Development/Plasticity/Repair

Tlx1 and Tlx3 Coordinate Specification of Dorsal Horn Pain-Modulatory Peptidergic Neurons

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The dorsal spinal cord synthesizes a variety of neuropeptides that modulate the transmission of nociceptive sensory information. Here, we used genetic fate mapping to show that Tlx3$^+$ spinal cord neurons and their derivatives represent a heterogeneous population of neurons, marked by partially overlapping expression of a set of neuropeptide genes, including those encoding the anti-opioid peptide cholecystokinin, pronociceptive Substance P (SP), Neurokinin B, and a late wave of somatostatin. Mutations of *Tlx3* and *Tlx1* result in a loss of expression of these peptide genes. Brn3a, a homeobox transcription factor, the expression of which is partly dependent on Tlx3, is required specifically for the early wave of SP expression. These studies suggest that Tlx1 and Tlx3 operate high in the regulatory hierarchy that coordinates specification of dorsal horn pain-modulatory peptidergic neurons.

Key words: **dorsal spinal cord; peptidergic neurons; Tlx3; cell fate specification; transcriptional regulation; pain**

Introduction

The dorsal horn of the spinal cord is an integrative center that processes and transmits somatic sensory information. Morphological and functional studies have revealed a tremendous diversity of dorsal horn neurons (Christensen and Perl, 1970; Lima and Coimbra, 1986; Todd and Spike, 1993; Han et al., 1998; Grudt and Perl, 2002; Todd and Koerber, 2006). Diversity of the dorsal horn neurons is further suggested by the expression of neuropeptides, including the opioid-like peptides Dynorphin (DYN) and enkephalin (ENK), the anti-opioid peptide cholecystokinin (CCK), the tachykinin peptides Substance P (SP) and Neurokinin B (NKB), somatostatin (SOM), and others (Marti et al., 1987; Todd and Spike, 1993; Todd and Koerber, 2006; Polgar et al., 2006). Functionally, neuropeptides modulate the transmission of somatic sensory information, particularly those involved with pain perception (Kajander et al., 1990; Xu et al., 1993; Wang et al., 2001; Wiesenfeld-Hallin et al., 2002).

The past decade has seen important progress in understanding dorsal horn neuron development (Caspary and Anderson, 2003; Helms and Johnson, 2003; Fitzgerald, 2005; Ma, 2006). Signals derived from the roof plate pattern the dorsal neural tube, such that precursors are divided into distinct compartments along the dorsoventral axis (Caspary and Anderson, 2003; Helms and Johnson, 2003). Early born neurons dorsal horn interneurons 1–6 (DI1–DI6) migrate ventrally and settle in deep dorsal horn laminas, whereas late born neurons (DIL_A and DIL_B) settle in superficial dorsal horn laminas (Caspary and Anderson, 2003; Helms and Johnson, 2003). With the exception of DI1–DI3 neurons, most dorsal horn neurons express Lbx1 at embryonic day 11.5 (E11.5) to E13.5 (Gross et al., 2002; Müller et al., 2002). Lbx1$^+$ neurons are divided into two populations, based on their nonoverlapping expression of the homeobox proteins Pax2 (DI4, DI6, and DIL_A) versus Tlx3 plus Lmx1b (DI5 and DIL_B) (Gross et al., 2002; Müller et al., 2002; Cheng et al., 2004). A set of transcription factors (TFs) acts to specify the excitatory versus the inhibitory neuron cell fates (Cheng et al., 2004, 2005; Glasgow et al., 2005; Mizuguchi et al., 2006; Hori et al., 2008). Lbx1 determines a basal GABAergic inhibitory neuron cell fate (Cheng et al., 2005). The homeobox proteins GSH1 and GSH2 control the expression of Tlx3, which in turn antagonizes Lbx1 to determine the glutamatergic excitatory neuron cell fate (Cheng et al., 2004, 2005; Mizuguchi et al., 2006). Ptf1a acts in combination with RBPjk (recombination signal binding protein for Igκ J region) to suppress Tlx3 expression and to promote GABAergic differentiation (Glasgow et al., 2005; Mizuguchi et al., 2006; Hori et al., 2008).

Despite this progress, transcriptional regulation of neuropeptides in the developing spinal cord is poorly understood. In this study, we used genetic fate mapping to show that Tlx3$^+$ neurons

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or their derivatives express a set of neuropeptides, including SP, CCK, NKB, and a late wave of SOM. Accordingly, expression of these peptide genes is eliminated in mice that lack *Tlx3* and *Tlx1*. Mechanistically, Tlx1 and Tlx3 activate a variety of downstream transcription factors, including the homeobox protein Brn3a, which controls the expression of an early wave of SP expression. Tlx1/3 therefore act as master regulators that coordinate the development of dorsal horn excitatory peptidergic neurons.

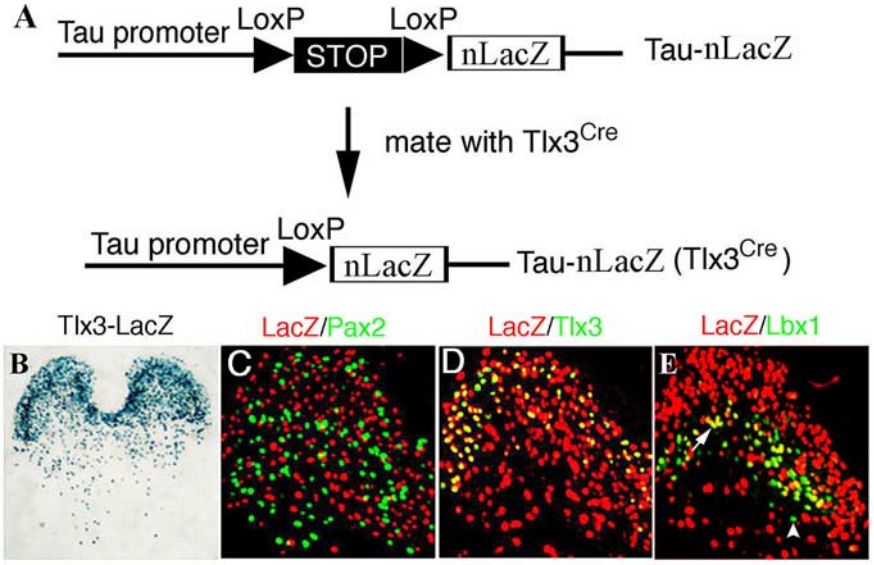

Figure 1. Persistent and transient Tlx3 expression revealed by fate mapping. ***A***, *Tau-nLacZ* reporter mice were crossed with *Tlx3*^{Cre} mice, allowing the removal of the "*STOP*" cassette, flanked with *loxP* sites (triangles), by Cre-mediated recombination. Subsequently, the expression of nLacZ protein is driven from the pan-neuronal *Tau* promoter. ***B–E***, Transverse sections through thoracic P7 spinal cord of *Tau-nLacZ*(*Tlx3*^{Cre}) mice. ***B***, X-gal staining marking the distribution of Tlx3^{+} neurons and their derivatives. ***C–E***, Double staining of the nLacZ protein product (***C–E***, red) plus Pax2 (***C***, green), Tlx3 (***D***, green), or Lbx1 (***E***, green, arrowhead) proteins. Neurons coexpressing nLacZ and Tlx3 (***D***) or Lbx1 (***E***, arrow) appear yellow. All Tlx3^{+} neurons showed either strong or weak expression of nLacZ (***D***) (data not shown).

Materials and Methods

Animals. The generation of *Tlx1* and *Tlx3* mutant mice, *Lbx1* mutant mice, FLPe deleter mice, and *Brn3a* mutant mice has been described previously (Roberts et al., 1994; Rodriguez et al., 2000; Shirasawa et al., 2000; Gross et al., 2002; Quina et al., 2005). The generation of *Tlx3*^{Cre} knock-in mice is described in supplemental Figure 1 (available at www.jneurosci.org as supplemental material). To fate map Tlx3-expressing neurons, the *Tlx3*^{Cre} knock-in mice were then crossed with Cre-dependent *Rosa26-lacZ* reporter mice (Soriano, 1999), as described in supplemental Figure 2 (available at www.jneurosci.org as supplemental material). *Tlx3*^{Cre} mice were also crossed with another Cre-dependent reporter line, *Tau-nLacZ* (Hippenmeyer et al., 2005), as described in Figure 1. In all timed matings, the morning that vaginal plugs were observed was considered to be E0.5. All animal procedures are contained in protocols reviewed and approved by the Animal Care Committees at the Dana-Faber Cancer Institute, Harvard Medical School.

In situ *hybridization and immunostaining.* Detailed methods for single- and double-color *in situ* hybridization (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) have been described previously (Qian et al., 2001). The following mouse *in situ* probes were amplified with gene-specific sets of PCR primers and cDNA templates prepared from postnatal day 0 (P0) or P7 mouse brain/spinal cords, including *Sst* (NM_009215; 0.5 kb), *Tac1* (NM_009311; 0.85 kb), *CCK* (NM_031161; 0.34 kb), *Pdyn* (NM_018863; 0.7 kb), *Penk1* (NM_001002927; 0.63 kb), and *Tac2* (D14423; 0.44 kb). Chick *Tac1* (BI395005; 0.4 kb) was amplified from cDNA from E10 chick spinal cord. *In situ* probes for dorsal horn functional genes (supplemental Fig. 7, available at www.jneurosci.org as supplemental material) were described previously (Qian et al., 2002; Cheng et al., 2004). To produce double-color *in situ* hybridization (supplemental Fig. 4, available at www.jneurosci.org as supplemental material), the first *in situ* hybridization signal [purple, with nitroblue tetrazolium (NBT)/5-bromo-4-chlor-indolyl-phosphate (BCIP) substrates] was photographed, followed by the development of the second signal [brown, with INT (iodonitro tetrazolium)/BCIP as substrates]. This sequential photographic process is helpful in determining whether a cell expresses a single gene or two genes.

The following antibodies were used for single or double immunostaining: rabbit anti-Pax2 antibody (Zymed Laboratories, South San Francisco, CA), rabbit anti-Brn3a antibody (E. Turner, University of California, San Diego, La Jolla, CA), and guinea pig anti-Lbx1, rabbit anti-Tlx3, and guinea pig anti-Tlx3 antibodies (T. Müller and C. Birchmeier, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany).

For *in situ* hybridization combined with fluorescent immunostaining, *in situ* hybridization was first performed without proteinase K treatment. After posthybridization washing, Tlx3, Pax2, Lbx1, or Brn3a proteins were detected by incubation with appropriate antibodies and then with Alexa-488-conjugated secondary antibody (1:200; Invitrogen, Carlsbad, CA) in PBS plus 0.1% Tween 20 solution. After the fluorescent signals were photographed, sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody, followed by development of the *in situ* hybridization signal with NBT/BCIP substrates. The bright-field views of the *in situ* hybridization images were inverted and then merged with the fluorescent images. This process avoids the masking of low-level fluorescent signals by nonfluorescent *in situ* signals.

Immunostaining combined with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining was also performed sequentially, with the immunostaining done first. The bright-field images of X-gal staining were inverted and then merged with the immunofluorescence images, thus avoiding the masking of low-level fluorescent signals by β-galactosidase (lacZ) staining signals. *In situ* hybridization combined with X-gal staining was performed by a similar sequential process, with the X-gal staining performed and photographed first.

In ovo *electroporation.* For electroporation studies in chick embryos, a cDNA fragment encoding a Myc-tagged mouse Brn3a fusion protein was cloned to the *RCASBP* chick viral expression vector (Morgan and Fekete, 1996) to produce the construct *RCAS-Brn3a*. The purified plasmid DNA was resuspended at concentrations of 5 μg/μl. *RCAS-Brn3a* plus a *GFP* expression vector, *pCAX-IRES-GFP* or *pCAX-GFP* (Gross et al., 2002), were coinjected into the spinal neural tubes of E2 chick embryos. After electroporation, the embryos were incubated at 39°C for an additional 72–120 h (E5–E7). Embryos with a high level of green fluorescent protein (GFP) fluorescence were fixed, and changes in the expression of genes of interest in the spinal cord were analyzed.

Cell counting. To count neurons that express *Tac1*, *Sst*, *Tac2*, and *Pemk1* per thoracic spinal section of E14.5 or E18.75 wild-type and *Tlx1/3* double mutants, three sets of thoracic transverse sections from three pairs of wild-type and mutant embryos (14 μm thickness) were hybridized with probes derived from the cDNAs for each peptide. Positive cells with clear nuclear morphology in the dorsal spinal cord were counted. Values were presented as mean ± SD. The differences in values were considered to be significant at $p < 0.05$ by Student's *t* test. To determine the percentage of Tlx3^{+}, Pax2^{+}, or Lbx1^{+} neurons that express a peptide gene, we again only counted those isolated cells with clear nuclear morphology.

Results

Generation of *Tlx3*^{Cre} knock-in mice

Tlx3 exhibits dynamic expression in the developing spinal cord (Qian et al., 2002). To follow the fate of those neurons derived from *Tlx3*$^{+}$ cells, we generated a *Tlx3*^{Cre} knock-in mouse line, in

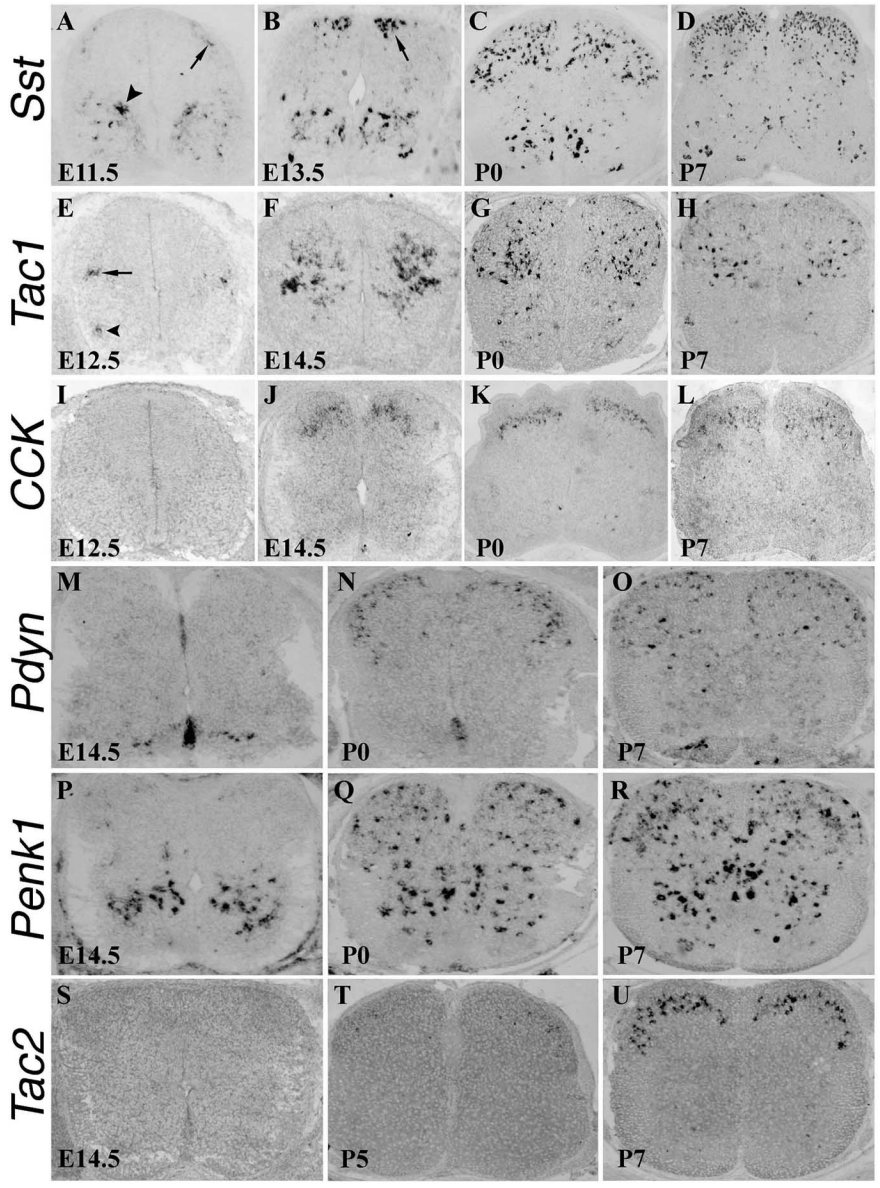

Figure 2. Expression of peptide precursor genes in the developing spinal cord. ***A–U***, *In situ* hybridization was performed on sections through thoracic spinal cord at various developmental stages using various peptide genes as the probes. ***A–D***, At E11.5, *Sst* expression was initiated in the dorsal spinal cord (***A***, arrow) but was already well established in the ventral spinal cord (***A***, arrowhead). By E13.5, a patch of *Sst*-expressing cells emerged in areas lateral to the dorsal midline (***B***, arrow). By P0, numerous *Sst*-expressing cells were present throughout the dorsal horn (***C***), and by P7, *Sst* expression was enriched in the superficial laminas (***D***). ***E–H***, At E12.5, *Tac1* expression was observed in the intermediate spinal cord area (***E***, arrow) and in the ventral horn at forelimb levels (***E***, arrowhead). By E14.5, *Tac1* expression was greatly expanded (***F***). From P0 to P7, most *Tac1*-expressing cells still occupied deep laminas, with the remaining few scattering through superficial laminas (***G***, ***H***). ***I–L***, *CCK*-expressing cells first emerged at E14.5 in intermediate dorsal horn laminas, and the expression persists at P0–P7. ***M–R***, *Pdyn*-expressing and *Penk1*-expressing cells emerged in the ventral spinal cord at E14.5 (***M***, ***P***) and then expanded to the dorsal horn from E16.5 to P0 (***N***, ***Q***) (data not shown). From P0 and on, *Pdyn*-expressing cells were enriched in superficial laminas (***N***, ***O***), and *Penk1*-expressing cells were scattered throughout the spinal cord (***Q***, ***R***). ***S–U***, *Tac2*-expressing neurons emerged from P5 to P7 in an intermediate layer.

which the *Cre* recombinase gene was inserted into the first coding exon of the *Tlx3* locus (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). To determine whether $Tlx3^{Cre}$ expression faithfully reflects *in vivo Tlx3* expression, we crossed $Tlx3^{Cre}$ mice with a Cre-dependent lacZ reporter line, *ROSA26-LacZ* (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (Soriano, 1999). In *ROSA26-LacZ*($Tlx3^{Cre}$) mice, Cre-mediated removal of a transcriptional termination cassette allows a constitutive expression of the lacZ protein product, β-galactosidase (Soriano, 1999). Consequently, all derivatives that undergo successful Cre-mediated DNA recombination are labeled by X-gal staining (also called lacZ staining). Examined at E11.5, *ROSA26-LacZ*($Tlx3^{Crei}$) embryos exhibited a lacZ staining pattern that matched endogenous *Tlx3* expression revealed by whole-mount *in situ* hybridization (supplemental Fig. 2, available at www.jneurosci.org as supplemental material), demonstrating that $Tlx3^{Cre}$ mice are an effective tool for fate-mapping experiments.

Persistent and transient Tlx3 expression in different dorsal horn laminas

To facilitate fate-mapping experiments, we next crossed $Tlx3^{Cre}$ mice with another Cre-dependent reporter mouse line, *Tau-nLacZ*, with the resulting double heterozygous mice referred to as *Tau-nLacZ*($Tlx3^{Cre}$) mice. After Cre-mediated removal of a transcriptional termination cassette, this reporter gene encodes β-galactosidase linked to a nuclear localization signal (nLacZ) and is driven from the pan-neuronal *Tau* promoter (Fig. 1*A*) (Hippenmeyer et al., 2005). In *Tau-nLacZ*($Tlx3^{Cre}$) mice at P7, X-gal staining showed that $nLacZ^+$ neurons were enriched in the dorsal spinal cord but also present in small numbers in the ventral spinal cord (Fig. 1*B*). $Tlx3^+$ cells normally give rise to glutamatergic neurons that are intermingled with inhibitory interneurons marked by the expression of Pax2 (Cheng et al., 2004). Consistent with this, virtually no $nLacZ^+$ neurons coexpressed Pax2 (Fig. 1*C*), providing a key validation of the fidelity of nLacZ expression in *Tau-nLacZ*($Tlx3^{Cre}$) mice. Double staining of Tlx3 protein and nLacZ showed that neurons with persistent Tlx3 expression ($nLacZ^+$;$Tlx3^+$) were enriched in the superficial dorsal horn, whereas neurons with transient Tlx3 expression ($nLacZ^+$;$Tlx3^-$) were distributed throughout the spinal cord but are enriched in areas from deep dorsal horn laminas to the ventral spinal cord (Fig. 1*D*). Transient Tlx3 expression in a subset of dorsal horn neurons is consistent with the previous finding that Tlx3 expression is switched off in DI3 and a portion of DI5 neurons that settle in deep dorsal horn laminas (Qian et al., 2002).

Most dorsal horn neurons, including $Tlx3^+$ excitatory neurons and $Pax2^+$ inhibitory neurons, develop from $Lbx1^+$ cells (Gross et al., 2002; Müller et al., 2002). In P7 spinal cord of *Tau-nLacZ*($Tlx3^{Cre}$) mice, Lbx1 protein, however, was virtually absent in a majority of $nLacZ^+$ neurons (Fig. 1*E*), as well as in most $Pax2^+$ neurons (data not shown), implying a transient Lbx1 expression in most dorsal horn neurons. Residual $Lbx1^+$ neurons

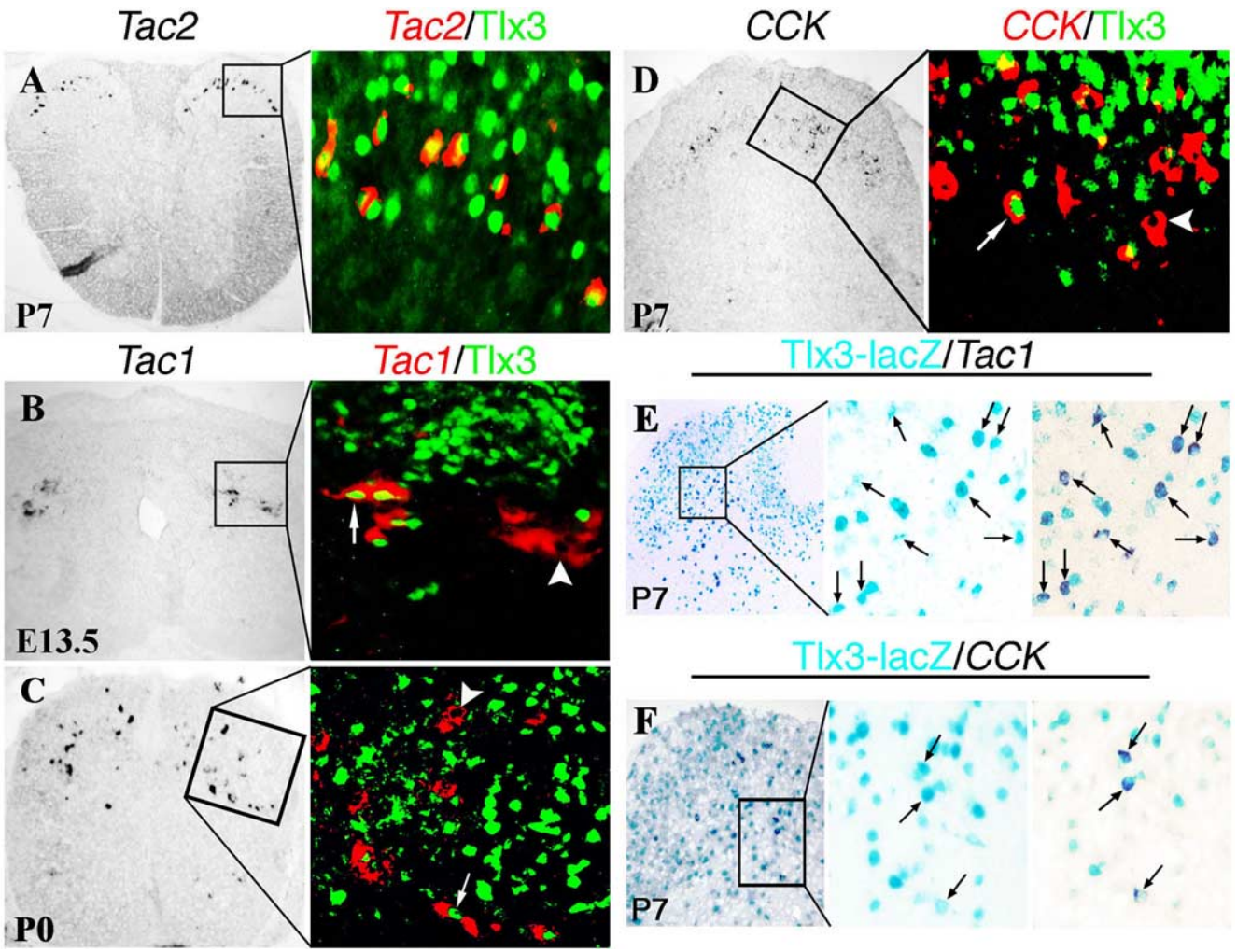

Figure 3. Expression of *Tac2*, *Tac1*, and *CCK* in Tlx3$^+$ neurons or their derivatives. ***A–D***, Double staining of Tlx3 protein (green) with *Tac2*, *Tac1*, or *CCK* mRNAs (red) on thoracic spinal sections at indicated stages. Note the consistent coexpression of *Tac2* with Tlx3 (***A***), whereas *Tac1* and *CCK* were expressed in both Tlx3$^+$ (arrows) and Tlx3$^-$ neurons (arrowheads). Only cells with clear nuclear morphology were analyzed. ***E***, ***F***, A combination of lacZ staining and *in situ* hybridization. Thoracic spinal sections of P7 *Tau-nLacZ(Tlx3^{Cre})* mice were first subjected to lacZ staining (blue). After photographing, the sections were then subjected to *in situ* hybridization (purple staining). Note a coexpression of lacZ with *Tac1* (***E***, arrows) or *CCK* (***F***, arrows).

were located in an intermediate dorsal horn lamina, most of which derive from Tlx3$^+$ neurons, as indicated by the coexpression of Lbx1 and nLacZ (Fig. 1*E*, arrows). In summary, both Tlx3 and Lbx1 exhibit dynamic expression in the developing spinal cord.

Tlx3$^+$ neurons/derivatives and Pax2$^+$ neurons express two distinct sets of peptide genes

We next examined the expression of the following six peptide genes: *Tachykinin 1* (*Tac1*) encoding the precursor for the SP and Neurokinin A, *Tachykinin 2* (*Tac2*) encoding the precursor for the NKB, *CCK* encoding the precursor for CCK peptides, *Somatostatin* (*Sst*) encoding the precursor for SOM, *Prodynorphin* (*Pdyn*) encoding the precursor for DYN, and *Preproenkephalin 1* (*Penk1*) encoding the precursor for ENK.

Figure 2 shows the spatial and temporal expression patterns of these peptide genes. Several features are noteworthy. First, expression of different peptide genes is established at distinct developmental stages. In the dorsal spinal cord, *Sst* expression starts at E11.5, followed by *Tac1* expression at E12.5, *CCK* expression at E14.5, *Pdyn* and *Penk1* expression at E16.5–P0, and finally *Tac2* expression at P5–P7 (Fig. 2) (data not shown). Second, as reported previously (Todd and Spike, 1993), each peptide gene exhibits a unique lamina-specific expression pattern (Fig. 2). Specifically, in the P7 spinal cord, *Sst* expression is enriched in superficial laminas but is also widely distributed, *Tac2* and *CCK* expression is confined to the intermediate laminas, *Tac1* expression is enriched in the deep laminas, *Pdyn* expression is enriched in superficial laminas, and *Penk1* expression is widely distributed (Fig. 2).

To better understand the relationship between transcriptional regulators and neuropeptide phenotype in the dorsal spinal cord, we undertook a series of double-staining experiments that combined *in situ* hybridization with peptide cDNAs as the probes and immunostaining with Tlx3 or Pax2 antibodies (Figs. 3, 4). *Tac2* expression was confined to a subset of Tlx3$^+$ neurons in intermediate laminas of P7 dorsal spinal cord (Fig. 3*A*). Only a portion of *Tac1*-expressing neurons expressed Tlx3 at E13.5 (Fig. 3*B*). At P0 or P7, ~22.5% (124 of 551) of *Tac1*-expressing neurons and 21.2% (95 of 442) of *CCK*-expressing neurons coexpressed Tlx3 (Fig. 3*C*,*D*). However, in P7 *Tau-nLacZ(Tlx3^{Cre})* fate-mapping mice, *Tac1* and *CCK* expression was confined exclusively to nLacZ$^+$ neurons (Fig. 3*E*,*F*), implying that all *Tac1*-expressing and *CCK*-

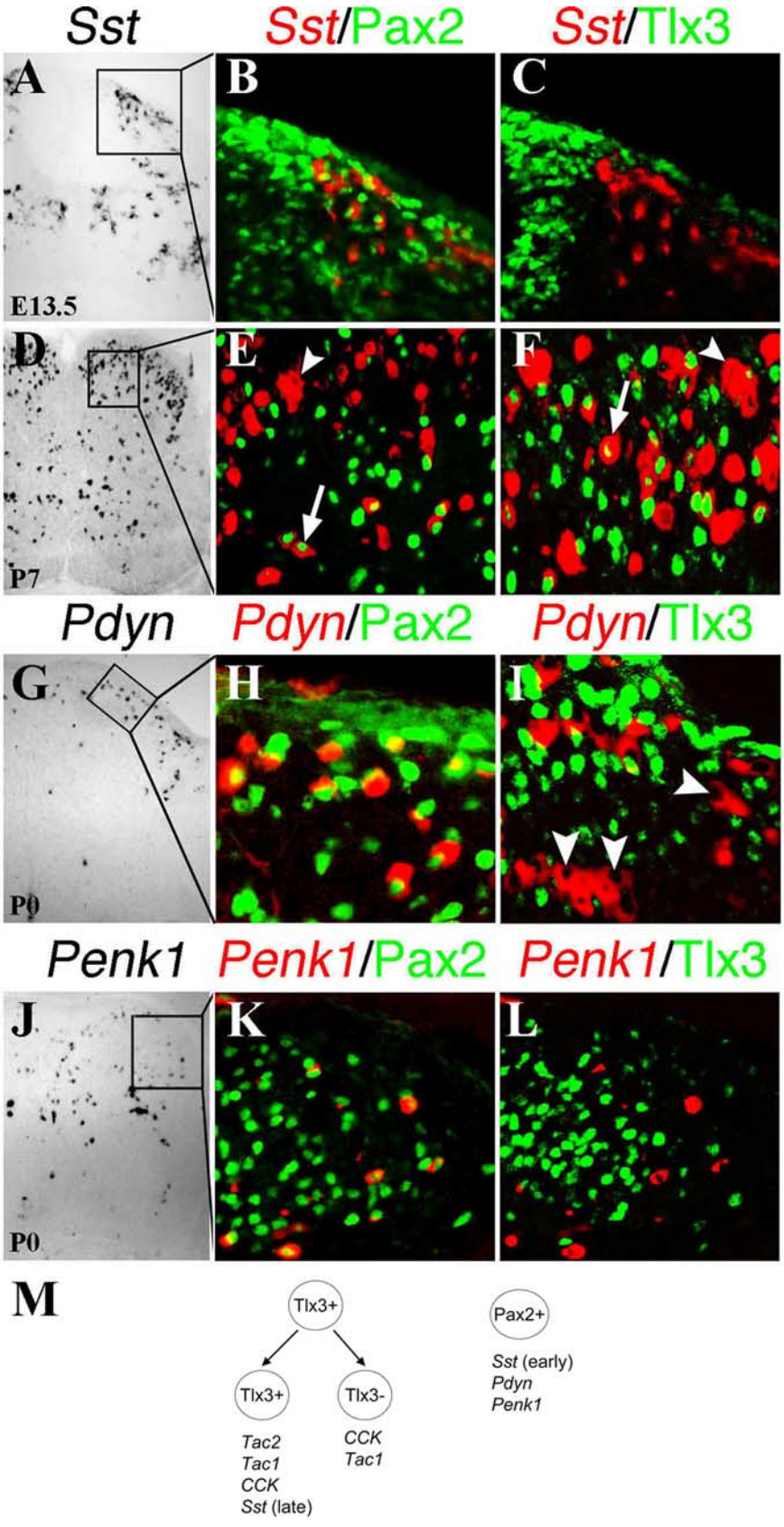

Figure 4. Expression of *Sst*, *Pdyn*, and *Penk1* in Pax2$^+$ or Tlx3$^+$ neurons. ***A–L***, Double staining of Pax2 protein (***B***, ***E***, ***H***, ***K***, green) or Tlx3 protein (***C***, ***F***, ***I***, ***L***, green) with *Sst* (***A–F***, red), *Pdyn* (***G–I***, red), or *Penk1* (***J–L***, red) mRNA on spinal sections at indicated stages. Bright-field *in situ* hybridization signals were converted into red pseudo-fluorescent signals. ***A–C***, Note a colocalization of *Sst* with Pax2 (***B***) but not with Tlx3 (***C***) in E13.5 lumbar dorsal horn. ***D–F***, In P7 dorsal horn, Pax2 was expressed in deep lamina *Sst*-expressing neurons (***E***, arrow) but not in superficial *Sst*-expressing neurons (***E***, arrowhead). At this stage, a portion of *Sst*-expressing neurons in superficial laminas coexpressed Tlx3 (arrow). Fate mapping showed that the remaining expressing neurons are derived from Tlx3$^+$ neurons, but Tlx3 expression is transient in most of these peptidergic neurons.

Sst exhibited a more complex expression pattern. At E13.5, ~65.3% (68 of 104) of *Sst*-expressing cells in the dorsal spinal cord expressed Pax2 (Fig. 4*B*), but none of them expressed Tlx3 (Fig. 4*C*). At P7, cells coexpressing Pax2 and *Sst* were confined to deep laminas (Fig. 4*E*). At this stage, a new population of *Sst*-expressing neurons was detected in superficial laminas that coexpressed Tlx3 (Fig. 4*F*). Further examination of *Sst* expression in *TaunLacZ(Tlx3^{Cre})* mice showed that a majority of *Sst*-expressing neurons in superficial laminas were nLacZ$^+$ (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) and were thus derived from Tlx3$^+$ neurons. Therefore, early and late waves of *Sst*-expressing neurons are primarily associated with Pax2$^+$ and Tlx3$^+$ neurons (and their derivatives), respectively, although some *Sst*-expressing neurons may develop from cells that do not express Pax2 or Tlx3. *Pdyn* and *Penk1* were expressed in neurons that coexpressed Pax2 (Fig. 4*H*,*K*) but not Tlx3 (Fig. 4*I*,*L*).

In summary, Tlx3$^+$ neurons or their derivatives express *Tac1*, *Tac2*, *CCK*, and a late wave of *Sst*, whereas Pax2$^+$ neurons express a different set of peptide genes, including *Pdyn*, *Penk1*, and an early wave of *Sst* (summarized in Fig. 4*M*). Double-color *in situ* hybridizations further showed that *Tac1*, *Tac2*, *CCK*, and *Sst* exhibited partially overlapping expression patterns (supplemental Fig. 4, available at www.jneurosci.org as supplemental material), thereby revealing a tremendous diversity of dorsal horn peptidergic neurons.

Tlx1 and *Tlx3* are required for peptide gene expression

We next analyzed peptide gene expression in mice that lacked both *Tlx3* and its related gene *Tlx1*, because *Tlx3* and *Tlx1* exhibit a partial redundancy in cervical and thoracic spinal cord (Cheng et al., 2004). Expression of *Tac1* and *CCK* in the dorsal spinal cord was virtually eliminated in *Tlx1/3*$^{-/-}$ mice, from E13.5 to E18.75 (Figs. 5*A–D*, 6). Because increased

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Tlx3-negative *Sst*-expressing neurons in superficial laminas (***F***, arrowhead) were derived primarily from Tlx3$^+$ neurons (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). ***G–L***, Colocalization of *Pdyn* and *Penk1* with Pax2 (***H***, ***K***) but not Tlx3 (***I***, ***L***). Arrows in ***I*** indicate *Pdyn*-expressing cells with clear nuclear morphology that lack Tlx3 expression. ***M***, Schematics show two distinct set of peptides that are associated with Tlx3$^+$ neurons (and their derivatives) and Pax2$^+$ neurons, respectively.

cell death has not been observed in *Tlx1/3*$^{-/-}$ spinal cords during embryonic development (Qian et al., 2002), the simplest interpretation of these results is that *Tlx1/3* are required to establish these peptidergic transmitter phenotypes.

Tlx1/3, however, exerted both negative and positive effects on *Sst* expression. At E14.5, the number of *Sst*-expressing neurons in dorsal thoracic spinal cord increased by fivefold in *Tlx1/3*$^{-/-}$ embryos compared with wild-type embryos (Fig. 5*I*,*J*,*M*), and most of these *Sst*-expressing neurons were confined to the intermediate and deep dorsal laminas (Fig. 5*J*). We previously reported that there is a marked increase of Pax2^{+} neurons in *Tlx1/3*$^{-/-}$ spinal cord (Cheng et al., 2004). Surprisingly, a double staining of *Sst* and Pax2 showed that only 28.2% of *Sst*-expressing neurons in E14.5 *Tlx1/3*$^{-/-}$ dorsal horn coexpressed Pax2 (supplemental Fig. 5, available at www.jneurosci.org as supplemental material), implying that most of these ectopic *Sst*-expressing neurons were derived from *Tlx1/3*$^{-/-}$ cells that are incapable of switching on Pax2 expression. A potential source could be DI3 interneurons that express Tlx3, but not Lbx1, which is required for Pax2 expression (Helms and Johnson, 2003; Fitzgerald, 2005).

By E18.75, *Sst* expression in the superficial dorsal horn, which is primarily derived from *Tlx3*$^{+}$ neurons, was eliminated in *Tlx1/3*$^{-/-}$ mice (Fig. 5*K*,*L*), whereas *Sst* expression in deep laminas was not affected (Fig. 5*L*). As a result of this, there is a fivefold reduction in the number of *Sst*-expressing neurons in the dorsal spinal cord of E18.75 *Tlx1/3*$^{-/-}$ mice compared with wild-type mice (Fig. 5*M*). These data suggest a dual function of *Tlx1/3*: activating and repressing *Sst* expression in superficial and deep dorsal horn laminas, respectively.

Expression of *Pdyn* and *Penk1*, which are confined to Pax2^{+} cells in wild-type embryos, did not exhibit obvious changes in E18.75 *Tlx1/3*$^{-/-}$ mice (Fig. 5*E–H*). The number of *Penk1*-expressing cells per dorsal horn section at thoracic axial levels was 115 ± 12 in wild-type mice and 123 ± 17 in *Tlx1/3*$^{-/-}$ mice ($p > 0.5$). The numbers of *Pdyn*-expressing cells in E18.75 dorsal spinal cord were also comparable, 65 ± 6 in wild-type mice versus 69 ± 4 in *Tlx1/3*$^{-/-}$ mice. However, the distribution of *Pdyn*-expressing cells may have been slightly affected, with an apparent increase of the density of *Pdyn*-expressing cells in the superficial laminas (Fig. 5, compare *H* and *G*). We previously showed that mutations of *Tlx1* and *Tlx3* result in a transformation of glutamatergic neurons into Pax2^{+} GABAergic neurons (Cheng et al., 2004). The lack of a significant increase of *Pdyn*-expressing and *Penk1*-expressing neurons suggests an incomplete switch in cell fate.

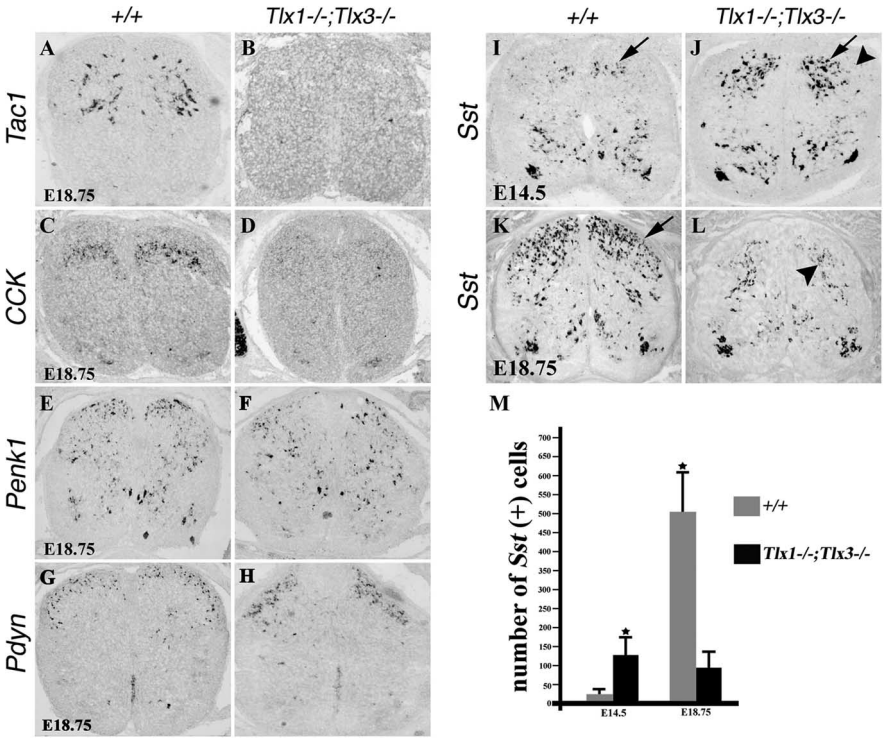

Figure 5. Loss of peptide gene expression in *Tlx1/3*$^{-/-}$ mice. ***A–L***, *In situ* hybridization was performed on sections through wild-type or *Tlx1/3*$^{-/-}$ thoracic spinal cords at E14.5 or E18.75. ***A–D***, Note a loss of *Tac1* and *CCK* expression in mutants. ***E–H***, *Penk1* and *Pdyn* expression was not reduced (as indicated by quantitative data; see Results). ***I***, ***J***, *Sst* expression was expanded in the deep dorsal horn of E14.5 *Tlx1/3*$^{-/-}$ embryos (arrows). However, no *Sst* expression was detected in the most superficial dorsal horn (***J***, arrowhead). ***K***, ***L***, *Sst* expression was lost in the superficial dorsal horn of E18.75 *Tlx1/3*$^{-/-}$ mice (arrow), whereas expression in deep dorsal horn laminas persisted (***L***, arrowhead). ***M***, Quantitative data showed the numbers of *Sst*-expressing neurons per wild-type or *Tlx1/3*$^{-/-}$ dorsal spinal cord section.

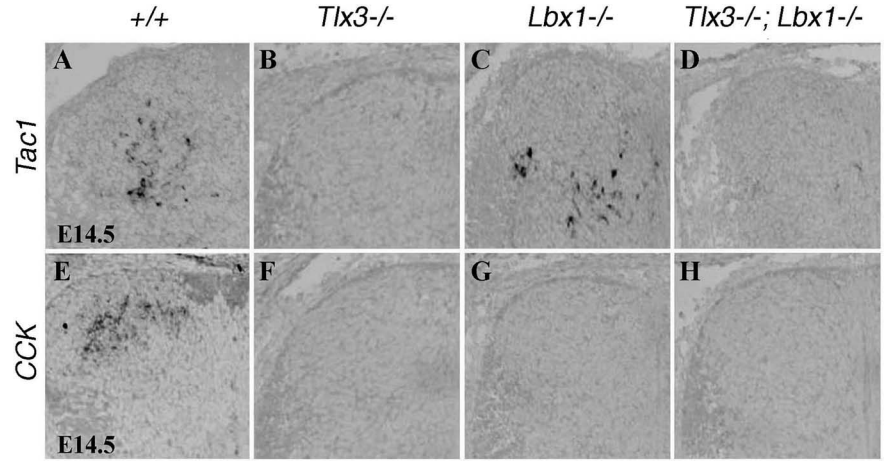

Figure 6. *Lbx1* is required for *CCK* expression. ***A–H***, *In situ* hybridization was performed on sections of E14.5 lumbar spinal cords with indicated genotypes, with *Tac1* and *CCK* as the probes.

In summary, *Tlx1/3* are required to establish the expression of a set of peptide genes, including *Tac1*, *CCK*, and *Sst*.

Tlx1/3 use distinct pathways to control peptide and glutamate transmitters: a requirement of *Lbx1* for *CCK* expression

Tlx1/3 acts to antagonize Lbx1 to specify the glutamatergic transmitter phenotype in dorsal horn excitatory neurons (Cheng et al., 2005). Accordingly, a loss of the expression of *Slc17a6*, which encodes the vesicular glutamate transporter 2 (VGLUT2) and the specific marker for dorsal horn glutamatergic neurons, in

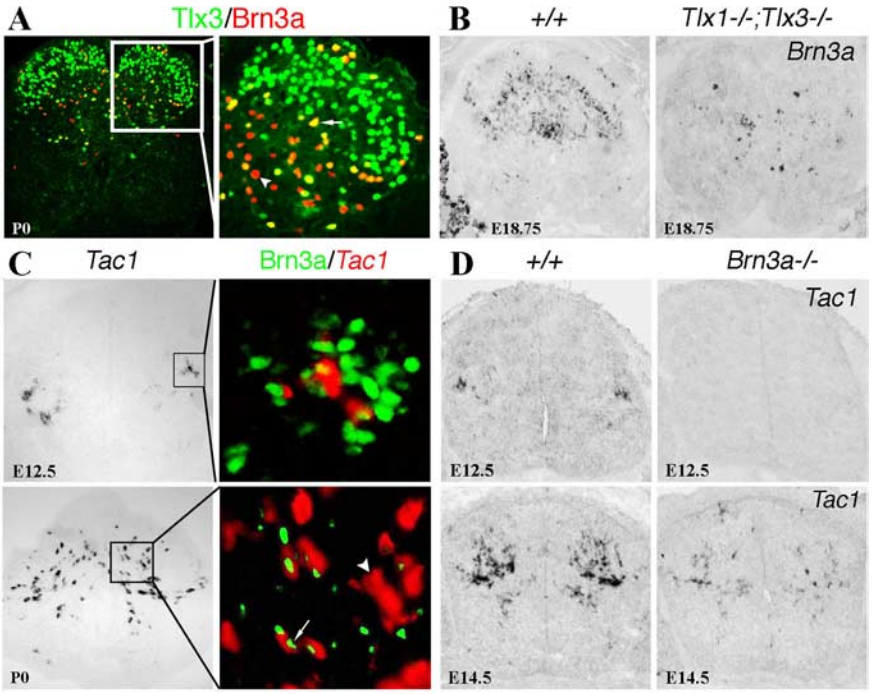

Figure 7. Brn3a controls an early wave of *Tac1* expression. ***A***, Double staining of Tlx3 (green) and Brn3a (red, arrowhead) protein in P0 thoracic spinal cord. Coexpressing cells appear in yellow (arrow). ***B***, *In situ* hybridization with the *Brn3a* probe on sections of the thoracic spinal cords of E18.75 wild-type and *Tlx1/3*$^{-/-}$ mice. Note a reduction of *Brn3a* expression in the mutant spinal cord. ***C***, Double staining of Brn3a protein (green) and *Tac1* mRNA (red) on sections of lumbar E12.5 and P0 wild-type spinal cords. Note that *Tac1* was expressed exclusively in Brn3a^{+} neurons at E12.5 but in both Brn3a^{+} (arrow) and Brn3a^{-} (arrowhead) cells at P0. ***D***, *In situ* hybridization of *Tac1* on sections through lumbar wild-type or *Brn3a*$^{-/-}$ spinal cords at indicated developmental stages.

Tlx3$^{-/-}$ embryos is restored in *Tlx3*$^{-/-}$;*Lbx1*$^{-/-}$ embryos (Cheng et al., 2005). To determine whether peptide transmitter phenotypes are established in a similar way, we analyzed peptide gene expression in *Tlx3*$^{-/-}$ and *Lbx1*$^{-/-}$ single knock-out mice and *Tlx3*$^{-/-}$;*Lbx1*$^{-/-}$ double knock-out mice at E14.5. E14.5 was chosen, because cell death occurs after E14.5 in the caudal spinal cord of *Lbx1* mutant mice (Gross et al., 2002; Cheng et al., 2005). In addition, lumbar spinal cords were analyzed because *Tlx3*, but not *Tlx1*, operates at this axial level (Cheng et al., 2005).

Expression of *Tac1* was eliminated in E14.5 *Tlx3*$^{-/-}$ mice (Fig. 6, compare *A* and *B*) but not affected in *Lbx1*$^{-/-}$ mice (Fig. 6, compare *A* and *C*). Furthermore, unlike a restoration of *VGLUT2* expression (Cheng et al., 2005), *Tac1* expression was not recovered in *Tlx3*$^{-/-}$;*Lbx1*$^{-/-}$ mice (Fig. 6*D*), suggesting that Tlx3 controls *Tac1* expression through an *Lbx1*-independent pathway. More surprisingly, *CCK* expression was eliminated in *Tlx3*$^{-/-}$ and *Lbx1*$^{-/-}$ single knock-out mice and in *Tlx3*$^{-/-}$;*Lbx1*$^{-/-}$ double knock-out mice (Fig. 6*E–H*), suggesting that both *Lbx1* and *Tlx3* are required for the expression of *CCK*. Tlx3 therefore uses distinct pathways to specify glutamate and peptide transmitters.

Brn3a is required for the early wave of *Tac1* expression

Tlx1/3 are required for the expression of a set of transcription factors in the dorsal spinal cord (Qian et al., 2002). We hypothesized that Tlx1/3 might use these downstream transcription factors to control the expression of peptide genes. To test this hypothesis, we examined the expression of neuropeptide genes in mice with a null mutation of the *Pou4f1* gene, encoding the Brn3a homeobox transcription factor (Quina et al., 2005).

Brn3a was expressed primarily in deep dorsal horn laminas and to a lesser extent in the most superficial laminas in P0 spinal cord (Fig. 7*A*,*B*). Double immunostaining showed that only a portion of Brn3a^{+} neurons coexpressed Tlx3 (Fig. 7*A*, arrow). This is consistent with previously demonstrated Brn3a expression in early born DI1 and DI2 interneurons that lack *Tlx3* expression (Gowan et al., 2001; Qian et al., 2002; Helms and Johnson, 2003). Accordingly, *Brn3a* expression was essentially, but not completely, eliminated in *Tlx1/3*$^{-/-}$ mice (Fig. 7*B*).

Because *Tac1* is also expressed predominantly in deep dorsal horn laminas, we performed a double staining of Brn3a protein and *Tac1* mRNA in the developing spinal cord. We found that at E12.5, virtually all *Tac1*-expressing neurons in the intermediate level of the spinal cord coexpressed Brn3a, but only a fraction of Brn3a^{+} neurons exhibited *Tac1* expression (Fig. 7*C*). From E12.5 to P0, a new population of *Tac1*-expressing neurons that did not express Brn3a emerged (Fig. 7*C*).

Consistent with this expression pattern, *Tac1* expression was virtually eliminated in the caudal spinal cord of E12.5 *Brn3a*$^{-/-}$ embryos (Fig. 7*D*), but only reduced in E14.5 *Brn3a*$^{-/-}$ spinal cord (Fig. 7*D*), suggesting a specific role of *Brn3a* in controlling the early wave of *Tac1* expression.

Because of incomplete loss of *Brn3a* expression in *Tlx1/3*$^{-/-}$ mice (Fig. 7*B*), two distinct models may explain a loss of *Tac1* expression in both *Tlx1/3*$^{-/-}$ and *Brn3a*$^{-/-}$ mice. First, Tlx1/3 and Brn3a act in a cascade to control *Tac1* expression (in other words, *Tac1* is established in cells in which Brn3a expression is dependent on Tlx1/3). Second, Tlx1/3 and Brn3a act in combination, meaning that *Tac1* expression is established in Tlx3^{+};Brn3a^{+} neurons in which Brn3a expression is independent of Tlx3. To distinguish these models, we analyzed *Tac1* and *Brn3a* expression in E12.5 wild-type and *Tlx1/3*$^{-/-}$ embryos. At this stage, *Tac1* expression was confined to a lateral region in the middle of the wild-type spinal cord (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). In *Tlx1/3*$^{-/-}$ embryos, expression of both *Tac1* and *Brn3a* was eliminated from this lateral region, whereas *Tlx3*-independent *Brn3a* expression was located in a dorsomedial area (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). These data are more consistent with the first model that Tlx3 and Brn3a act sequentially to control *Tac1* expression.

Expression of other *Tlx3*-dependent genes, including *CCK*, *Sst*, the *Gria2* glutamate receptor gene (Cheng et al., 2004), and the *TRPC3* transient receptor potential channel gene (Li et al., 2006), was not grossly affected in *Brn3a* mutants (supplemental Fig. 7, available at www.jneurosci.org as supplemental material), suggesting a specific role of *Brn3a* in controlling the early wave of *Tac1* expression.

To determine whether *Brn3a* is sufficient to promote *Tac1* expression, we performed gain-of-function analyses by using chick electroporation technique (Itasaki et al., 1999). Electroporation of a Brn3a expression vector, *RCAS-Brn3a*, in E2 chick neural tubes resulted in an induction of *Tac1* expression at E5 (Fig. 8*B*) and even more at E7 (Fig. 8*D*). Electroporation with

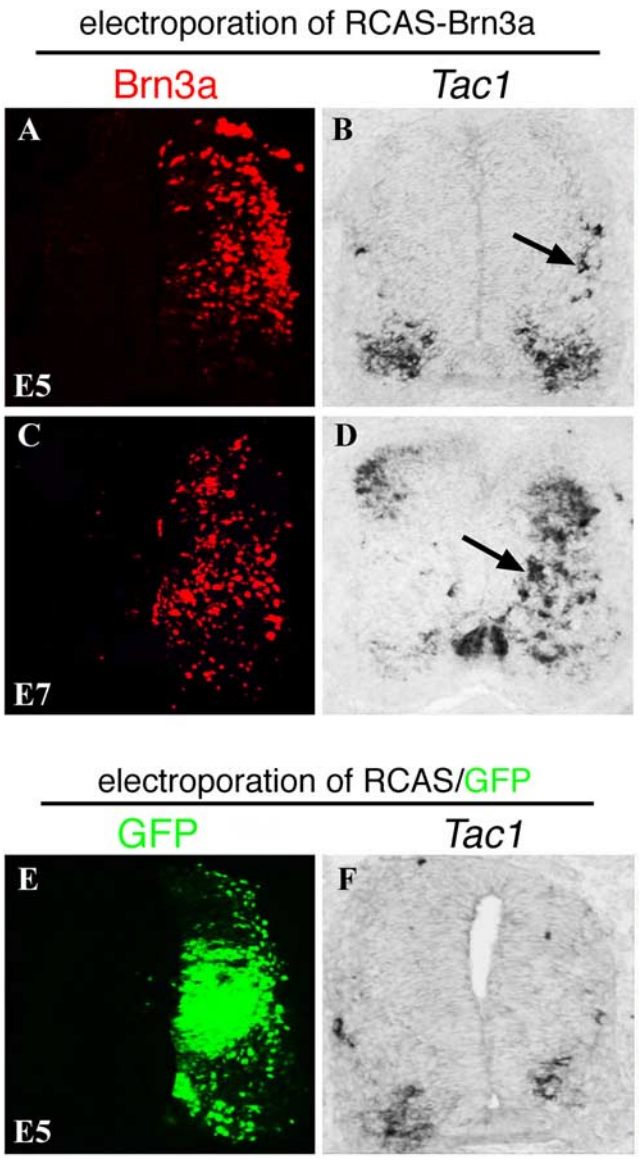

Figure 8. Brn3a induces *Tac1* expression in the chick neural tube. ***A–D***, Electroporation of *RCAS-Brn3a* (plus *pCAX-GFP*, not shown) into the right side of chick neural tubes at E2, followed by analysis of the expression of exogenous Brn3a protein by immunostaining (***A***, ***C***) and *Tac1* expression by *in situ* hybridization at E5 (***A***, ***B***) or E7 (***C***, ***D***). Arrows indicate ectopic *Tac1* expression. ***E***, ***F***, Electroporation with control vectors (*RCAS* plus *pCAX-GFP*, referred to as *RCAS/GFP*). GFP expression (***E***) was used to monitor electroporation efficacy. Note a lack of *SP* induction (***F***).

control vectors did not affect *Tac1* expression (Fig. 8*E*,*F*), suggesting that *Tac1* induction by *RCAS-Brn3a* electroporation was not caused by side effects associated with proviral vector electroporation (Hermann and Logan, 2003). As in the case with wild-type mouse spinal cord (Fig. 7*C*), only a portion of Brn3a$^+$ neurons coexpressed *Tac1* (data not shown), suggesting that Brn3a needs a specific cellular context to activate *Tac1*.

Discussion

Ontogeny of dorsal horn peptidergic neurons

This study suggests that dorsal horn peptidergic neurons emerge from distinct neuronal populations. Expression of *Tac1*, *Tac2*, *CCK*, and the late wave of *Sst* is confined to Tlx3$^+$ neurons or their derivatives, and the development of these peptidergic neurons is compromised in mice that lack *Tlx3* and *Tlx1*. Expression of *Pdyn*, *Penk1*, and a portion of early wave *Sst* is restricted to Pax2$^+$ neurons, and their development is independent of *Tlx1* or *Tlx3*. Our data also suggest that some *Sst*-expressing neurons may develop from cells that do not express Tlx3 or Pax2. Tlx3 and Pax2 are associated with excitatory and inhibitory neurons, respectively, at least at embryonic stages (Cheng et al., 2004). Consistently, neurons that produce Neurokinin B (the product of *Tac2*), Substance P (the product of *Tac1*), and a late wave of SOM (the product of *Sst*) belong to glutamatergic excitatory neurons (Proudlock et al., 1993; Todd et al., 2003; Todd and Koerber, 2006; Polgar et al., 2006). Also consistent with an association with Pax2$^+$ neurons, a small number of SOM$^+$ neurons located in the deep dorsal horn are inhibitory neurons (Proudlock et al., 1993).

Separate genetic controls of glutamate and peptide transmitter phenotypes

Tlx1/3 are known to antagonize Lbx1 to control the expression of VGLUT2, the vesicular glutamate transporter and the specific marker for dorsal horn glutamatergic neurons (Todd et al., 2003; Cheng et al., 2004; Fremeau et al., 2004). Loss of VGLUT2 expression in *Tlx3* mutant mice is restored in *Tlx3*$^{-/-}$;*Lbx1*$^{-/-}$ double mutants (Cheng et al., 2005). However, expression of the *Tlx1/3*-dependent peptide genes is not restored in *Tlx3*$^{-/-}$;*Lbx1*$^{-/-}$ double mutants, implying that Tlx1/3 use distinct pathways to coordinate glutamate and peptide transmitters. A separate control of these transmitters is supported by the fact that all dorsal horn excitatory neurons use glutamate as a fast transmitter, whereas individual peptide transmitters are confined to a small subset of dorsal horn neurons.

The development of *Tlx1/3*-dependent peptidergic neurons is subject to complex genetic control. The early, but not late, *Tac1* expression is dependent on *Brn3a*. Moreover, Tlx1/3 can exert both positive and negative effects on *Sst* expression in different dorsal horn lamina. A surprising result is that *CCK* expression is dependent on both *Lbx1* and *Tlx3*, despite that Tlx3 antagonizes Lbx1 to control *VGLUT2* expression. One potential solution for these seemly conflicting Tlx3 activities is that glutamate and CCK transmitter phenotypes are established at distinct stages. *VGLUT2* expression is established soon after cells exit from the cell cycle, and at this stage Tlx3 acts to remove an inhibitory effect of Lbx1 on *VGLUT2* expression (Cheng et al., 2005). *CCK* expression, however, is established at E14.5 (Fig. 2), when Tlx3 protein has been extinguished in most *CCK*-expressing neurons (supplemental Fig. 8, available at www.jneurosci.org as supplemental material). Our hypothesis is that Tlx3 extinguishment allows Lbx1 to escape a Tlx3-mediated inhibition, and Lbx1 might in turn act together with an unknown *Tlx3*-dependent event (established at earlier stages) to control *CCK* expression.

Tlx1 and Tlx3 orchestrate a set of downstream transcription factors to specify dorsal horn neuron subtypes

Our findings argue that Tlx1/3 act as "master regulators" in coordinating dorsal horn excitatory neuron development. Virtually all known functional genes that are preferentially expressed in glutamatergic neurons within the dorsal spinal cord are eliminated in *Tlx1/3*$^{-/-}$ mice, including *VGLUT2* (Cheng et al., 2004), the glutamate receptor gene *Gria2* (Kerr and Todd, 1998; Cheng et al., 2004), the channel gene *TRPC3* (Li et al., 2006), and a set of peptide genes described in this study. *Tlx1/3* activate a set of downstream transcription factors, some of which appears to control a portion of *Tlx1/3*-dependent differentiation programs (Fig. 9). For example, Brn3a is required for the early wave of *Tac1* expression but is dispensable for the expression of other *Tlx1/3*-dependent genes (supplemental Fig. 6, available at www.

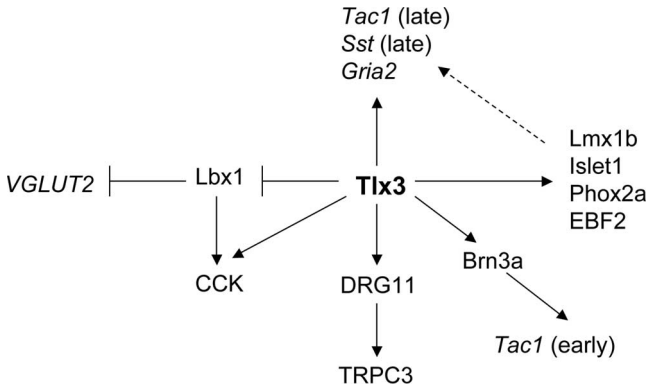

Figure 9. Tlx1/3 coordinate dorsal horn neuron development. Tlx3 acts to antagonize Lbx1 to control *VGLUT2* expression and the glutamatergic transmitter phenotype. Both *Tlx3* and *Lbx1* are required for *CCK* expression, and transient *Tlx3* expression may allow Lbx1 to work together with a putative *Tlx3*-dependent downstream event to control *CCK* expression (see Discussion). Tlx3 acts via Brn3a to control the early wave of *Tac1* expression, but *Brn3a* is not required for the expression of other *Tlx3*-dependent genes such as *CCK*, *Sst*, *TRPC3*, or *Gria2*. Tlx3 acts via DRG11 to control the expression of *TRPC3* (Li et al., 2006), but DRG11 is not required for the expression of any *Tlx3*-dependent peptide genes or *Gria2* (C. Lopes and D. Lima, unpublished data). *Tlx3* is required for the expression of many other dorsal horn transcription factors such as Islet1, Phox2a, EBF2 [wrongly called EBF3 in our previous paper (Qian et al., 2002)], and Lmx1b (Qian et al., 2002). These downstream factors may control other unknown downstream events or control the expression of those *Tlx3*-dependent but *Brn3a/DRG11*-independent target genes such as late-wave *Tac1*, late-wave *Sst*, or *Gria2* (dashed arrow).

jneurosci.org as supplemental material). DRG11, encoded by the homeobox gene *Prrxl1* (Saito et al., 1995; Chen et al., 2001), is required for the expression of *TRPC3* (Li et al., 2006) but not *Gria2* or any category I peptide genes (C. Lopes and D. Lima, unpublished data). Finally, other *Tlx1/3*-dependent transcription factors such as Islet1, Phox2a, and early B-cell factor 2 (EBF2) are all expressed in a fraction of dorsal horn neurons (Tiveron et al., 1996; Qian et al., 2002; Li et al., 2006), and therefore they likely contribute to specification of other specialized dorsal horn neuron subtypes. One challenging unsolved question is to understand how Tlx1/3 are able to activate distinct downstream differentiation programs in distinct dorsal horn neuron contexts.

Modular control of the development of the mammalian nervous system

One important concept in developmental biology is that specification of individual neuronal cell types is controlled by a unique combination of transcription factors (TFs), or combinatorial TF codes (Shirasaki and Pfaff, 2002; Thor and Thomas, 2002). However, after late neuronal phenotypes are analyzed, it becomes increasingly evident that a TF code established in newly born neurons specifies more than one neuronal cell type. As aforementioned, a set of peptidergic neurons and other excitatory neurons in the superficial dorsal horn develop from DI5/DIL_B neurons that share the same TF code, by coexpressing Tlx3 and Lmx1b (Gross et al., 2002; Müller et al., 2002; Cheng et al., 2004), and Tlx3 coordinates the development of these neurons. In the ventral spinal cord, Engrailed1$^+$ V1 interneurons are composed of multiple neuron subtypes involved with locomotion controls (Sapir et al., 2004; Alvarez et al., 2005). In dorsal root ganglia, the Runx1 runt domain transcription factor is required for the development of a variety of nociceptive sensory neurons (Chen et al., 2006; Ibanez and Ernfors, 2007; Marmigere and Ernfors, 2007; Woolf and Ma, 2007). The emerging theme is that a TF code established in newly formed neurons coordinates specification of a heterogeneous group of neurons that perform related physiological functions, thereby implying a modular control of the development of the mammalian nervous system.

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