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Original Article

Lmx1b controls peptide phenotypes in serotonergic and dopaminergic neurons

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Serotonin (5-HT) neurons synthesize a variety of peptides. How these peptides are controlled during development remains unclear. It has been reported that the co-localization of peptides and 5-HT varies by species. In contrast to the situations in the rostral 5-HT neurons of human and rat brains, several peptides do not coexist with 5-HT in the rostral 5-HT neurons of mouse brain. In this study, we found that the peptide substance P and peptide genes, including those encoding peptides thyrotropin-releasing hormone, enkephalin, and calcitonin gene-related peptide, were expressed in the caudal 5-HT neurons of mouse brain; these findings are in line with observations in rat and monkey 5-HT neurons. We also revealed that these peptides/peptide genes partially overlapped with the transcription factor Lmx1b that specifies the 5-HT cell fate. Furthermore, we found that the peptide cholecystokinin was expressed in developing dopaminergic neurons and greatly overlapped with *Lmx1b* that specifies the dopaminergic cell fate. By examining the phenotype of *Lmx1b* deletion mice, we found that *Lmx1b* was required for the expression of above peptides expressed in 5-HT or dopaminergic neurons. Together, our results indicate that Lmx1b, a key transcription factor for the specification of 5-HT and dopaminergic transmitter phenotypes during embryogenesis, determines some peptide phenotypes in these neurons as well.

Keywords Lmx1b; neurotransmitter; peptide; serotonergic neuron; dopaminergic neuron

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Introduction

The central serotonergic (5-HT) system is an important modulator of neural circuitry and controls a wide variety of physiological processes and behaviors [1]. This system contains nine small clusters of neurons, B1–B9, in midbrain and hindbrain [2,3]. The rostral division (nuclei B4–B9) has cell bodies located in midbrain and rostral pons and provides innervation primarily to forebrain targets. Cells of caudal division (nuclei B1–B3) are located primarily in the medulla oblongata and provide descending projections primarily to the spinal cord [4]. Because 5-HT innervates nearly all levels of central nervous system [3], it is not surprising that the dysfunction of 5-HT system is associated with many neurological and psychiatric disorders, such as aggression, impulsivity, and depression [5].

Although 5-HT neurons share common biosynthetic pathways, they are actually a highly heterogeneous population, which is reflected by the diversity of their anatomical characteristics, axonal morphologies, electrophysiological properties, and genetic regulation [6–8]. The diversity of 5-HT neurons is further suggested by the expression of peptides, including substance P (SP), thyrotropin-releasing hormone (TRH), enkephalin (ENK), and calcitonin gene-related peptide (CGRP) [9].

During embryogenesis, 5-HT neurons develop from the most ventral hindbrain in proximity to the floor plate [10,11]. Extrinsic signaling molecules and intrinsic transcription factors are required for the neurogenesis and maturation of 5-HT neurons [12,13]. Although much progress has been made in understanding the genetic control of 5-HT neurons, the transcriptional regulation of neuropeptides in developing 5-HT neurons is poorly understood.

Lmx1b, a LIM-homeodomain transcription factor, was initially characterized as a critical regulator controlling dorsoventral patterning in developing limbs [14]. In the nervous system, Lmx1b has been shown to specify serotonergic neurons in the developing hindbrain [15,16].

A recent study has revealed that several peptides, such as galanin (GAL), corticotropin-releasing factor (CRF), and SP, do not coexist with 5-HT in the dorsal raphe nucleus (DRN) neurons of mice as they do in rats and humans [17]. It is still unknown whether the variation in peptide expression across species in rostral 5-HT neurons is present in

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caudal raphe 5-HT neurons. Three studies, including ours, have previously shown that the transcription factors that determine glutamatergic or GABAergic cell fate also selectively control the expression of distinct sets of peptides in glutamatergic or GABAergic neurons of developing dorsal spinal cord [18–20]. Therefore, it is of interest to examine whether *Lmx1b* that specifies 5-HT cell fate during mouse development also determines the expression of peptides which are localized in 5-HT neurons. Here, we reported that four peptide genes encoding SP, TRH, ENK, and CGRP were expressed in caudal 5-HT neurons, and Lmx1b was required for their proper expression. A similar phenomenon of the coexistence of peptide cholecystokinin (CCK) in dopaminergic neurons has been reported in rat brain [21]. In this study, we found that *Cck*, a peptide gene, was expressed in developing dopaminergic neurons in the mouse brain, and its proper expression required the presence of *Lmx1b* that specifies the dopaminergic cell fate.

Materials and Methods

Animals

The generation of *Lmx1b* mutant mice has been described previously [14]. Genotyping for *Lmx1b* mutant mice was conducted as described previously [15]. For all timed matings, the morning when vaginal plugs were observed was considered embryonic day (E) 0.5.

In situ hybridization

Single- and double-color *in situ* hybridization experiments were performed following the methods described previously [22,23]. The *in situ* probe *Tac1* (NM_009311, 0.86 kb) was amplified with a gene-specific set of polymerase chain reaction primers and cDNA templates prepared from embryonic brain/spinal cord. Other *in situ* probes have been described previously [24,19] or were obtained from Dr Qiufu Ma (Dana-Farber Cancer Institute, Boston, USA).

For double-color *in situ* hybridization, the purple signal [with Nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates] was developed first, followed by the development of reddish brown signal [with 2-(4-iodophenyl)-5-(4-nitrophenyl)-3-phenyltetrazolium chloride (INT)/BCIP substrates] [23].

Immunostaining and double staining

Immunostaining was performed as described previously [22]. For double-staining *in situ* hybridization combined with immunostaining, *in situ* hybridization was performed first with proteinase K at a lower concentration (1–2 μg/ml) and in shorter digestion time (3–7 min). After NBT/BCIP color development, immunostaining procedures to detect tryptophan hydroxylase (TPH), Lmx1b, 5-HT, SP, ENK, and tyrosine hydroxylase (TH) proteins were conducted. The following first and second antibodies were used: mouse anti-TPH (1 : 200; Sigma-Aldrich, St Louis, USA); mouse anti-Lmx1b (1 : 200), which was generated by immunizing mouse with synthetic peptide according to the website of Dr Thomas Jessell's lab (http://sklad.cumc.columbia.edu/jessell); rabbit anti-5-HT (1 : 500; ImmunoStar, Hudson, USA); rabbit anti-SP (1 : 500; ImmunoStar); rabbit anti-methionine ENK (1 : 500; ImmunoStar); rabbit anti-TH (1 : 500; Pel-Freez, Rogers, USA); goat anti-mouse Cy3 (1 : 500; Jackson ImmunoResearch Laboratories, West Grove, USA); and goat anti-rabbit Alexa-488 (1 : 300; Invitrogen, Carlsbad, USA). For confocal analysis, the fluorescent signals from the middle part (~8 μm) of the cryosections (12–14 μm) were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Oberkochen, Germany) and projected with software (Zeiss LSM Image Browser). The *in situ* signals were photographed under transillumination and a representative image was used as *in situ* signal. To generate the confocal images, the bright-field images of *in situ* hybridization signals were converted into pseudo-red fluorescent color and then merged with the fluorescent images using Adobe Photoshop software.

Results

Expression of peptide genes in 5-HT neurons

5-HT neurons are not a homogenous population. The neurochemical diversity of 5-HT neurons was revealed by the co-localization of a variety of neuropeptides in 5-HT neurons [9]. Furthermore, the co-localization of neuropeptides with 5-HT was shown to be variable across species. An extensive co-localization study was conducted to examine the chemical neuroanatomy of the DRN and adjacent structures of mouse brain [17]. In contrast to observations in the rat and human DRN [25–27], the results of that study revealed that no co-localization of the peptides GAL, CRF, and SP with 5-HT was detected in mouse DRN neurons [17].

In 5-HT neurons at the caudal level of rats and monkeys, it has been reported that several peptides, such as SP, TRH, ENK, and CGRP, coexist with 5-HT [28–32]. Whether these peptides are expressed in the caudal 5-HT neurons of mouse brain remains unclear. We examined the expression of following peptide genes: *Tachykinin 1* (*Tac1*), which encodes the precursor for SP; *Thyrotropin releasing hormone* (*Trh*), which encodes precursor for TRH; *Preproenkephalin* (*Penk*), which encodes precursor for ENK; and *Calcitonin gene-related peptide, alpha* (*Calca*), which encodes precursor for αCGRP. **Fig. 1** showed the spatial and temporal expression patterns of these peptide genes in raphe region at the caudal level. The expression of *Tac1* and *Calca* emerged at ~E13.5, whereas the expressions of *Trh* and *Penk* were initiated at ~E12.5 in raphe areas. From E14.5 to P0, all of these peptide genes were expressed in raphe region.

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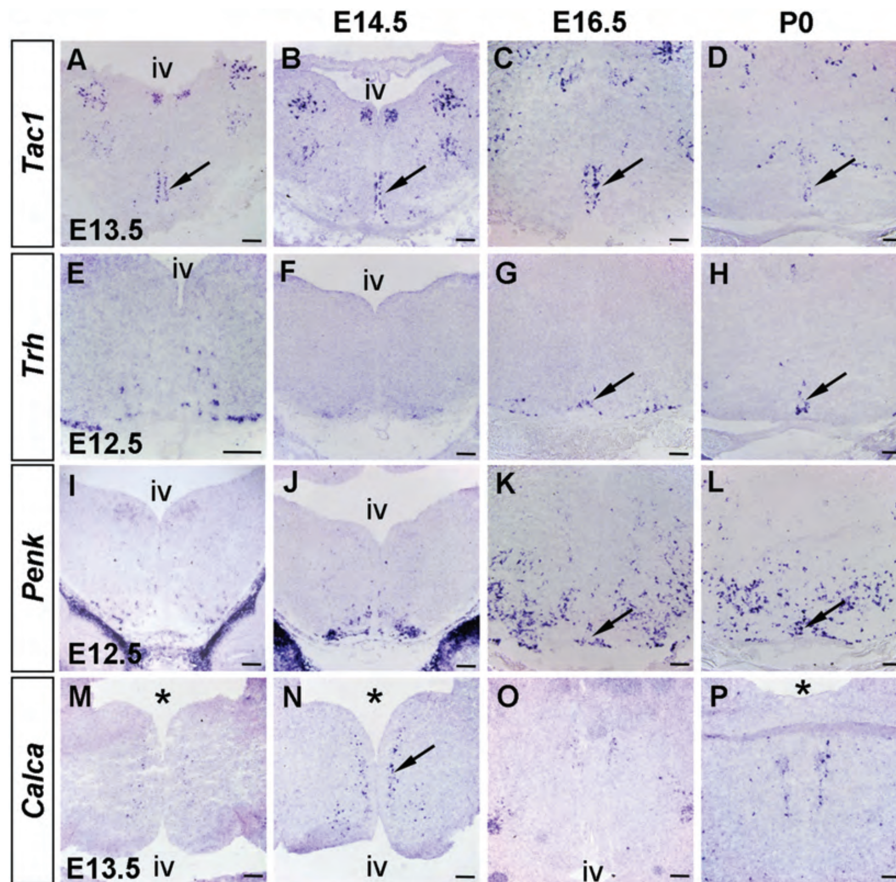

Figure 1 Expression of peptide precursor genes in the developing raphe nucleus *In situ* hybridization was performed on coronal sections of mouse brains at various developmental stages using peptide genes as probes. (A–D) *Tac1* was expressed in medulla oblongata. From E13.5, the expression of *Tac1* was detected and enriched in the caudal raphe nucleus near midline (arrows). (E–H) *Trh* was first expressed in the pons at E12.5 in a scattered pattern. At E16.5 and P0, the expression of *Trh* was enriched in the ventral surface of the pons, and a subset of Trh^+ cells was found near the midline (arrows). (I–L) Expression of *Penk* in the pons. *Penk* was weakly expressed at E12.5; an intense signal was detected in the ventral half of the pons from E14.5 to P0. A subset of $Penk^+$ cells was found surrounding the midline near the ventral surface of the pons (arrows). (M–P) Two strips of *Calca*-expressing cells were found located at the junction between the pons and the rostral part of cerebellum primordium at E14.5 (arrow). '*' Indicates the aqueduct; 'iv' indicates the fourth ventricle. Bar = 100 μm.

To determine whether SP, TRH, ENK, and CGRP peptides were expressed in embryonic 5-HT neurons, we performed double-staining experiments on E14.5 or E16.5 mouse brain sections in which peptide SP was detected by immunostaining. The peptide genes *Trh*, *Penk*, and *Calca* were detected by *in situ* hybridization and 5-HT neurons were detected by TPH or 5-HT immunostaining. Results showed that many 5-HT neurons expressed SP [arrows, **Fig. 2(A,B)**], a moderate number of 5-HT neurons expressed *Trh* [arrows, **Fig. 2(E,F)**], and a few 5-HT neurons expressed *Penk* [arrows, **Fig. 2(I,J)**] or *Calca* [arrows, **Fig. 2(M,N)**].

Two studies, including ours, have revealed that transcription factor Lmx1b is expressed in 5-HT neurons [15,16]. To determine whether the peptides SP, TRH, ENK, and CGRP co-localize with Lmx1b in developing 5-HT neurons, we performed double-staining experiments on E12.5 or E14.5 mouse brain sections in which the peptide SP was detected by immunostaining, peptide genes *Trh*, *Penk*, and *Calca* were detected by *in situ* hybridization and Lmx1b was detected by immunostaining. The results showed that peptide SP [**Fig. 2(C,D)**], and the peptide genes *Trh* [**Fig. 2(G,H)**], and *Penk* [**Fig. 2(K,L)**] were co-localized with Lmx1b. In contrast, most $Calca^+$ neurons in the presumptive raphe area had downregulated the expression of Lmx1b [**Fig. 2(O,P)**].

The peptide SP and peptide genes *Trh* and *Penk* were expressed in mouse medullary 5-HT neurons at comparable levels [**Fig. 2(A–L)**]. To determine whether this peptide and these genes co-localize in same neurons, we performed double-staining experiments on mouse brain sections at E14.5 in which *Trh* mRNA was detected by *in situ* hybridization and peptides SP and ENK were detected by immunostaining. Results showed that a few neurons expressed *Trh* and SP and that many neurons expressed both *Trh* and ENK [**Fig. 2(Q–T)**]. We also performed double-staining experiments on mouse brain sections at P0 in which *Penk* mRNA was detected by *in situ* hybridization and SP was detected by immunostaining. The results showed that

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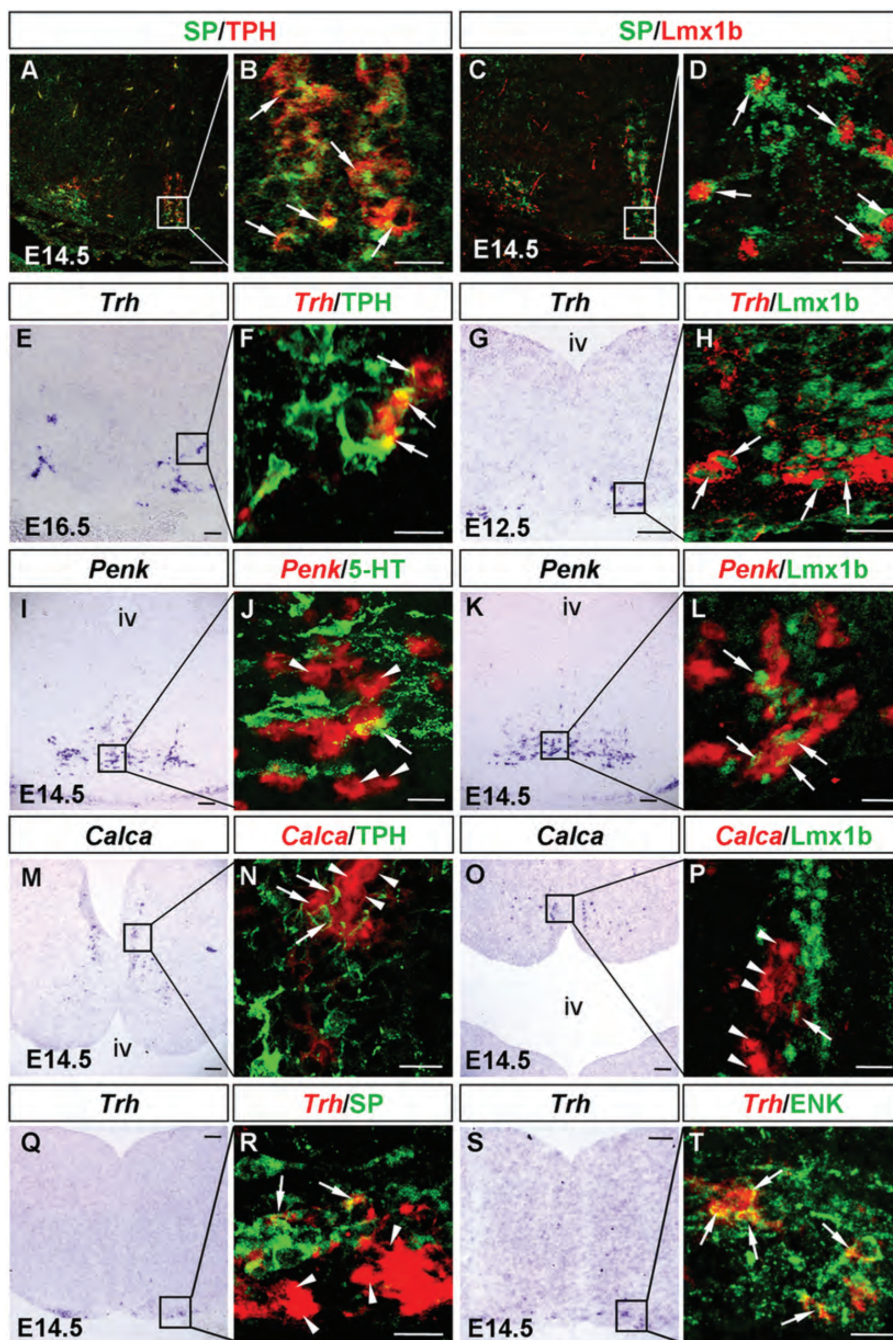

Figure 2 Characterization of SP$^+$, *Trh*$^+$, *Penk*$^+$, and *Calca*$^+$ neurons in raphe nucleus (A–D) Double staining for SP protein (green) and serotonergic marker TPH protein (red) or Lmx1b protein (red) was performed on coronal sections of the medulla oblongata of E14.5 wild-type embryos. Panels B and D represent the boxed areas in A and C and most of the SP$^+$ neurons co-expressed TPH or Lmx1b (arrows). (E–P) Double staining for TPH protein (F, N, green), 5-HT (J, green) or Lmx1b protein (green, H, L, P) and *Trh* (red, F, H), *Penk* (red, J, L), or *Calca* (red, N, P) mRNA on coronal sections of hindbrain at indicated stages. Panels F, H, J, L, N, and P were confocal images indicating the boxed areas in E, G, I, K, M, and O, respectively. Bright-field *in situ* hybridization signals were converted into red pseudocolor signals. Arrows showed the co-localization of *Trh* with TPH (F) or Lmx1b (H). Few *Penk*$^+$ neurons were 5-HT$^+$ (arrows and arrowheads, J), whereas a number of *Penk*$^+$ neurons expressed Lmx1b (arrows, L). A few *Calca*$^+$ neurons co-expressed TPH, and the majority of *Calca*$^+$ neurons had downregulated Lmx1b expression (arrows and arrowheads; N, P). (Q–T) Double staining for SP (green) or ENK protein (green) and *Trh* mRNA (red) on coronal sections of the hindbrain at E14.5. Panels R and T were confocal images indicating the boxed were in Q and S. Bright-field *in situ* hybridization signals were converted into red pseudocolor signals. *Trh* and SP exhibited modest overlap (arrows, R), whereas *Trh* and ENK exhibited major overlap and co-localized with each other (arrows, T). 'iv' indicates the fourth ventricle Bar = 100 μm (A, C, E, G, I, K, M, O, Q, S), 20 μm (B, D, F, H, J, L, N, P, R, T).

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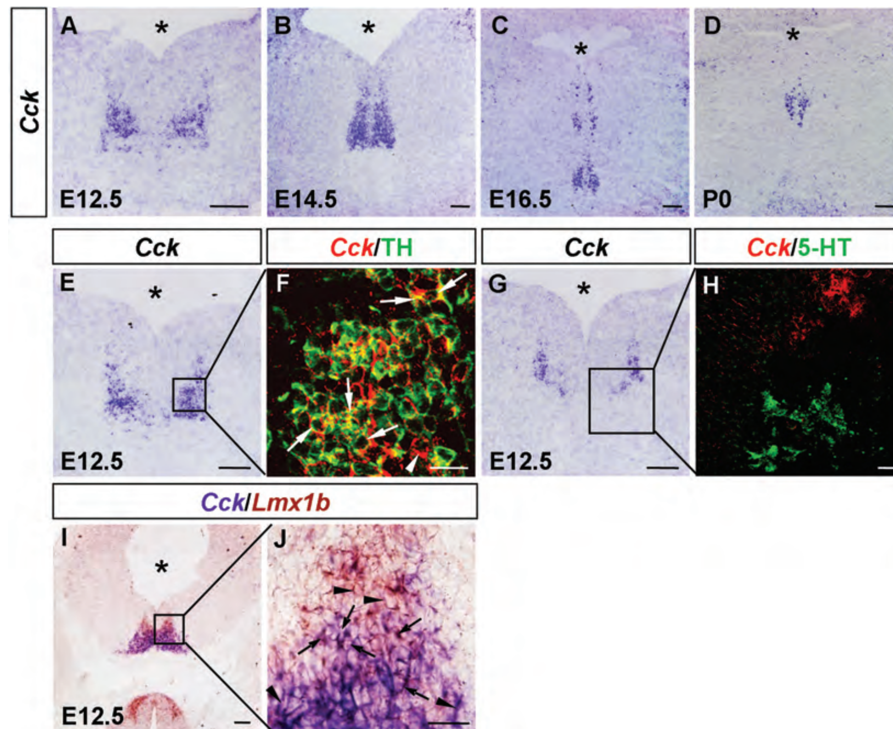

Figure 3 Characterization of *Cck*$^+$ neurons in the vPAG (A–D) *Cck* was expressed in the ventral periaqueductal gray of the mouse brain from E12.5 to P0. The location of *Cck*$^+$ cells was near the midline. (E–H) Double staining of TH protein (green) or 5-HT (green) and *Cck* mRNA (red) on coronal sections of the midbrain at E12.5. Panels F and H were confocal images indicating the boxed areas in E and G. Bright-field *in situ* hybridization signals were converted into red pseudocolor signals. Most of *Cck*$^+$ neurons co-expressed TH (arrows, F). However, *Cck*$^+$ neurons did not express 5-HT. (I–J) Double-color *in situ* hybridization of *Cck* (purple, developed with NBT/BCIP substrates) and *Lmx1b* (brown, developed with INT/BCIP substrates) mRNA was performed on coronal sections of the midbrain at E12.5. Panel J represents a higher magnification of the boxed region in I. Arrows showed the partial overlapping pattern of *Cck* and *Lmx1b* (J). '*' Indicates the aqueduct. Bar = 100 μm (A–E, G, I), 20 μm (F, H, J).

almost no neurons co-expressed *Penk* and SP (data not shown). Further triple-staining experiments in which *Trh* was detected by *in situ* hybridization and SP + TPH or ENK + TPH were detected by immunostaining revealed that few 5-HT neurons expressed *Trh* and SP or *Trh* and ENK simultaneously at E14.5 (data not shown). More studies are warranted to determine whether two peptides are co-localized in 5-HT neurons simultaneously at embryonic stages [31,33,34].

Expression of peptide gene *Cck* in dopaminergic neurons

In addition to 5-HT neurons, it is well known that peptides coexist with other neurotransmitters [35]. For example, peptide CCK has been reported to coexist with TH in the ventral periaqueductal gray (vPAG) of rat brain [21]. To determine whether CCK is expressed in the vPAG of developing mouse brain, we examined the expression of *Cck*, which encodes the precursor for CCK, by performing *in situ* hybridization experiments. Results showed that *Cck* was indeed expressed in the vPAG region of the developing mouse brain from E12.5 to P0 [**Fig. 3(A–D)**].

To determine the identities of the cells in which *Cck* was expressed in developing mouse vPAG region, we performed double-staining experiments in which *Cck* mRNA was detected by *in situ* hybridization and the dopaminergic neuronal marker TH and the 5-HT neuronal marker 5-HT were detected by immunostaining. Results showed that *Cck* was extensively expressed in subsets of dopaminergic neurons, whereas *Cck* was not expressed in 5-HT neurons in the vPAG area [**Fig. 3(E–H)**]. *Lmx1b* has previously been reported to control the development of dopaminergic neurons [36–38]. Therefore, we examined whether *Cck* co-localized with *Lmx1b* by performing double-color *in situ* hybridization experiments. Result showed that many neurons expressed both *Cck* and *Lmx1b* in vPAG region [**Fig. 3(I,J)**].

Lmx1b is required for peptide gene expression in 5-HT and dopaminergic neurons

Studies on peptidergic fates in developing dorsal spinal cord have revealed that peptidergic and neurotransmitter phenotypes can be determined by the same set of transcription factors [18–20]. Because *Lmx1b* specifies 5-HT and dopaminergic cell fate [15,16,36–38], peptide genes *Tac1*, *Trh*, *Penk*, and *Calca* were expressed in 5-HT neurons and *Cck* in dopaminergic neurons (**Figs. 2** and **3**), we examined whether *Lmx1b* controls the expression of the peptide genes

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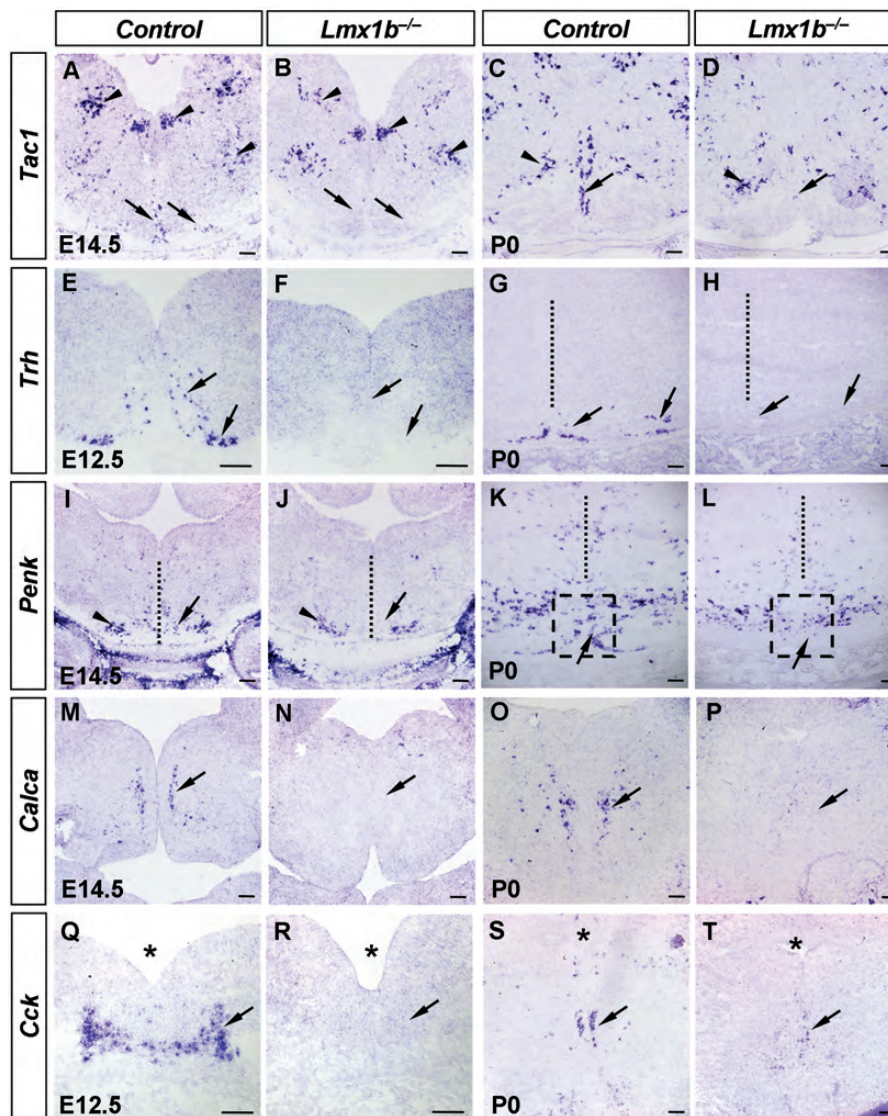

Figure 4 ***Lmx1b*** **is required for the expression of** ***Tac1*****,** ***Trh*****,** ***Penk*****, and** ***Calca*** **in raphe nucleus and the expression of** ***Cck*** **in vPAG region** *In situ* hybridization was performed on coronal sections of wild-type or *Lmx1b*$^{-/-}$ hindbrains at E12.5, E14.5, or P0. Arrows showed the specific loss of *Tac1* (A−D), *Trh* (E−H), *Penk* (I−L), and *Calca* (M−P) expressions in raphe nucleus and the loss of *Cck* (Q−T) expression in the ventral periaqueductal gray of *Lmx1b*$^{-/-}$ embryos. Bar = 100 μm.

in 5-HT and dopaminergic neurons. *In situ* hybridization results showed that the expressions of these peptide genes *Tac1*, *Trh*, *Penk*, and *Calca* in raphe area and the expression of peptide gene *Cck* in vPAG region were abolished or markedly reduced in *Lmx1b* deletion mice at E12.5, E14.5, and P0 [**Fig. 4(A−T)**].

Discussion

It has been well established that peptides usually coexist with classic neurotransmitters in the nervous system [35]. In 5-HT neurons at the rostral level, the expressions of several peptides, including GAL, CRF, and SP, have been reported in rat and human DRN neurons, whereas no coexistence of these peptides with 5-HT in mouse DRN has been detected [17,25−27]. In rat and monkey, peptides SP, TRH, ENK, and CGRP have been reported to coexist with 5-HT in 5-HT neurons at the caudal level [28−32]. In this study, we showed that these peptides also co-localized with 5-HT in developing mouse brain at the caudal level (**Fig. 2**). Furthermore, we observed that the peptide gene *Cck* was expressed in developing dopaminergic neurons in this mouse (**Fig. 3**), which is in line with observations in rats [21].

Studies on the regulation of peptide gene expression in the dorsal spinal cord have revealed that transcription factors which specify glutamatergic and GABAergic neurotransmitter phenotypes also determine the expression of co-localized peptide genes [18−20]. It has been reported that the transcription factor *Lmx1b* specifies 5-HT and dopaminergic cell fate [15,16,36−38]. Therefore, it is of

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interest to examine whether *Lmx1b* controls the expression of peptide genes which coexist with 5-HT or dopamine. Our results revealed that *Lmx1b* indeed controlled the expression of peptide genes *Tac1*, *Trh*, *Penk*, and *Calca* which co-localized with 5-HT, and *Lmx1b* was also shown to control the expression of *Cck* that co-localized with dopamine (**Fig. 4**). Although the expressions of peptide genes *Tac1* [**Fig. 4(A–D)**] and *Penk* [**Fig. 4(I–L)**] in raphe area were controlled by *Lmx1b*, their expression in close brainstem were not affected by *Lmx1b* deletion. Furthermore, it is noteworthy that most *Calca*$^{+}$ neurons did not express Lmx1b at E14.5, whereas its expression was abolished in *Lmx1b* deletion mice [**Fig. 2(P)**, **Fig. 4(M,N)**]. It has been reported that specific deletion of *Lmx1b* in 5-HT neurons results in the loss of 5-HT neuron-specific gene expression [39]. However, it has previously been proposed that the severe reduction of DA neurons in *Lmx1b* deletion embryos is primarily due to an earlier role of *Lmx1b* in regulating the expression of *Fgf8* and *Wnt1* in isthmic organizer and that the lack of phenotypic effects on the specification and differentiation of DA progenitors in *Lmx1b* deletion mice is the result of compensation exerted by *Lmx1a* [38]. Further studies are needed to clarify whether the complete loss of *Calca* was caused by a cell non-autonomous function of *Lmx1b* in midbrain–hindbrain patterning or an intrinsic function of *Lmx1b* in 5-HT neurons. Therefore, although the expression of peptide genes in 5-HT or dopaminergic neurons was controlled by transcription factor *Lmx1b*, the underlying mechanism may be different. Lastly, since a number of *Trh*$^{+}$, *Penk*$^{+}$, and *Calca*$^{+}$ neurons did not express TPH or 5-HT, a complete loss or marked reduce of their expression in *Lmx1b* deletion mice suggested that *Lmx1b* might also control the development of non-5-HT neurons in these regions (**Figs. 2** and **4**).

Above results provide more evidence that the neurotransmitter and peptide phenotypes of single neurons may be specified by the same set of transcription factors. It will be of interest to examine whether additional similar cases will be found. Identifying core transcription factors that determine transmitter and peptide phenotypes in same cells may help generate specific types of neurons for potential functional and therapeutic studies.

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