Research report

Expression of the LIM-homeodomain gene Lmx1a in the postnatal mouse central nervous system

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The LIM-homeodomain transcription factor Lmx1a plays critical roles in roof plate formation as well as in cell fate determination of midbrain dopaminergic neurons during embryonic development, but its function in the adult brain remains unknown. In the present study, as the first step in exploring its function in adult brain, we examined the expression of Lmx1a in the mouse central nervous system (CNS) from birth to adulthood by in situ hybridization. Lmx1a was expressed at high levels in the posterior hypothalamic area, supramammillary nucleus, ventral premammillary nucleus, subthalamic nucleus, ventral tegmental area, compact part of the substantia nigra and parabrachial nucleus from birth to adulthood, and co-localized with its paralogue Lmx1b in these regions. On the other hand, Lmx1a expression in the cochlear nuclei, medial cerebellar nucleus and superior vestibular nucleus was only observed until postnatal day (P) 30 and showed no colocalization with Lmx1b. Lmx1a-expressing neurons in the ventral midbrain were dopaminergic as evidenced by co-expression with tyrosine hydroxylase in these regions. Furthermore, Lmx1a expression was also found in the choroid plexuses and ependymal cells, although its expression was only detected during the first two postnatal weeks. These results suggest that Lmx1a may be involved in postnatal development as well as in maintenance of some aspects of normal brain function.

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1. Introduction

LIM homeodomain transcription factors are involved in regional patterning, cell fate specification and cell differentiation during embryonic development [2,15]. Among the 13 family members present in vertebrates, the Lmx subgroup consists of two paralogues: Lmx1a and Lmx1b. During early embryonic development, Lmx1b is a key regulator of a number of developmental processes, including dorsoventral patterning of vertebrate limbs [3,6,11,16,24].

Differentiation of anterior eye structures [23], formation of kidney glomerular basement membranes [3,10–12,20], and the inductive activity of the isthmic organizer [14], as well as differentiation and survival of hindbrain serotonergic neurons and spinal dorsal horn neurons [8,9]. For its paralogue, Lmx1a (which is mutated in dreher mice and rats [17,19]) is required for the formation of the roof plate and roof plate-derived structures in the developing central nervous system (CNS), such as dorsal spinal cord interneurons [4,18], the cerebellum [17,19] and the cerebral cortex [5]. Additionally, Lmx1a is expressed in the ventral midbrain where it is required for the fate differentiation of midbrain dopaminergic neurons [1,21].

To begin exploring the role of Lmx1a in the postnatal CNS, we have investigated the expression of Lmx1a mRNA in the mouse brain from birth to adulthood. Our comprehensive mapping showed that persistent Lmx1a expression was restricted to the caudal hypothalamic region, ventral midbrain (i.e. dopaminergic neurons) and parabrachial region, and co-localized with Lmx1b in these regions. On the other hand, Lmx1a expression in choroid plexuses and ependymal cells was only present in the first two weeks of postnatal life, and its expression in the cerebellum, cochlear nucleus and superior vestibular nucleus disappeared by P30. These results suggest that Lmx1a may play a role in postnatal development as well as in the maintenance of normal functions in specific neuronal populations in the brain.

Abbreviations: 3V, third ventricle; 4V, forth ventricle; Ch, cerebellum; cp, cerebrum peduncle; Cx, cerebral cortex; DC, dorsal cochlear nucleus; DM, dorsomedial hypothalamic nucleus; DT, dorsal tegmental nucleus; f, fornix; f, fimbria of the hippocampus; GrC, granular layer of the cochlear nucleus; Hi, hippocampus; L, lateral hypothalamic area; LS, lateral septal nucleus; LV, lateral ventricle; Med, medial cerebellar nucleus; PB, parabrachial nucleus; PH, posterior hypothalamic area; PMV, ventral premammillary nucleus; Pr5, principal sensory trigeminal nucleus; SGI, superficial glial zone of the cochlear nucleus; SNC, compact part of the substantia nigra; SNR, reticular part of the substantia nigra; SO, superior olivary complex; St, striatum; STh, subthalamic nucleus; SuM, supramammillary nucleus; Th, thalamus; VC, ventral cochlear nucleus; VTA, ventral tegmental area.

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Fig. 1. (A–C) Lmx1a mRNA is observed in the posterior hypothalamic (PH) area, subthalamic (STh) nucleus and ependymal cells of the third ventricle (3V) at P0. (B) and (C) show higher magnification of (A). (D–H) A few Lmx1a-expressing neurons are seen in the dorsomedial hypothalamic nucleus (DM) and perifornical area surrounding the fornix (f), whereas comparatively more are located in the subthalamic nucleus at P7 (D–F) and P12 (G and H). (E) and (F) show higher magnification of (D) and (H) shows higher magnification of (G). (I–I′′) Double labeling of Lmx1a mRNA (magenta in I′′) and Lmx1b protein (green in I′′) in the subthalamic nucleus at P7. Arrowheads point to neurons expressing only Lmx1a mRNA; the arrow points to a neuron expressing only Lmx1b; double arrows point to neurons expressing both Lmx1a and Lmx1b. cp, cerebral peduncle; Hi, hippocampus; Th, thalamus; LV, lateral ventricle. Scale bars: 150 μm (A, D and G); 50 μm (B, C, E, F and H); 30 μm (I′, applies to I–I′′).

2. Materials and methods

2.1. Animals and section preparation

C57B6 mice were sacrificed at different postnatal stages (P0, P7, P12, P30, P60 and P180; n = 4 for each stage). Mice were deeply anesthetized with sodium pentobarbital (70 mg/kg, i.p.) and perfused transcardially with 0.01 M phosphate buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain and spinal cord were removed and transversely sectioned into 14-μm thick slices with a cryostat after cryoprotection with 25% sucrose in PBS.

2.2. In situ hybridization

Lmx1a full-length cDNA was isolated using primers designed based on the sequence of the mouse gene (Genbank accession number X81406; forward: ATGGTG-GAAGCGCTGAAG, reverse: CTCAAGAGGTGAAATAGGAATTC) and sub-cloned into the pGEM-T vector. Digoxigenin-labelled Lmx1a anti-sense riboprobes were synthesized and used for in situ hybridization on brain sections as described previously [14]. A sense riboprobe was also generated and used as a negative control (see Fig. 2G). Anatomical identification and nomenclature were adapted from the mouse brain atlas of Paxinos and Franklin [22].
Fig. 2. (A–C) Lmx1a mRNA is observed in the ventral tegmental area (VTA), compact part of the substantia nigra (SNC), supramammillary nucleus (SuM) and ventral pre-mammillary nucleus (PMV) at P0. (B) and (C) show higher magnification of (A). (D–F) Expression of Lmx1a in the ventral tegmental area, compact part of the substantia nigra and supramammillary nucleus at P12 (D and E) and P180 (F). (E) shows higher magnification of (D). (G) No in situ signal is observed when the sense RNA probe is used in place of the anti-sense Lmx1a riboprobe. (H–H′′) Double immunostaining of TH (green in H′′) and Lmx1a (red in H′′) in the ventral midbrain at P7. The arrow points to a neuron expressing only Lmx1a; the arrowhead points to a neuron expressing only TH; double arrows point to neurons co-expressing Lmx1a and TH. SNR, reticular part of the substantia nigra. Scale bars: 150 μm (A and D); 50 μm (B, C, E, F and H); 25 μm (H′′, applies to H–H′′).

2.3 In situ hybridization and immunohistochemistry double labeling

In situ hybridization of Lmx1a was performed first as described in [14] but without proteinase K treatment. After completing in situ hybridization procedures, sections were immunostained with the primary antibodies rabbit anti-Lmx1b (1:1000) [7], or rabbit anti-Th1 (1:2500, a gift from Dr. R. Hevner, University of Washington, Seattle, WA, USA) in PBS containing 0.3% Triton X-100 and 2% normal donkey serum overnight at 4 °C. Sections were then treated with biotinylated donkey anti-rabbit IgG antibody (1:400; Jackson Immunoresearch, USA) for 2 h and Cy2-conjugated streptavidin (1:1000; Jackson Immunoresearch) for 1 h. In situ hybridization signals were imaged under bright field illumination and immunofluorescence signals were captured under epifluorescent light. For overlays, in situ hybridization signals were pseudo-colored magenta.

2.4 Double immunolabeling of Lmx1a and tyrosine hydroxylase

Double labeling of Lmx1a with tyrosine hydroxylase (TH) was performed by incubating P12 sections with both rabbit anti-Lmx1a (1:2000; a gift from Dr. M.
Fig. 3. (A–I) *Lmx1a* mRNA is observed in the parabrachial (PB) nucleus, superficial glial zone of the cochlear (SGl) nucleus, granular layer of the cochlear (GrC) nucleus, dorsal cochlear (DC) nucleus and granule cell layer of the paraflocculus (arrowheads in F and I) of the cerebellum (Cb) at P0 (A–C), P7 (D–F) and P12 (G–I). Arrows (F and I) indicate *Lmx1a* expression in the choroid plexus in the later recess of the fourth ventricle. Note that *Lmx1a* expression is observed in the paraflocculus but not other areas of the cerebellum. (B) and (C) show higher magnification of (A); (E) and (F) show higher magnification of (D); (H) and (I) show higher magnification of (G). (J–J") Double labeling of *Lmx1a* mRNA (magenta in J") and *Lmx1b* protein (green in J") in the parabrachial nucleus at P7. Arrowheads point to neurons expressing only *Lmx1a*; the arrow points to a neuron expressing only *Lmx1b*; double arrows point to neurons expressing both *Lmx1a* and *Lmx1b*. Pr5, principal sensory trigeminal nucleus; SO, superior paraolivary nucleus; VC, ventral cochlear nucleus. Scale bars: 150 μm (A, D and G); 50 μm (B, C, E, F, H and I); 30 μm (J", applies to J–J").

German, UCSF Diabetes Center, CA, USA) and mouse anti-TH (1:5000; Sigma, USA) antibodies overnight at 4°C. Signals were visualized by incubating sections with biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories, USA) for 2 hours followed by a 3-hour incubation with a mixture of Cy3-conjugated streptavidin (1:1000; Jackson Immunoresearch), and Cy2-labeled donkey anti-mouse IgG (1:200; Jackson Immunoresearch).

2.5. Cell counting

Cell counting of *Lmx1a*- and *Lmx1b*- or TH-positive neurons was performed on every fifth 16-μm thick coronal sections covering the given brain regions (e.g. posterior hypothalamic region, subthalamic nucleus, supramammillary nucleus, ventral tegmental area and compact part of the substantia nigra) from four animals at P7 and P12. Cell counts were pooled and proportion of double-labeled neurons in whole neuronal population was calculated. All data values are presented as mean ± S.D.

3. Results

Expression of *Lmx1a* mRNA was restricted to a few regions in the postnatal CNS, including the hypothalamus, midbrain and hindbrain. *Lmx1a* expression persisted in most of these regions from birth to adulthood. We focused our initial characterization of *Lmx1a*
Fig. 4. (A–E) *Lmx1a* mRNA is observed in the choroid plexus (arrows) and ependymal cells (arrowheads) of the lateral ventricle (LV) (A, B and D), third ventricle (B and C) and fourth ventricle (E) at P0. Note that *Lmx1a* is also expressed in the medial cerebellar nucleus (Med) (E). (C) and (D) shows higher magnification of (B). (F and G) No *Lmx1a* expression is detectable in the choroid plexus (arrow) and ependymal cells (arrowheads) of the third ventricle (F) or lateral ventricle (G) at P7. (H–H′′) Double labeling of *Lmx1a* mRNA (magenta in H′′) and Tbr1 protein (green in H′′) in the medial cerebellar nucleus at P12. Double arrows point to neurons expressing both *Lmx1a* and Tbr1. Scale bars: 150 μm (A, D and G); 50 μm (B, C, E, H and I); 30 μm (H′, applies to H–H′′).

expression in the postnatal CNS at P0–P7 because *in situ* signals were strongest and most broadly distributed in neonates relative to older mice.

In the hypothalamus, many strongly labeled neurons were observed at P0 and P7 in the posterior hypothalamic area, subthalamic nucleus (Fig. 1A–F), supramammillary nucleus and ventral premammillary nucleus (Fig. 2A and C and data not shown). As *Lmx1b* is also expressed in these regions [7], we examined whether both paralogues are expressed in the same populations of neurons. Double labeling of *Lmx1b* protein and *Lmx1a* mRNA revealed that *Lmx1a* and *Lmx1b* expression overlapped well in these regions, and the majority of labeled neurons expressed both (n = 4 for each; 70.2 ± 6.7% in the posterior hypothalamic region; 92.6 ± 8.5% in the subthalamic nucleus; 68.5 ± 7.3% in the supramammillary nucleus; Fig. 1I–I′′ and data not shown). *Lmx1a* expression remained stable in the posterior hypothalamic areas and the subthalamic nucleus (Fig. 1G and H) at all stages examined (until P180), although *in situ* signals progressively weakened (data not shown). Note that no *Lmx1a* mRNA was detected in the olfactory bulb, cerebral cortex, hippocampus or striatum (Fig. 4A and B).

In the P0 midbrain, *Lmx1a* mRNA was only observed in the ventral tegmental area and the compact part of the substantia nigra (Fig. 2A and B). Similar to the hypothalamus, double labeling showed that 70.4 ± 6.5% of *Lmx1a*-expressing cells also expressed *Lmx1b* in these regions (n = 4; data not shown). During early embryonic development, *Lmx1a* is expressed in precursors of midbrain dopaminergic neurons as well as differentiating dopaminergic neurons where it is thought to be required for the specification of
dopaminergic cell fate [1,2]. Double immunostaining showed that Lmx1a continued to be expressed in TH-positive neurons in the ventral midbrain at early postnatal stages (Fig. 2H–H′). 71.5 ± 6.8% of Lmx1a-positive neurons were co-labeled with TH, and double-labeled neurons corresponded to 68.6 ± 7.0% of TH-positive neurons (n = 4). Thus, Lmx1a is expressed in the midbrain dopaminergic neurons in the postnatal mouse brain. Although the staining intensity diminished with age, Lmx1a remained expressed in the ventral midbrain until P180 (Fig. 2D–F).

In the P0–P7 hindbrain, expression of Lmx1a mRNA was found in the parabrachial nucleus (Fig. 3A, B, D and E), medial cerebellar nucleus (Fig. 4E) and superior vestibular nucleus (data not shown). Expression in the medial cerebellar nucleus and superior vestibular nucleus was confirmed by co-labeling with Tbr-1 (Fig. 4H–H′ and data not shown), a marker of the deep cerebellar nucleus and superior vestibular nucleus [26]. Lmx1a transcripts were also found in the granular layer and superficial glial zone of the cochlear nucleus, as well as in the dorsal cochlear nucleus (Fig. 3A, C, D and F). Lmx1a and Lmx1b were co-expressed in the parabrachial nucleus (Fig. 3J–J′), but not in other hindbrain regions where Lmx1a expression was found. While no other cerebellar nuclei expressed Lmx1a mRNA at P0 or P7, expression initiated in the internal granule cell layer of the paraflocculus of the cerebellum by P12 (Fig. 3D, F, G, I and data not shown). Expression in the parabrachial nucleus (Fig. 3G and H) persisted until P180, whereas in the cerebellum (i.e. medial cerebellar nucleus and granular cells of the paraflocculus), cochlear nucleus and superior vestibular nucleus Lmx1a expression disappeared by P30 (data not shown). In situ hybridization did not reveal Lmx1a expression in other regions of the hindbrain and spinal cord at all stages analyzed.

In addition to the brain regions mentioned above, Lmx1a was also expressed in ependymal cells and choroid plexus throughout the ventricular system, including the lateral, third and fourth ventricles at P0 (Figs. 1A and B, 4A–E). However, by P7, Lmx1a expression was restricted to the choroid plexus of the fourth ventricle (Fig. 3F), and was not observed in the ependyma or other choroid plexuses (Fig. 4F and G). Expression in the fourth ventricle persisted to P12 (Fig. 3I), but was undetectable by P30 (data not shown).

4. Discussion

In the present study, we examined the distribution of Lmx1a mRNA in the postnatal mouse CNS by in situ hybridization. Expression of Lmx1a in the posterior hypothalamic area, subthalamic nucleus, midbrain dopaminergic neurons and parabrachial nucleus persisted from birth to adulthood, whereas its expression in the cerebellum, cochlear nucleus, superior vestibular nucleus and neuronal cells (ependymal cells and choroid plexus) extinguished within the first month of postnatal life. These data suggest that Lmx1a expression is developmentally regulated, and that it may contribute to the maturation, function and maintenance of the postnatal and adult brain. Interestingly, Lmx1a and Lmx1b were co-expressed in the posterior hypothalamic area, subthalamic nucleus, ventral midbrain and parabrachial nucleus, suggesting that the two paralogues may function in a combinatorial manner in the postnatal brain.

Lmx1a has been shown to be expressed in the basal plate of the hypothalamus in the embryonic brain [13], and we found that Lmx1a expression in the posterior hypothalamic area, superior mamillary nucleus and ventral premammillary nucleus as well as subthalamic nucleus is maintained into postnatal life. Lmx1a mRNA is observed in the deep cerebellar nuclei at embryonic stages [13], but was only localized in the medial cerebellar nucleus after birth. In addition, we found Lmx1a to be expressed in the cochlear nucleus and superior vestibular nucleus from birth and until P30.

Expression in these areas has not been observed in studies of the embryonic mouse brain, either because these nuclei are too small to be clearly identified in the embryonic brain or Lmx1a expression is developmentally regulated in these regions.

Previous studies have shown that Lmx1a plays an important role in the development of roof plate-derived or -associated brain structures [4,17,19]. Furthermore, embryonic expression of Lmx1a in the cortical hem (a local organizer for patterning of the cerebral cortex) [13] is likely necessary for normal development of the cerebral cortex, which is malformed in dreher mice and rats [17,19]. Consistent with the critical role of Lmx1a in brain morphogenesis, recent studies have indicated that Lmx1a is required and sufficient for the differentiation of midbrain dopaminergic neurons during embryonic development [1,21]. In the present study, we found that Lmx1a was expressed in midbrain dopaminergic neurons from birth to adulthood, and was co-expressed with Lmx1b in the ventral tegmental area and compact part of the substantia nigra. Because Lmx1b is also required for the differentiation of dopaminergic neurons [25], it is of interest to determine whether these two transcription factors are required for the postnatal maturation, survival or functioning of midbrain dopaminergic neurons, and to assess whether they operate in an independent, redundant or combinatorial manner. Lmx1a expression is present in the choroid plexuses at embryonic stages [13]. Our data showed that in addition to choroid plexuses, Lmx1a mRNA was also expressed in ependymal cells surrounding the cerebral ventricles during the first two postnatal weeks. Although the function of Lmx1a in these structures is unknown, it is possible that Lmx1a is involved in the formation and postnatal maturation of the choroid plexuses as well as the normal secretion of cerebrospinal fluid. A detailed analysis of these structures and cerebrospinal fluid in dreher mice and rats is needed.

Conflict of interest

We declare that we have no competing financial interests.

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