

Tlx3 Controls Cholinergic Transmitter and Peptide Phenotypes in a Subset of Prenatal Sympathetic Neurons

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The embryonic sympathetic nervous system consists of predominantly noradrenergic neurons and a very small population of cholinergic neurons. Postnatal development further allows target-dependent switch of a subset of noradrenergic neurons into cholinergic phenotype. How embryonic cholinergic neurons are specified at the prenatal stages remains largely unknown. In this study, we found that the expression of transcription factor *Tlx3* was progressively restricted to a small population of embryonic sympathetic neurons in mice. Immunostaining for vesicular acetylcholine transporter (VACHT) showed that *Tlx3* was highly expressed in cholinergic neurons at the late embryonic stage E18.5. Deletion of *Tlx3* resulted in the loss of *Vacht* expression at E18.5 but not E12.5. By contrast, *Tlx3* was required for expression of the cholinergic peptide vasoactive intestinal polypeptide (VIP), and somatostatin (SOM) at both E12.5 and E18.5. Furthermore, we found that, at E18.5 these putative cholinergic neurons expressed glial cell line-derived neurotrophic factor family coreceptor *Ret* but not tyrosine hydroxylase (Ret^+/TH^-). Deletion of *Tlx3* also resulted in disappearance of high-level *Ret* expression. Last, unlike *Tlx3*, *Ret* was required for the expression of VIP and SOM at E18.5 but not E12.5. Together, these results indicate that transcription factor *Tlx3* is required for the acquisition of cholinergic phenotype at the late embryonic stage as well as the expression and maintenance of cholinergic peptides VIP and SOM throughout prenatal development of mouse sympathetic neurons.

Introduction

The sympathetic nervous system is a major component of the autonomic nervous system that maintains the homeostasis of the internal environment during diverse physiological conditions (Cannon, 1939; Janig and McLachlan, 1992). Mature mammalian and avian sympathetic ganglia are composed of two types of neurons. The vast majority of sympathetic neurons are noradrenergic and express molecular markers, such as tyrosine hydroxylase (TH) and dopamine β -hydroxylase (DBH). By contrast, a small subset of sympathetic neurons use acetylcholine as transmitter and can be discriminated by the expression of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VACHT) (Elfvin et al., 1993; Ernsberger and Rohrer, 1999). Diversity of mature sympathetic neurons is also indicated by the restricted expression of peptides in distinct subpopulations (Elfvin et al., 1993; Gibbins, 1995). How the diversity of the sym-

pathetic neurons is generated during development is a major question for studying neural development (Le Douarin and Kalcheim, 1999; Howard, 2005; Huber, 2006).

Substantial progress has been made in understanding the extrinsic signals and intrinsic transcription factors that control the acquisition of noradrenergic phenotype in sympathetic neurons (Apostolova and Dechant, 2009; Cane and Anderson, 2009; Rohrer, 2011). Transcription factors *Phox2b* and *Hand2* are required to maintain the noradrenergic traits, whereas *Gata3* is required for the survival of differentiated noradrenergic neurons (Schmidt et al., 2009; Coppola et al., 2010; Tsarovina et al., 2010). For cholinergic differentiation in sympathetic neurons, there are two modes of how sympathetic neurons acquire their cholinergic phenotype (Ernsberger and Rohrer, 1999; Francis and Landis, 1999; Ernsberger, 2001). In the classic postnatal target-dependent cholinergic differentiation, the sudomotor neurons and the neurons that innervate the periosteum initially express noradrenergic phenotype, but acquire the cholinergic phenotype while losing functional adrenergic properties after contacting the target tissue (Landis, 1990; Asmus et al., 2000). The switch from noradrenergic to cholinergic phenotype is initiated by signals released from the target (e.g., sweat glands) (Apostolova and Dechant, 2009; Cane and Anderson, 2009). The glycoprotein (gp)130 receptor that is expressed in sympathetic neurons is indispensable for the target-dependent acquisition of cholinergic phenotype (Stanke et al., 2006). Prenatal expression of cholinergic properties has also been found in a very small population of developing sympathetic neurons of rodents and chick (Ernsberger et al., 1997; Schäfer et al., 1997; Schütz et al., 1998). Further studies revealed that transcription factor *Phox2b* and *Ret*, the

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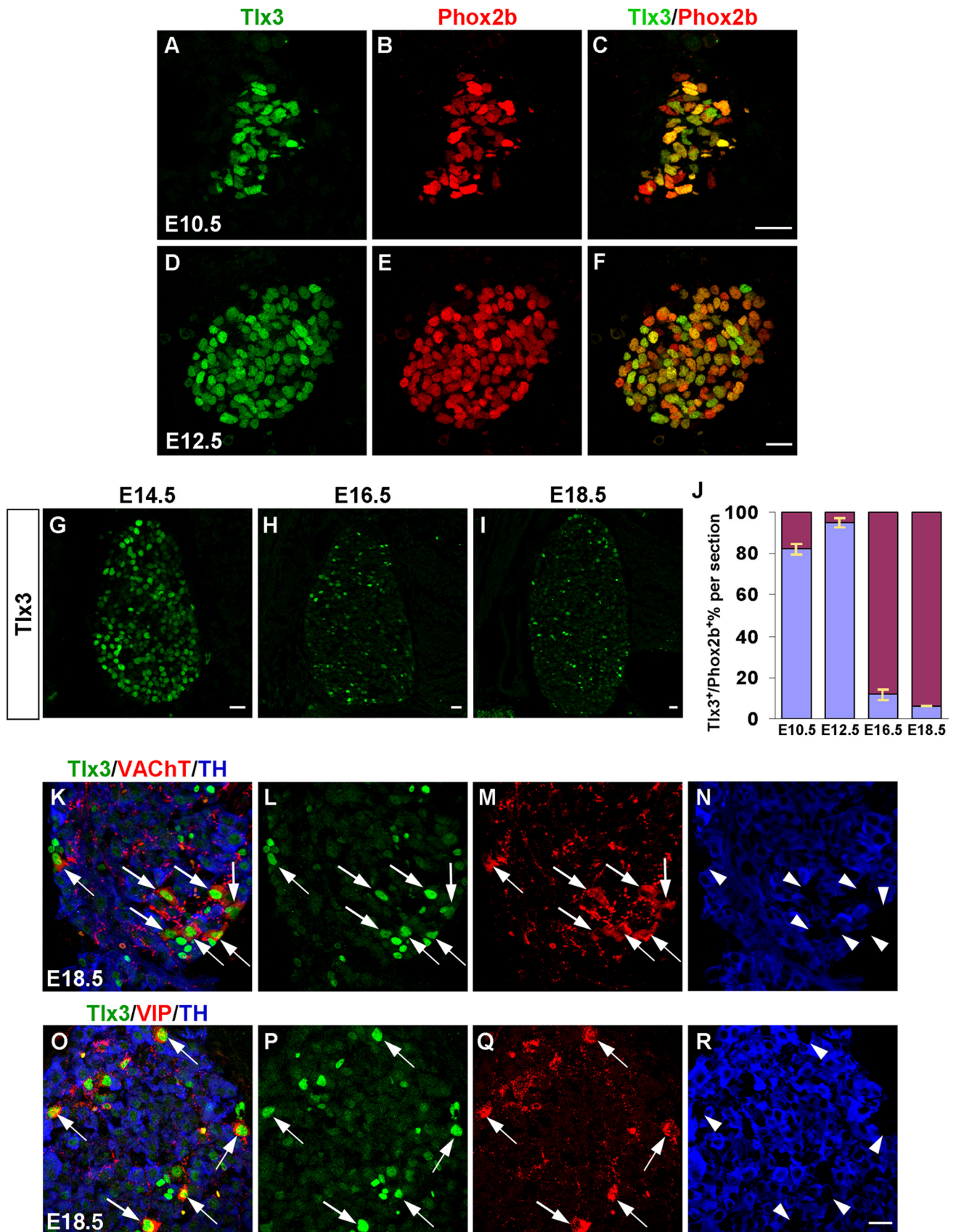


Figure 1. Expression of Tlx3 in the embryonic sympathetic ganglia of mouse. *A–I*, Immunostaining of Tlx3 was performed on transverse sections of the primary sympathetic chain from wild-type mice at E10.5 (*A*), and stellate ganglia at E12.5 (*D*), E14.5 (*G*), E16.5 (*H*), and E18.5 (*I*). Double staining of Tlx3 and Phox2b was performed at E10.5 (*A–C*) and E12.5 (*D–F*). *J*, The number of Tlx3⁺ cells was determined in sympathetic ganglia at E10.5, E12.5, E16.5, and E18.5 relative to the total number of Phox2b⁺ cells. *K–R*, Triple immunostaining of Tlx3, TH, and VAcHT (*K–N*) or VIP (*O–R*) was performed on sections of stellate ganglia at E18.5. Arrows (*K–M*, *O–Q*) and arrowheads (*N*, *R*) showed the majority of VAcHT⁺/TH[−] and VIP⁺/TH[−] cells coexpressed Tlx3. Scale bars: *A–F*, *K–R*, 10 μm; *G–I*, 20 μm.

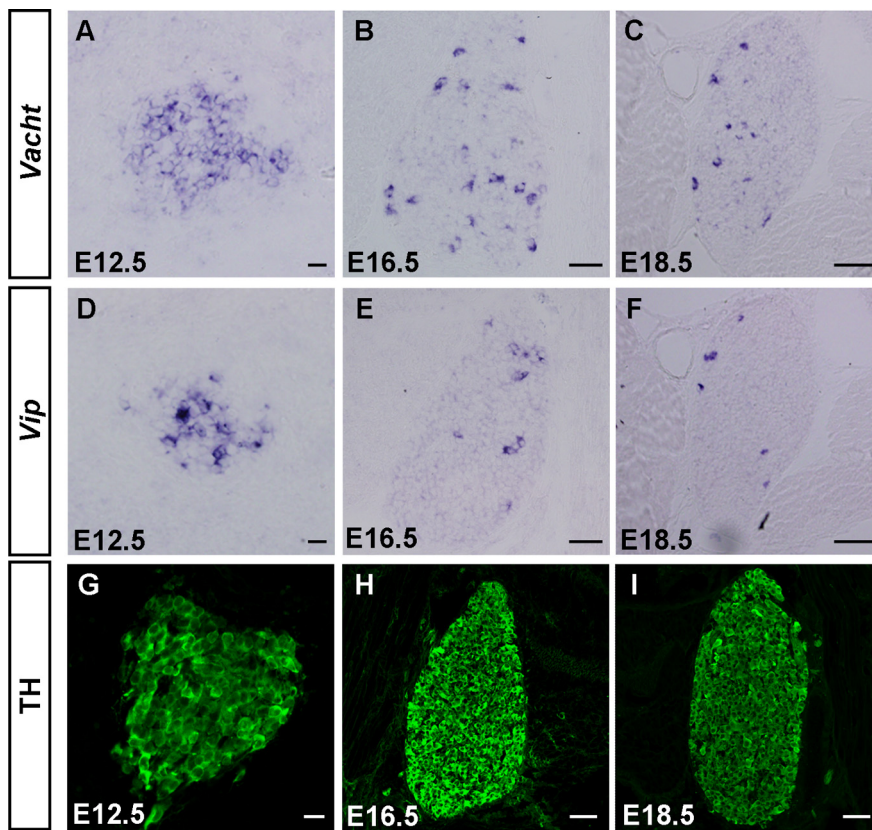


Figure 2. Different expression patterns of cholinergic and noradrenergic neuronal markers during embryonic sympathetic development. **A–F**, *In situ* hybridization was performed on sections of stellate ganglia from wild-type mice at E12.5, E16.5, and E18.5 with *Vacht* (**A–C**), and *Vip* (**D–F**) as the probes. Note a progressively restricted expression pattern of *Vacht*. **G–I**, Immunostaining of TH was performed on sections of stellate ganglia from wild-type mice at E12.5, E16.5, and E18.5. The persistently widespread expression of TH was observed during embryonic sympathetic development. Scale bars: **A, D, G**, 20 μm ; **B, C, E, F, H, I**, 50 μm .

glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs) coreceptor, are required for this embryonic cholinergic differentiation (Bureau et al., 2004; Huber and Ernsberger, 2006). However, the molecular mechanism that controls the development of embryonic cholinergic neurons remains largely unknown.

The homeodomain transcription factor *Tlx3* and *Tlx1* determine glutamatergic over GABAergic cell fates and the expression of corresponding peptides and transmitter receptors in the dorsal spinal cord (Cheng et al., 2004, 2005; Xu et al., 2008; Guo et al., 2012; Hu et al., 2012). Here, we report that *Tlx3* is enriched in the cholinergic subset of sympathetic neurons at the late embryonic stage and is required for their proper phenotypic development.

Materials and Methods

Mouse strains. The generation of *Tlx3* mutant mice was described previously (Shirasawa et al., 2000). *Ret* mutant mice were kindly donated by Dr. Frank Costantini (Columbia University Medical Center, New York, NY) and obtained from The Jackson Laboratory (Schuchardt et al., 1994). In all timed mating using both male and female mice, the morning that vaginal plugs were observed was designated as E0.5. Genotyping for *Tlx3* mutant mice was done as described previously (Qian et al., 2002). Wild-type *Ret* allele was identified with the information provided by The Jackson Laboratory and the mutant *Ret* allele was amplified with the following primers that produce a 651 bp product: 5'-AGTCTC-CAAGGCAATTCAACG-3' and 5'-GCCCAATAGCAGCCAGTCC-3'. All animal procedures are contained in protocols reviewed and approved

by the Animal Care Committee at the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

In situ hybridization and immunostaining. *In situ* hybridization experiments were performed as described previously (Huang et al., 2010). Riboprobe for mouse *Vacht* (NM_021712, 1.14 Kb) and *Vip* (NM_011702, 1.04 kb) were used. *Sst in situ* probe was described previously (Huang et al., 2008).

Immunostaining was performed as described previously (Huang et al., 2010). The following antibodies were used: rabbit anti-*Tlx3* antibody (1:400) (Huang et al., 2010), guinea pig anti-*Tlx3* (1:1000, kind gift from C. Birchmeier, Max Delbrück Center for Molecular Medicine, Berlin, Germany), goat anti-VACHT (1:50, Santa Cruz Biotechnology), rabbit anti-VACHT (1:500, SYSY), goat anti-VIP (1:200, Santa Cruz Biotechnology), goat anti-SOM (D-20; 1:300, Santa Cruz Biotechnology), mouse anti-TH (1:500, Sigma-Aldrich), rabbit anti-TH (1:500, Pel-Freez), goat anti-Ret (1:25, R&D Systems), goat anti-Phox2b (1:50, Santa Cruz Biotechnology), rabbit anti-SOM (H-106) (1:50, Santa Cruz Biotechnology), mouse anti-CGRP (1:500, Sigma-Aldrich).

Cell counting. Stellate ganglion from three pairs of control and mutant embryos (10 μm) were analyzed with various molecular markers. Immunoreactive cells with signal highly above the background were counted and the values were presented as mean \pm SEM. The differences in values were considered to be significant at $p < 0.05$ by Student's *t* test.

Results

Expression of *Tlx3* in sympathetic cholinergic neurons

To study the expression of transcription factor *Tlx3* in the developing sympathetic neurons, we performed immunostaining experiments on sympathetic ganglia of mouse embryos from E9.5 to E18.5. The first *Tlx3*⁺ cells were detectable at E10.5 and found to coexpress Phox2b, which is known to be expressed in early sympathetic progenitors (Pattyn et al., 1997, 1999), indicating that *Tlx3* was highly expressed in developing sympathetic ganglia (Fig. 1A–F). Approximately 82% and 95% Phox2b⁺ cells expressed *Tlx3* at E10.5 and E12.5, respectively (Fig. 1J). Over the next few days, *Tlx3* expression was progressively restricted to a small population of sympathetic neurons (Fig. 1G–I). At E16.5 and E18.5, the percentage of Phox2b⁺ neurons that expressed *Tlx3* reduced to 17% and 6%, respectively (Fig. 1J). This progressive restriction of *Tlx3* expression in sympathetic neurons was reminiscent of the restrictive expression pattern of cholinergic marker genes in rodent and chick sympathetic neurons at prenatal stages (Ernsberger et al., 1997; Schütz et al., 1998; Bureau et al., 2004; Huber and Ernsberger, 2006) (Fig. 2). Furthermore, gene expression studies performed in chicken sympathetic ganglia have shown that *Tlx3* is among the genes upregulated by factors that induce cholinergic neuronal differentiation *in vitro* (Apostolova et al., 2007). To determine whether *Tlx3* is associated with cholinergic neuronal differentiation in developing sympathetic ganglia, we performed triple staining of the stellate ganglia at embryonic stages, where a small population of cholinergic neu-

rons has been observed at the prenatal stages (Schäfer et al., 1997; Schütz et al., 1998). VACHT and ChAT are well known molecular markers for cholinergic neurons (Ernsberger and Rohrer, 1999). *In situ* hybridization studies have shown that the expression level of *Vacht* is higher than that of *Chat* in sympathetic neurons (Bureau et al., 2004). Thus, we focused on VACHT expression in this study. Immunostaining results showed that VACHT and TH were coexpressed in many neurons at E12.5, but in largely separated populations by E18.5 (Fig. 1*K–N* and data not shown). Meanwhile, the majority of VACHT⁺ neurons (82%) expressed *Tlx3* at E18.5 (Fig. 1*K–N*). Vasoactive intestinal polypeptide (VIP) is known to be expressed in cholinergic neurons of stellate ganglia in newborn mice (Stanke et al., 2000). Indeed, our immunostaining results showed that the majority of VIP⁺ neurons (80%) expressed *Tlx3* (Fig. 1*O–R*). Thus, most of the cholinergic neurons express *Tlx3* during late embryonic stages.

It has been reported that cholinergic sympathetic neurons in adult stellate ganglia that innervate sweat glands acquire cholinergic properties largely between P4 and P21 through target-dependent gp 130 signaling (Guidry and Landis, 1998; Ernsberger and Rohrer, 1999; Francis and Landis, 1999; Stanke et al., 2006). Those sudomotor neurons are characterized by the expression of calcitonin gene-related peptide (CGRP) (Landis and Fredieu, 1986; Anderson et al., 2006). To study whether *Tlx3* was expressed in the second-wave of cholinergic neurons, we performed immunostaining experiments on postnatal stellate ganglia. The results showed that *Tlx3* was clearly expressed in a subset of sympathetic neurons at P14 and P60 (Fig. 3*A,B*). Furthermore, double staining results showed that *Tlx3*⁺ neurons expressed CGRP at P14 and P30 (Fig. 3*C–H*). *Tlx3* also was nicely colocalized with VIP in sympathetic neurons: most *Tlx3*⁺ neurons (87%) expressed VIP and ~42% VIP⁺ neurons expressed *Tlx3* at P30 (Fig. 3*I–N*). Meanwhile, double staining results revealed that almost all of the VIP⁺ neurons coexpressed VACHT at P14 and P60 (data not shown). A recent study also showed that *Tlx3* was expressed in cholinergic neurons at P60 (Furlan et al., 2013). Together, *Tlx3* was expressed in the postnatal emergence of cholinergic neurons as well.

Tlx3 controls development of cholinergic neurons

To determine whether *Tlx3* was required for the development of cholinergic neurons in stellate ganglia, we examined the expres-

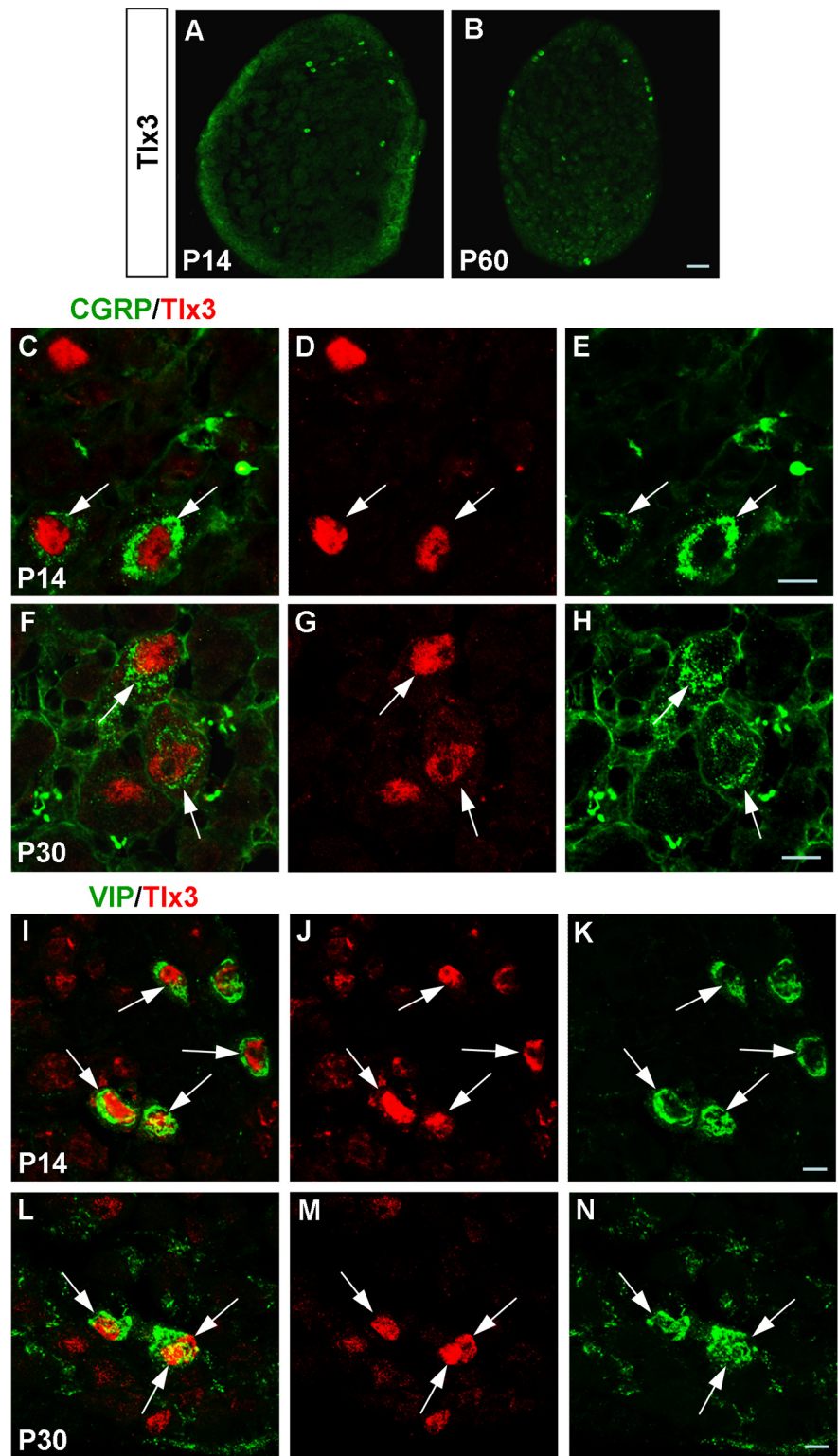


Figure 3. Expression of *Tlx3* in the postnatal sympathetic ganglia of mouse. **A, B**, Immunostaining of *Tlx3* was performed on sections of stellate ganglia from wild-type mice at P14 (**A**) and P60 (**B**). **C–H**, Double immunostaining of *Tlx3* and CGRP was performed on sections of stellate ganglia from wild-type mice at P14 (**C–E**) and P30 (**F–H**). **I–N**, Double immunostaining of *Tlx3* and VIP was performed on sections of stellate ganglia from wild-type mice at P14 (**I–K**) and P30 (**L–N**). Scale bars: **C–N**, 10 μm; **A, B**, 50 μm.

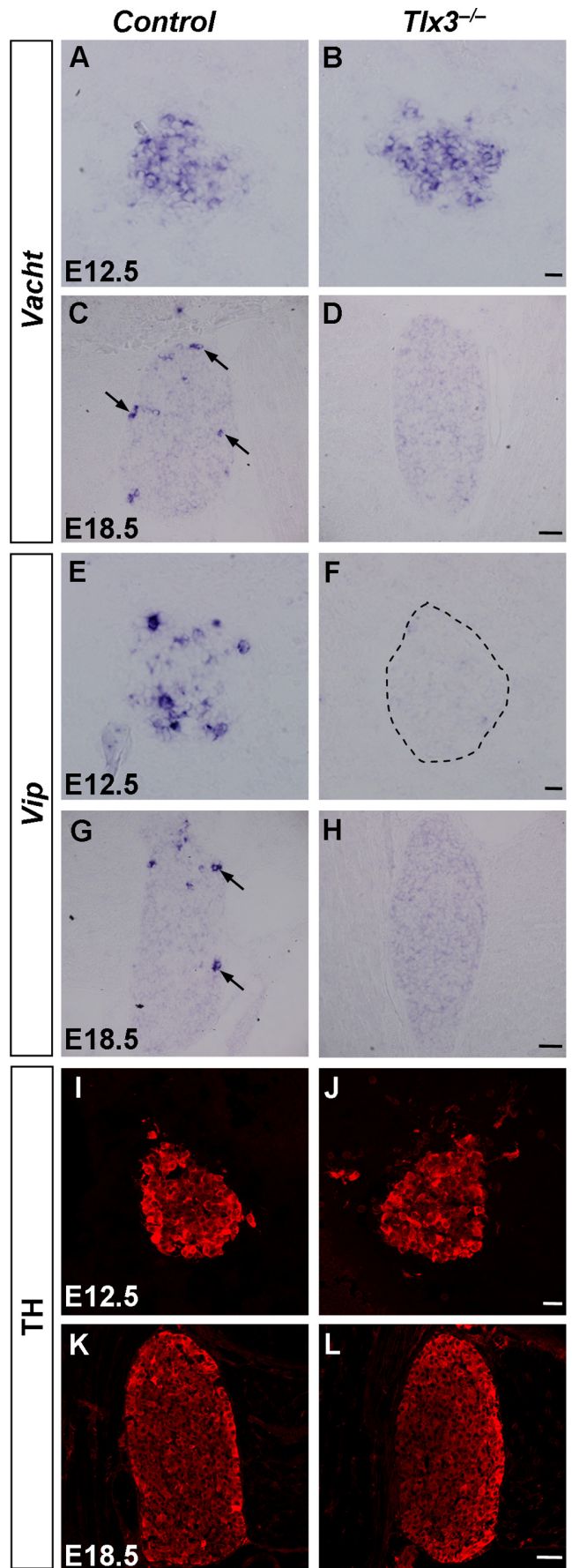
sion of *Vacht* and *Vip* in control (wild-type or *Tlx3*^{+/-}) and *Tlx3* deletion (*Tlx3*^{-/-}) mice by *in situ* hybridization. The results showed that the early expression of *Vacht* at E12.5 was similar between control and *Tlx3*^{-/-} mice, whereas the expression of

Vacht expression at E18.5 was abolished in *Tlx3*^{-/-} mice but not in control mice (Fig. 4A–D). In contrast, the expression of *Vip* was eliminated in *Tlx3*^{-/-} mice at both E12.5 and E18.5 (Fig. 4E–H). To determine whether *Tlx3* controlled the development of noradrenergic neurons in the stellate ganglion, we examined the expression of TH and transcription factors that are required for the development of noradrenergic neurons, including *Phox2b*, *Hand2*, *Gata3*, and *Insm1*, in the *Tlx3*^{-/-} mice at E12.5 and E18.5. The results showed that the expression of these molecular markers were largely unaffected when *Tlx3* was fully ablated (Figs. 4I–L, 5).

Some VIP-expressing neurons in the stellate ganglia of rat are also known to express the neuropeptide somatostatin (SOM) (Masliukov and Timmermans, 2004). To determine whether SOM or the gene *Sst* that encodes SOM was expressed in embryonic cholinergic neurons, we performed *in situ* hybridization and immunostaining experiments. The results showed that *Sst* was consistently expressed in a small portion of neurons in embryonic stellate ganglia (Fig. 6A–D). Furthermore, most of the VAcHT-expressing cholinergic neurons (83%) expressed SOM and ~64% of VIP⁺ neurons expressed SOM at E18.5 (Fig. 6E–L). We also found that most SOM⁺ neurons (89%) expressed *Tlx3* at E18.5 (Fig. 6M–P). Finally, we examined whether *Tlx3* was required for the expression of SOM or *Sst* and found that the expression of SOM or *Sst* at both E12.5 and E18.5 was eliminated in *Tlx3*^{-/-} mice (Fig. 6Q–T). Together, these results showed that *Tlx3* is required for the embryonic development of cholinergic neurons in the stellate ganglion by specifying the expression of *Vacht*, *Vip*, and SOM. The neonatal lethality of *Tlx3* mutant mice (Shirasawa et al., 2000) prevented us from studying whether *Tlx3* controls the development of the second-wave of cholinergic neurons at the postnatal stages.

Expression of *Ret* in developing stellate ganglia of *Tlx3* deletion mice

Ret has been shown to regulate cholinergic properties in embryonic mouse sympathetic neurons (Bureau et al., 2004). To determine the relationship between *Tlx3* and *Ret* in cholinergic development, we first examined whether they are colocalized in the same cells. Immunostaining results showed that the majority of Ret⁺/TH⁻ neurons (87%) expressed *Tlx3* in wild-type mice (Fig. 7A–D). Triple staining results further showed that most Ret⁺/TH⁻ neurons (92%) were indeed cholinergic neurons (Fig. 7E–H). Then we examined whether *Tlx3* was required for the expression of *Ret* in the putative cholinergic (Ret⁺/TH⁻) neurons by analyzing the *Tlx3*^{-/-} mice. Interestingly, the small number of putative cholinergic (Ret⁺/TH⁻) neurons disappeared, whereas the majority of Ret⁺/TH⁺ neurons were unaffected in *Tlx3*^{-/-} mice at E18.5 (Fig. 7I–L, arrows). It was unclear whether the disappearance of cholinergic (Ret⁺/TH⁻) neurons in *Tlx3*^{-/-} mice was due to the downregulation of high-level expression of *Ret* in these neurons or cell death. The relatively constant number of Ret⁺ neurons in control and *Tlx3* deletion mice (control: 70 ± 2 vs *Tlx3*^{-/-}: 71.3 ± 1.4 cells/section) suggested that Ret⁺/TH⁻ neurons may have changed their fate from cholinergic to noradrenergic phenotype (Ret⁺/TH⁺) by gaining the expression of TH (Fig. 7I,K). Because the number of Ret⁺/TH⁻ neurons was small (~8 neurons/section), it remains to be determined whether cell type switch had indeed occurred. Nevertheless, the above results indicated that *Tlx3* is required to maintain the high-level expression of *Ret* in embryonic cholinergic neurons in the sympathetic ganglion.



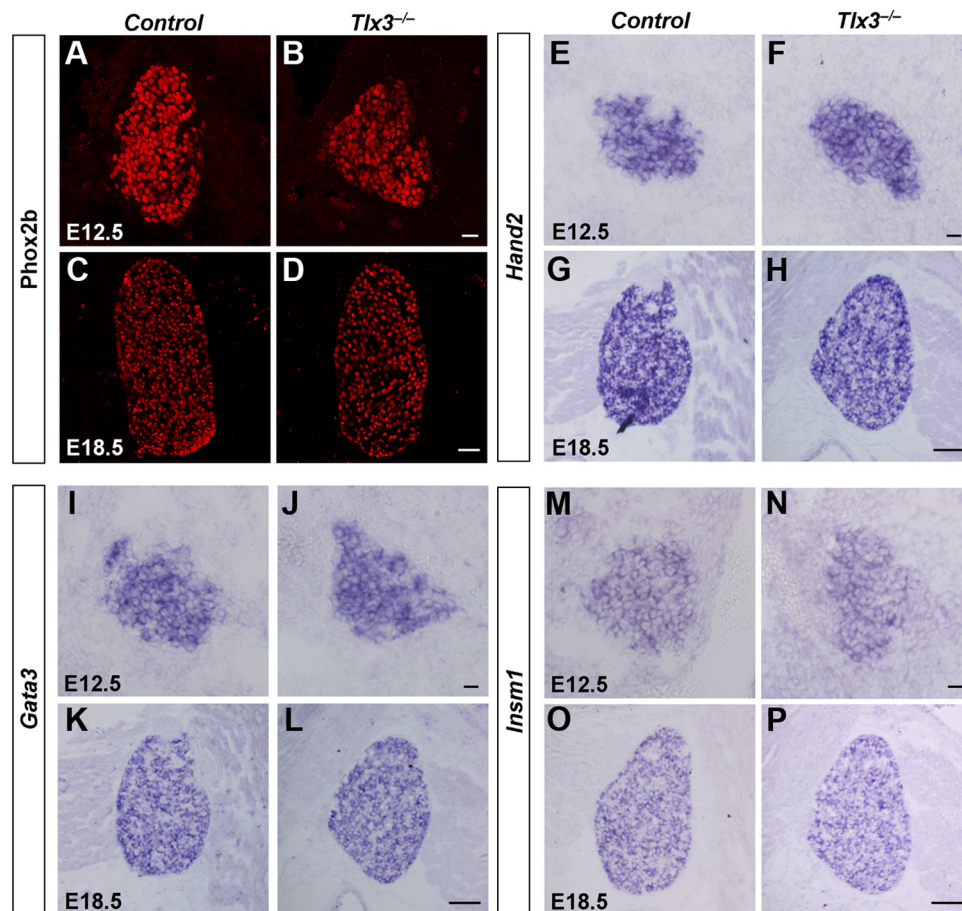


Figure 5. Expression of some molecular markers in *Tlx3*^{-/-} mice. **A–D**, Immunostaining of Phox2b was performed on sections of stellate ganglia from control and *Tlx3*^{-/-} mice at E12.5 and E18.5. **E–P**, *In situ* hybridization was performed on sections of stellate ganglia from control and *Tlx3*^{-/-} mice at E12.5 and E18.5 with *Hand2* (**E–H**), *Gata3* (**I–L**), and *Insm1* (**M–P**) as the probes. No apparent differences were seen in the control and *Tlx3*^{-/-} mice. Scale bars: **A, B, E, F, I, J, M, N**, 20 μ m; **C, D, G, H, K, L, O, P**, 50 μ m.

Ret controls peptide genes *Sst* and *Vip* in developing stellate ganglia

To determine whether *Ret* is required for the expression of peptides *Vip* and *Sst*, we performed *in situ* hybridization experiments. The results showed that the expression of *Vip* and *Sst* remained present in *Ret*^{-/-} mice at E12.5 whereas their expression was eliminated by E18.5 (Fig. 7*M–T*). This indicated that *Ret* coordinately controlled the expression of transmitter and peptide phenotypes in stellate ganglia at the late embryonic stage. This is distinct from the action of *Tlx3*, which controls the expression of these peptides since E12.5.

Discussion

In this study, we found that high-level expression of transcription factor *Tlx3* was widely present in early embryonic stellate ganglionic neurons and became progressively enriched in cholinergic neurons at late embryonic and postnatal stages. Analysis of the

Tlx3^{-/-} mice revealed that *Tlx3* was required for *Vacht* expression at E18.5 but not at E12.5, although it was required for the expression of cholinergic peptides VIP and SOM at both stages. Furthermore, we found that *Tlx3* controlled the expression of *Ret* at a high level, and *Ret* expression was required for the expression of *Vip* and *Sst* mRNAs at the late prenatal stage. These results shed light on the molecular mechanisms underlying differentiation of cholinergic neurons in the embryonic sympathetic nervous system.

Transcriptional control of neurotransmitter phenotypes in sympathetic ganglia

Diverse sympathetic neurons are derived from the multipotent neural crest progenitors (Goridis and Rohrer, 2002). For the postnatal acquisition of cholinergic phenotype in sympathetic neurons, it is well known that target-derived signals are responsible for the switch of the noradrenergic phenotype to the cholinergic fate (Landis, 1990; Asmus et al., 2000). However, cholinergic phenotype was observed in a small population of embryonic sympathetic neurons (Ernsberger et al., 1997; Schäfer et al., 1997; Schütz et al., 1998). The development of these embryonic cholinergic neurons is still poorly understood. It has been reported that transcription factor *Phox2b* was required for the early cholinergic differentiation at E11.5, because cholinergic markers *Chat* and *Vacht* were absent in the sympathetic ganglia of *Phox2b* knock-out mice (Huber and Ernsberger, 2006). Because the expression of noradrenergic markers and generic neuronal

Figure 4. Loss of cholinergic properties in stellate ganglia of *Tlx3*^{-/-} mice. **A–H**, *In situ* hybridization was performed on sections of stellate ganglia from control and *Tlx3*^{-/-} mice at E12.5 and E18.5 with *Vacht* (**A–D**), and *Vip* (**E–H**) as the probes. The *Vacht* and *Vip* signals were absent in *Tlx3*^{-/-} mice at E18.5. Note that the expression of *Vacht* was comparable in *Tlx3*^{-/-} versus control mice, whereas the expression of *Vip* was abolished in *Tlx3*^{-/-} mice early at E12.5. **I–L**, Immunostaining of TH was performed on sections of stellate ganglia from control and *Tlx3*^{-/-} mice at E12.5 and E18.5. No apparent difference was observed between *Tlx3* mutant and control mice. Scale bars: **A, B, E, F, I, J**, 20 μ m; **C, D, G, H, K, L**, 50 μ m.

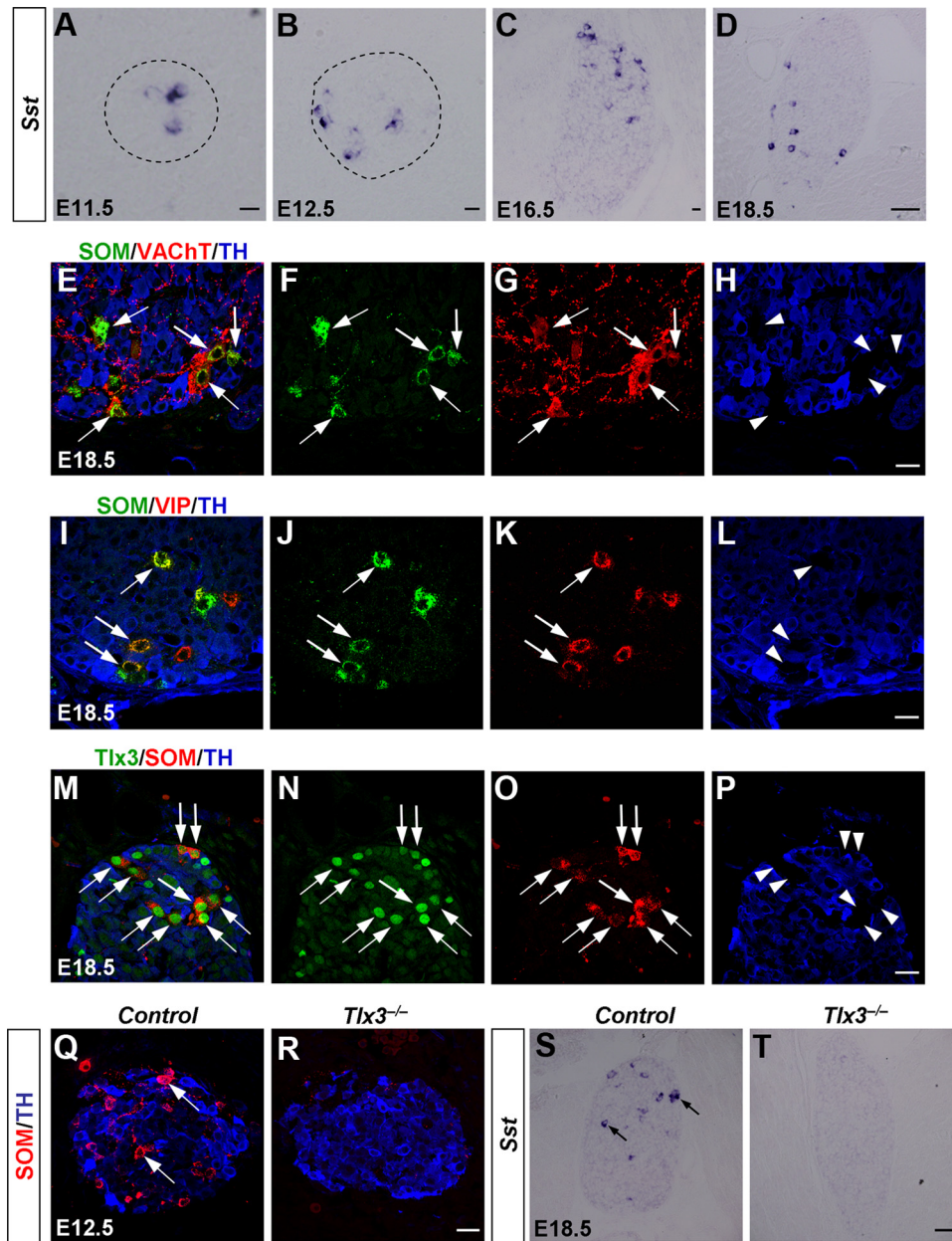


Figure 6. SOM or *Sst* was expressed in cholinergic sympathetic neurons and their expression was abolished in *Tlx3*^{-/-} mice. **A–D**, *In situ* hybridization was performed on sections of primary sympathetic chain at E11.5 (**A**), and stellate ganglia at E12.5 (**B**), E16.5 (**C**), and E18.5 (**D**) with *Sst* as the probe. Strong signals were detected in a small subset of neurons in the embryonic sympathetic ganglia. **E–H**, Triple immunostaining of SOM, VAcHt, and TH was performed on sections of stellate ganglia from wild-type mice at E18.5. The majority of SOM⁺ cells were VAcHt⁺/TH⁻ cholinergic neurons (arrows and arrowheads). **I–L**, Triple immunostaining of SOM, VIP, and TH was performed on sections of stellate ganglia from wild-type mice at E18.5. A large portion of VIP⁺/TH⁻ cells expressed SOM (arrows and arrowheads). **M–P**, Triple immunostaining of *Tlx3*, SOM, and TH was performed on sections of stellate ganglia from wild-type mice at E18.5. Most SOM⁺/TH⁻ cells expressed *Tlx3* (arrows and arrowheads). **Q–R**, Expression of SOM or *Sst* in the stellate ganglia from *Tlx3*^{-/-} mice. Double-staining of SOM and TH was performed on sections of E12.5 stellate ganglia from control and *Tlx3*^{-/-} mice (**Q, R**). *In situ* hybridization was performed on sections of stellate ganglia from control and *Tlx3*^{-/-} mice at E18.5 (**S, T**). Note a complete loss of expression of SOM or *Sst* in *Tlx3*^{-/-} mice at both E12.5 and E18.5. Scale bars: **E–R**, 10 μm; **A–C**, 20 μm; **D, S, T**, 50 μm.

properties were impaired in sympathetic neurons of these *Phox2b* knock-out mice (Pattyn et al., 1999), it is possible that a general deficiency of neuronal differentiation or cell death could underlie the absence of cholinergic markers in these mice (Huber and Ernsberger, 2006). Furthermore, a recent study reported that ongoing *Phox2b* expression was not required for the maintenance of cholinergic differentiation during embryogenesis (Coppola et al., 2010). *Ret* is also required for the acquisition of cholinergic properties in embryonic sympathetic neurons (Burau et al., 2004). In the present study, we showed that transcription factor *Tlx3* controls the cholinergic neuronal development and is required for

the expression of *Ret* at a high level (Figs. 4, 6, 7). Furthermore, we found that *Ret* controlled the expression of cholinergic peptide genes *Vip* and *Sst* in embryonic sympathetic neurons (Fig. 7*M–T*). The fate of prenatal cholinergic neurons in stellate ganglia and potential targets they innervate remain unknown (Guidry and Landis, 1998; Anderson et al., 2006; Schütz et al., 2008; Apostolova and Dechant, 2009; Cane and Anderson, 2009). Further studies on fate mapping and neuronal projection are needed to clarify these issues. What roles *Tlx3* plays in controlling the postnatal emergence of cholinergic neurons remains unknown. Because *Tlx3* knock-out mice die shortly after birth (Shi-

rasawa et al., 2000), it also will be of interest to study whether *Tlx3* is required for the acquisition of the postnatal cholinergic properties by analyzing the *Tlx3* conditional knock-out mice.

Differential regulation of transmitter and peptide in cholinergic neurons

The peptide VIP is expressed in the cholinergic neurons of newborn mice (Stanke et al., 2000). We showed here that the peptide SOM was expressed in embryonic cholinergic neurons as well (Fig. 6E–H). By analyzing the gene deletion mice, we found that the expression of *Vip* and *Sst* mRNAs required *Tlx3* but not *Ret* at the early embryonic stage (E12.5) (Figs. 4E, F, 6Q, R, 7M, N, Q, R), suggesting that *Tlx3* may be required for the initiation whereas *Ret* is only needed for the maintenance of VIP and SOM expression. On the other hand, *Tlx3* and *Ret* are both required for *Vacht* expression at the late (E18.5 or newborn mice) but not early (E12.5 or E13) embryonic stage (Bureau et al., 2004) (Fig. 4A–D), suggesting these genes are both required for the maintenance but not the initiation of *Vacht* expression. Thus, cholinergic transmitter and the peptide phenotypes in the prenatal sympathetic ganglia are differentially regulated. Such differential regulation was also observed in the target-dependent acquisition of cholinergic properties as well. In chick sympathetic ganglia, the timing of *Vip* expression correlates with the innervation of peripheral targets by sympathetic fibers (Ernsberger et al., 1997). Interestingly, the expression of *Vip* but not *Chat* in these sympathetic ganglia depends on the cytokines signaling through heterodimeric gp130/LIFR β receptors (Geissen et al., 1998; Duong et al., 2002). In contrast to that found in chick, gp130 signaling is essential for the postnatal target-dependent expression of both VIP and VACHT in sympathetic stellate ganglia of the mouse (Stanke et al., 2006). Two recent studies also reported differential dependence of the cholinergic loci *Vacht* and *Chat* vs *Vip* gene on *Satb2* and p38 activities that are essential for cholinergic *trans*-differentiation of sympathetic neurons (Apostolova et al., 2010; Loy et al., 2011). Thus, differential regulation of transmitter and neuropeptide phenotypes may be a general phenomenon in both embryonic and postnatal development of cholinergic neurons.

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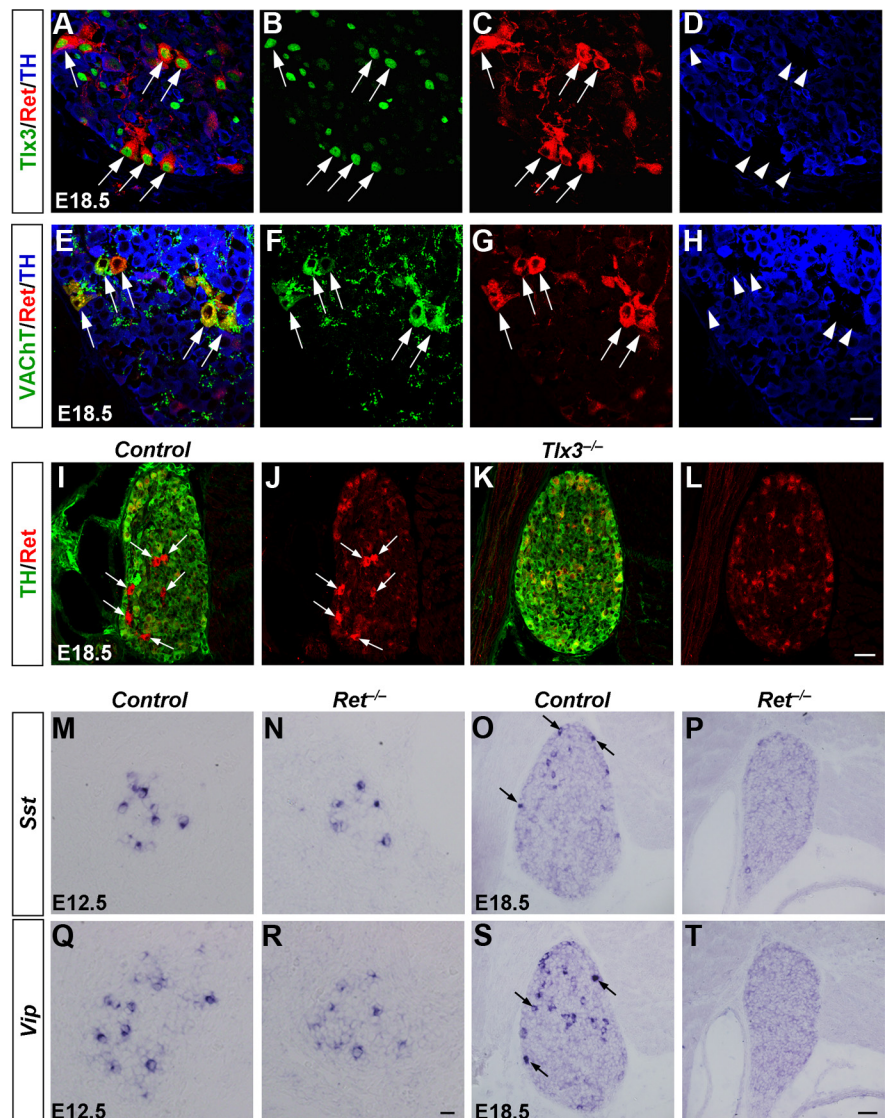


Figure 7. *Tlx3* and *Ret* in regulating the acquisition of cholinergic properties. **A–D**, Triple immunostaining of *Tlx3*, *Ret*, and *TH* was performed on sections of stellate ganglia from wild-type mice at E18.5. **E–H**, Triple immunostaining of VACHT, *Ret*, and *TH* was performed on sections of stellate ganglia from wild-type mice at E18.5. **I–L**, Double-staining of *Ret* and *TH* was performed on sections of stellate ganglia from control and *Tlx3*^{−/−} mice at E18.5 (**I, K**). The expression of *Ret* in the control and *Tlx3*^{−/−} mice was shown in (**J, L**). **M–T**, *In situ* hybridization was performed on sections of stellate ganglia from control and *Ret*^{−/−} mice at E12.5 and E18.5 with *Sst* (**M–P**), and *Vip* (**Q–T**) as the probes. Note that the expression of *Sst* and *Vip* was detectable in *Ret*^{−/−} mice at E12.5, but abolished at E18.5. Scale bars: **A–H**, 10 μ m; **M, N, Q, R**, 20 μ m; **I–L, O, P, S, T**, 50 μ m.

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