and ER $\beta$  in the spinal cord, it is necessary to know the evolution of the population of ER $\alpha$ - and ER $\beta$ -positive neurons during the course of development. Both ER $\alpha$  and ER $\beta$  are expressed in dorsal root ganglion (DRG) neurons during the early postnatal period, and both contribute to development and survival of these neurons (16). Developmental changes in the distribution of ER $\alpha$  immunoreactivity have been reported in neurons and fibers of rat prenatal and postnatal spinal cord (17), but knowledge of the distribution and function of ER $\beta$  in the spinal cord during embryogenesis is lacking. Our recent study (18) demonstrated

that ER $\beta$  is the predominant estrogen nuclear receptor in the brain during embryogenesis. At the protein level, ER $\beta$  is strongly expressed within laminated CNS structures, including the cerebral cortex, cerebellum, hippocampus, and olfactory bulb, and contributes to neuronal development in these areas (18).

The dorsal horn is also a cytoarchitecturally laminated region. The superficial laminae of the spinal cord (laminae I and II) receive primary afferent fibers that mostly convey nociceptive and thermoceptive inputs to associative and second-order neurons. Proper development of the superficial laminae of the dorsal horn is required for the function of sensory pathways (19–23). In mammals and birds, calbindin-D28K (CB)-positive and calretinin (CR)-positive neurons are mainly localized in the superficial areas of the dorsal horn, which are the sites for terminals of type A collaterals of primary afferents (24–29). Therefore, CB- and CR-positive neurons might be involved in spinal nociceptive processing, visceral regulation, and dorsal column sensory pathways.

In the present study, we demonstrate ER $\beta$  expression in the spinal cord during embryogenesis and use ER $\beta$  knockout (ER $\beta^{-/-}$ ) mouse embryos to explore a role of ER $\beta$  in dorsal horn morphogenesis, especially CB- and CR-positive interneurons located in the superficial layers. Given that this region of the dorsal horn processes nociceptive information, we extended our analysis of the targets of afferent projections to specific laminae in the dorsal spinal cord and expression of nociceptive receptors in DRG. These findings suggest that ER $\beta$  plays an important role in the dorsal horn development and thus affects sensory function and pain sensitivity.

## Results

### Pattern of ER $\beta$ and ER $\alpha$ Expression in the Developing Spinal Cord.

ER $\beta$  expression appeared in the embryonic spinal cord as early as embryonic day 13.5 (E13.5), and, at this time, most of the ER $\beta$ -positive cells were specifically localized in the superficial layers of the dorsal horn, with a few cells in the lateral and anterior part of mantle layer (Fig. 1A). At E14.5, ER $\beta$  expression in the spinal cord increased significantly. ER $\beta$ -positive neurons occupied laminae I–II. Distinct immunoreactive cells were also present in the lateral region of lamina V and near the central canal (Fig. 1B and C). At E15.5 and E16.5, strong ER $\beta$  expression was mainly seen in laminae I–II of the dorsal horn (Fig. 1D and E). Sagittal sections showed ER $\beta$  widely expressed in laminae I and II throughout the rostro-caudal part of the spinal cord, with the strongest signal

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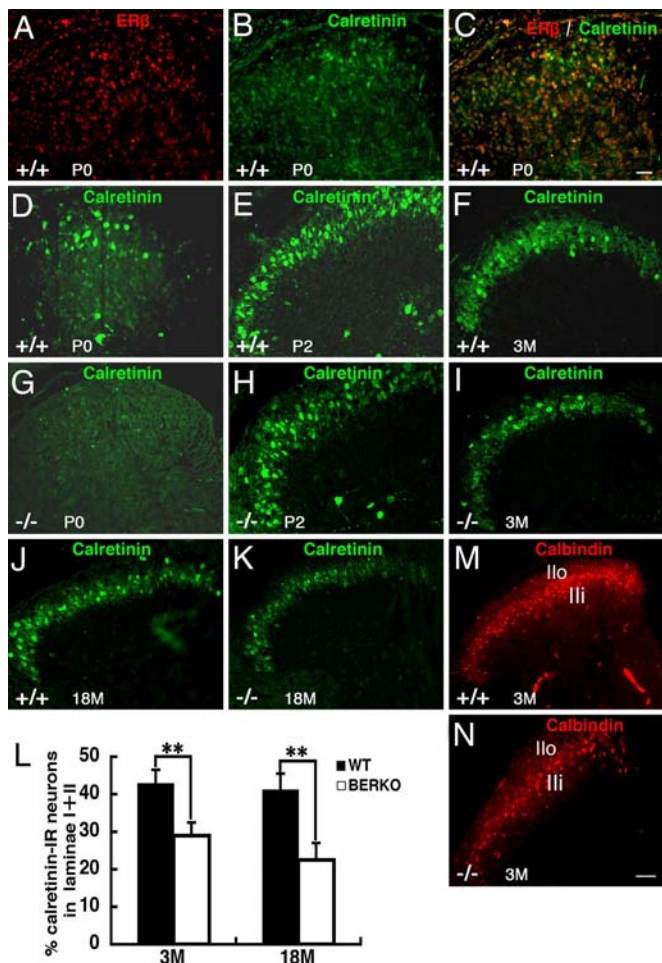
Conflict of interest statement: J.-Å.G. is a shareholder and consultant of Karo Bio AB.

Abbreviations: ER, estrogen receptor; DRG, dorsal root ganglion; CB, calbindin-D28K; CR, calretinin; Pn, postnatal day *n*; En, embryonic day *n*; NeuN, neuron-specific nuclear protein; SP, substance P; CGRP, calcitonin gene-regulated peptide; IB4, isolectin B4.

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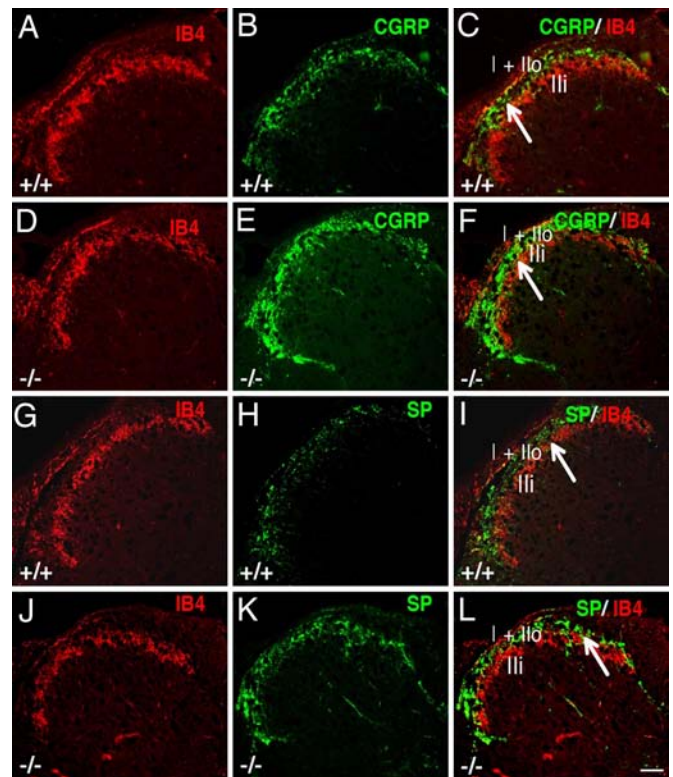




**Fig. 4.** Expression of calretinin and calbindin in the superficial layers of the dorsal horn in the lumbar region in postnatal WT and  $ER\beta^{-/-}$  female mice. (A–C) At P0, double staining for  $ER\beta$  and calretinin shows that, in the dorsal horn, most of the calretinin-positive neurons also express  $ER\beta$ . (D and G) There are fewer calretinin-positive cells in  $ER\beta^{-/-}$  mice (G) than in WT mice (D). (E and H) At P2, in  $ER\beta^{-/-}$  mice (H), there are fewer calretinin-positive cells in the medial part of lamina II compared with WT mice (E). (F and I–K) Expression of calretinin in lamina II is significantly lower in  $ER\beta^{-/-}$  mice (I and K) than WT mice (F and J) at 3 (F and I) and 18 (J and K) months of age. (L) The average percentage of calretinin-labeled cells in laminae I and II of spinal cord dorsal horn at 3 and 18 months of age is shown ( $n = 3$ ; error bar, SD; \*\*,  $P < 0.01$ , Student's  $t$  test).  $ER\beta^{-/-}$ . (M) In 3-month-old WT mice, calbindin is mainly localized in laminae I–II of the dorsal horn. (N) Decreased calbindin-labeled cells are seen in the outer layer II (Ilo) and lamina I of  $ER\beta^{-/-}$  mouse dorsal horn. (Scale bars: A–E, G, and H, 20  $\mu\text{m}$ ; F, I–K, M, and N, 50  $\mu\text{m}$ ).

superficial dorsal horn also expressed  $ER\beta$  (Fig. 4A–C). At this stage, few CR-positive cells were detected in the dorsal horn of  $ER\beta^{-/-}$  mice (Fig. 4G). At P2, most of the CR-labeled cells were localized in the lamina II, with some positive cells in lamina I (Fig. 4E); in  $ER\beta^{-/-}$  mice, there were fewer CR-positive cells in the medial part of lamina II (Fig. 4H). In the adult spinal cord, both CR and CB were strongly expressed in the lamina II; CR-positive cells mainly occupied the lateral part, whereas CB-positive cells mainly localized in the medial part (Fig. 4F, J, and M). In  $ER\beta^{-/-}$  mice, the number of CR-positive neurons in lamina II in mice at 3 and 18 months of age (Fig. 4I, K, and L) was much lower than that in age-matched WT mice. In  $ER\beta^{-/-}$  mice, a decrease in the number of CB-positive cells was also seen in the outer layer II (Ilo) at 3 months of age (Fig. 4N).

**Central Afferent Targeting Is Impaired in  $ER\beta$  Mutants.** The distribution of peptide-containing [substance P (SP)/calcitonin gene-



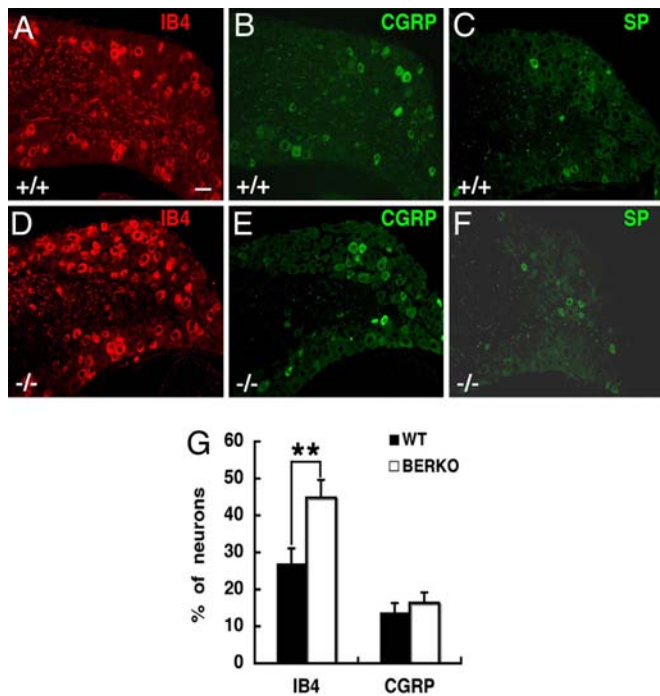
**Fig. 5.** Distribution of CGRP, SP, and IB4 in lumbar superficial dorsal horn of 3-month-old, female WT and  $ER\beta^{-/-}$  mice. (A, D, G, and J) IB4-labeled afferents terminate in inner lamina II and show similar expression pattern in  $ER\beta^{-/-}$  (D and J) and WT mice (A and G). (B, E, H, and K) Expression of CGRP and SP in superficial dorsal horn is higher in  $ER\beta^{-/-}$  (E and K) than in WT mice (B and H). (C, F, I, and L) Double labeling of IB4 and CGRP or SP clearly shows more CGRP or SP afferent innervation detected in lamina III in  $ER\beta^{-/-}$  mice (F and L) compared with WT mice (C and I). Arrows in C, F, I, and L indicate inner lamina II. (Scale bar: 20  $\mu\text{m}$ ).

regulated peptide (CGRP)] and nonpeptide-containing [isoleucine B4 (IB4)] thin primary afferents (C fibers) that terminate in laminae I–II was examined in female mice at 3 months of age. In WT mice, IB4<sup>+</sup> afferents project predominantly to inner lamina II (Iii) (Fig. 5A and G), whereas CGRP<sup>+</sup> and SP<sup>+</sup> peptidergic afferents predominantly project to lamina I and outer layer II (Ilo) and, to a lesser extent, to lamina III (Fig. 5B and H). Although the expression pattern of IB4 appeared similar in  $ER\beta^{-/-}$  (Fig. 5D and J) and WT mice (Fig. 5A and G), expressions of CGRP and SP in  $ER\beta^{-/-}$  mouse primary afferents (Fig. 5E and K) were higher than those in WT mice (Fig. 5B and H). Double labeling of IB4 and CGRP or SP clearly showed more CGRP and SP afferent innervation in lamina III in  $ER\beta^{-/-}$  (Fig. 5F and L) than in WT mice (Fig. 5C and I).

**Expression of Nociceptive Receptors in the DRG of  $ER\beta^{-/-}$  Female Mice at 3 Months of Age.** In addition to evaluating distribution of primary afferents in the dorsal horn of  $ER\beta^{-/-}$  mice, we also examined the CGRP-, IB4-, and SP-labeled cell bodies in lumbar (L4–5) DRG at 3 months of age. Expressions of CGRP and SP were similar in  $ER\beta^{-/-}$  (Fig. 6E and F) and WT mice (Fig. 6B and C), but the percentage of IB4-positive cells in DRG was significantly higher in  $ER\beta^{-/-}$  (Fig. 6D) than in WT mice (Fig. 6A and G).

## Discussion

In this study, we demonstrate that  $ER\beta$  is expressed in the spinal cord as early as E13.5. During middle and later embryonic ages,



**Fig. 6.** Expression of IB4, CGRP, and SP in the DRG of female WT and ER $\beta^{-/-}$  mice at 3 months of age. (A and D) The number of IB4-labeled cells in DRG is higher in ER $\beta^{-/-}$  mice (D) than in WT littermates (A). (B, C, E, and F) Expression of CGRP (B and E), and SP (C and F) is similar in ER $\beta^{-/-}$  (E and F) and WT mice (B and C). (G) The average percentages of DRG neurons expressing IB4 or CGRP in WT and ER $\beta^{-/-}$  female mice at 3 months of age are shown ( $n = 3$ ; error bar, SD; \*\*,  $P < 0.01$ , Student's  $t$  test). BERKO, ER $\beta^{-/-}$ . (Scale bar: 20  $\mu\text{m}$ .)

ER $\beta$  was strongly expressed in the dorsal horn, mainly in the laminae I and II. In contrast, ER $\alpha$ -positive neurons were not detected before E15.5, and the level of expression of ER $\alpha$  was lower than that of ER $\beta$ . Thus, ER $\beta$  is the predominant estrogen nuclear receptor involved in the development of the dorsal horn. At E15.5 and E16.5, in the dorsal horn, most of the ER $\alpha$ -positive cells also expressed ER $\beta$ . *In vitro* studies have reported that ER $\alpha$  and ER $\beta$  have the ability to heterodimerize when they coexist in the same nucleus and that hetero- and homodimers display different transcriptional activities (30–33). Colocalization of ER $\alpha$  and ER $\beta$  provides evidence that ER $\alpha$  and ER $\beta$  have the opportunity to interact *in vivo* within the spinal cord.

Our previous studies have demonstrated that ER $\beta$  is the predominant estrogen nuclear receptor in the brain during embryogenesis and that it contributes to embryonic and postnatal cortical development through influencing neuronal differentiation and migration (34, 35). In the cerebellum, ER $\beta$  expression occurs in each neuronal type at different postnatal days and is involved in the regulation of differentiation and maintenance of various types of neurons (36). In the present study, we demonstrated that there is retarded neuronal development in the dorsal horns of ER $\beta^{-/-}$  mice at E17.5. This was evident in the paucity of NeuN-labeled neurons in the dorsal horn and the loss of CR-positive neurons in the superficial layers. Double staining for ER $\beta$  and CR showed that, in the dorsal horn of WT neonates (P0), most of the CR-positive neurons in the superficial dorsal horn also expressed ER $\beta$ . In ER $\beta^{-/-}$  mice at P0, there were few CR-positive cells, indicating that the developmental neuronal deficit remained. Furthermore, ER $\beta$  is essential for interneuron survival throughout life because, in ER $\beta^{-/-}$  mice, there were abnormalities in distribution and number of interneurons in the adult spinal cord. The number of CR-labeled cells in lamina II in 3- and 18-month-old mice was much lower in ER $\beta^{-/-}$  than in

WT mice. There was also a decrease in CB-positive cells in the outer layer II (IIo) in ER $\beta^{-/-}$  mice at 3 months of age. The worsening of the neuronal losses with age suggests that ER $\beta$  is essential throughout life for maintenance of the integrity of sensory pathways in the spinal cord.

Our recent study (18) also demonstrated that ER $\beta$  is necessary for the development of CR-positive neurons in the embryonic mouse brain. All of these data suggest that ER $\beta$  can affect dorsal horn morphogenesis through modulating interneuron development of the superficial laminae of the dorsal horn.

To date, the exact function of CR and CB in the nervous system remains to be discovered. However, it has been proposed that the interneurons in the spinal cord that express these proteins may relate to sensory pathways (37–39). In the dorsal horn, DRG afferents innervate not only the secondary sensory neurons but also the lamina II interneurons, which can have either excitatory or inhibitory effects on the secondary sensory neurons such that pain signals can be modulated (40–42). Nociceptive neurons are specified early during development and precede the formation of synaptic contacts with their future peripheral or central targets (41). The establishment of laminae I and II is essential in the development and functional maturation of nociceptive circuits and subsequent processing of noxious and thermal sensitivity in mammals (43, 44). The level of ER $\beta$  has been implicated in altered stability of synaptic connections in the hippocampus (45). Therefore, CR- and CB-positive interneurons may contribute to establishing proper connections with the corresponding primary afferents.

We investigated the distribution of the primary afferents that target dorsal horn neurons. In 3-month-old WT mice, there were more CGRP- and SP-positive fibers in the dorsal horn of ER $\beta^{-/-}$  than in that of WT mice. In contrast, there was no difference in IB4-positive fibers between ER $\beta^{-/-}$  and WT mice. Peptidergic fiber terminals expressing SP and/or CGRP have been observed in laminae I–II at E18–19, whereas the IB4<sup>+</sup> subset of C fiber synaptic terminals appeared at P5 (46–49). Loss of ER $\beta$  had little effect on CGRP or SP in DRG neurons. We found that expression of CGRP and SP in lumbar (L4–5) DRG was similar in ER $\beta^{-/-}$  and WT mice but that there was higher IB4 expression in ER $\beta^{-/-}$  mice. On the basis of these data, we can infer that peptidergic fibers do carry peptides from the DRG to the dorsal horn but that, in ER $\beta^{-/-}$  mice, they have fewer interneurons in the dorsal horn with which to interact. This leads to accumulation of SP and CGRP in the afferent fibers.

A recent study has reported that ER $\beta$  401, a selective ER $\beta$  agonist, is antihyperalgesic in preclinical models of chemical-induced and acute inflammatory pain (50). Combining this information with our results, we can infer that endogenous ER $\beta$  receptor activation in the spinal cord in CR and CB neurons may be the site at which ER $\beta$  agonists can modulate pain sensitivity.

## Materials and Methods

**Animals and Tissue Preparation.** ER $\beta^{-/-}$  mice were generated as described in ref. 34. Heterozygous mice were used for breeding. ER $\beta^{+/-}$  female mice were mated overnight with ER $\beta^{+/-}$  males and inspected at 9:00 a.m. on the following day for the presence of vaginal plugs. Noon of this day was assumed to correspond to E0.5. All animals were housed in the Karolinska University Hospital Animal Facility (Huddinge, Sweden) in a controlled environment on a 12-h light/12-h dark illumination schedule and were fed a standard pellet diet with water provided ad libitum. To obtain embryos, pregnant mice were anesthetized deeply with CO<sub>2</sub> and were perfused with PBS followed by 4% paraformaldehyde (in 0.1 M PBS, pH 7.4). Embryos were taken out and put on ice, and spinal cords were dissected and postfixed in the same fixative overnight at 4°C. For the P0 and P2 pups, spinal cords were dissected and postfixed in 4% paraformaldehyde overnight at 4°C. Three- and 10-month-old mice were perfused individually

with PBS followed by 4% paraformaldehyde, and spinal cords and DRG were then removed and postfixed overnight. Sex was determined after direct visual inspection of the gonads with a dissecting microscope, and the tail and limbs were removed from each embryo for genotyping. Both male and female embryos were used to study ER $\beta$  expression, and there were no observable differences between them in our experiments. To explore ER $\beta$  function in the spinal cord, only female embryos and adult spinal cords were used in this study. After fixation, spinal cords and DRG were processed for either paraffin (6- $\mu$ m) or frozen (30- $\mu$ m) sections.

**Immunohistochemistry.** Paraffin sections were deparaffinized in xylene, rehydrated through graded alcohol, and processed for antigen retrieval by boiling in 10 mM citrate buffer (pH 6.0) for 2 min. The sections were incubated in 0.5% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min at room temperature to quench endogenous peroxidase and then were incubated in 0.5% Triton X-100 in PBS for 30 min. To block nonspecific binding, sections were incubated in 3% BSA for 1 h at 4°C. For ER $\beta$  staining, retrieval was improved by incubating the sections with 0.15 units/ml  $\beta$ -galactosidase for 2 h. Sections were then incubated with anti-ER $\beta$  1:200, anti-calretinin 1:2,000, anti-NeuN 1:200, anti-ER $\alpha$  1:200, anti-CGRP 1:500, anti-IB4 1:200, or anti-SP 1:100 in 1% BSA and 0.1% Triton X-100 overnight at room temperature. BSA replaced primary antibodies in negative controls. After washing, sections were incubated with the corresponding secondary antibodies in 1:200 dilutions for 2 h at room temperature. The Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used for the avidin-biotin complex (ABC) method according to the manufacturer's instructions. Peroxidase activity was visualized with 3,3'-diaminobenzidine (DAKO, Carpinteria, CA). The sections were lightly counterstained with hematoxylin, dehydrated through an ethanol series to xylene, and mounted. For immunofluorescence, slides were directly mounted in Vectashield antifading medium (Vector Laboratories). The sections were examined under a Zeiss (Göttingen, Germany) fluorescence microscope with filters suitable for selectively detecting the fluorescence of FITC (green) and Cy3 (red) or were examined under a light microscope. For colocalization, images from the

same section but showing different antigen signals were overlaid. Frozen sections were processed for detecting ER $\beta$  and ER $\alpha$  expression in 10-month-old spinal cords and for detecting calbindin in 3-month-old spinal cord. Sections were blocked for 30 min with 1% H<sub>2</sub>O<sub>2</sub> and followed by 10% normal serum, were rinsed three times with PBS, and were incubated overnight with the antibodies ER $\beta$  1:500, ER $\alpha$  1:500, and calbindin 1:1,000. These sections were processed further with biotinylated secondary antibodies for ER $\beta$  and ER $\alpha$  and with Cy3-labeled antimouse IgG for calbindin. Visualization was done with 3,3'-diaminobenzidine or with a fluorescence microscope.

**Data Analysis.** Stained spinal cord and DRG sections (10–12 sections for each mouse) were examined under a fluorescence microscope, and images were captured under  $\times 20$  magnification. Percentage of CR-immunopositive cells in laminae I–II and percentages of IB4 and CGRP in DRG were calculated. Estimates of the number of CR-immunoreactive cells in laminae I–II of lumbar spinal cords and IB4- and CGRP-labeled cells in L4–5 DRG were made based on the counts of the 10 images showing the highest number of labeled neurons. Statistical analysis was performed using Student's *t* test.

**Chemicals and Antibodies.** We purchased  $\beta$ -galactosidase and biotin-conjugated IB4 from Sigma-Aldrich (St. Louis, MO). The following antibodies were used: rabbit polyclonal anti-calretinin from Swant, (Bellinzona, Switzerland), mouse anti-calbindin and rabbit anti-CGRP from Sigma-Aldrich, rabbit polyclonal anti-ER $\alpha$  from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse anti-NeuN from Chemicon (Temecula, CA). The chicken polyclonal anti-ER $\beta$  503 was produced in our laboratory (18), Cy3 anti-SABC, Cy3 anti-mouse, and FITC anti-rabbit antibodies were from Jackson ImmunoResearch (West Grove, PA), and biotinylated goat anti-rabbit IgG and rabbit anti-chicken/turkey IgG were from Zymed (South San Francisco, CA).

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