# 8
# Transcriptional Control of the Development of Central Serotonergic Neurons

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**Abstract**

The central serotonergic neurons [5–hydroxytryptamine (5–HT) neurons] are several clusters of neurons located bilaterally along the midline and in the adjacent reticular formation of the brainstem. These neurons project widely to almost every part of the brain and spinal cord, and thereby modulate a variety of developmental processes and animal behaviors. A deregulation of the 5–HT level in the central nervous system might contribute to numerous psychiatric disorders, including fear, aggression, depression, and anxiety, and to pain modulation. The recent identification of 5–HT-specific transcription factors and the genetic manipulation of these factors in mice have begun to unveil molecular mechanisms underlying the specification, differentiation, survival, and maintenance of the central 5–HT neurons. This chapter summarizes some of recent advances about transcriptional control of 5–HT neuron development, and highlights the results from gene-targeting experiments in mice. The discussion concentrates on two classes of transcription factors. One class, represented by *Nkx2.2* and *Mash1*, is expressed in 5–HT progenitor cells residing within the ventricular zone. The second class, represented by *Lmx1b* and *Pet1*, is expressed in 5–HT postmitotic neurons only. The comparison of different mutant lines that lack an individual transcription factor has permitted us to speculate about their relationship during the development of 5–HT neurons. Finally, behavioral study of knockout mice with impaired 5–HT systems have shown the involvement of some transcription factors in the etiology of psychiatric abnormalities.

## 8.1
## Introduction

The 5–hydroxytryptamine (5–HT) system in the central nervous system (CNS) comprises several groups of morphologically distinct neurons that are mainly distributed in the midline region of the brainstem along the rostrocaudal neuroaxis. They are clustered in the raphe nuclei of the brainstem, and some are dispersed in the adjacent reticular formation (Tork, 1990; Jacobs and Azmitia, 1992). One widely used

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nomenclature for central 5–HT neurons was originally proposed by Dahlstrom and Fuxe (1964), who classified them into nine clusters (B1–B9) on the basis of their anatomical architecture and location (Fig. 8.1). Caudal 5–HT neurons (B1–B4) are located in the medulla oblongate, whereas rostral 5–HT (B5–B9) neurons reside in the pons and the caudal-most part of the midbrain. Another commonly used nomenclature for 5–HT neurons is based on their anatomical architecture in the raphe nuclei of the brainstem (Tork, 1990; Jacobs and Azmitia, 1992) (Table 8.1). The 5–HT neurons probably possess the most complex projection network in the CNS: three major descending 5–HT efferents emanating from B1–B4 neurons project to the spinal cord, whereas B5–B9 neurons send their efferents through five major ascending routes to almost every region of the CNS (Tork, 1990; Jacobs and Azmitia, 1992). These neurons release 5–HT that interacts with at least 14 5–HT receptors, most of which are G-coupled receptors to initiate a downstream second message signal transduction pathway (Martin et al., 1998; Pauwels, 2000).

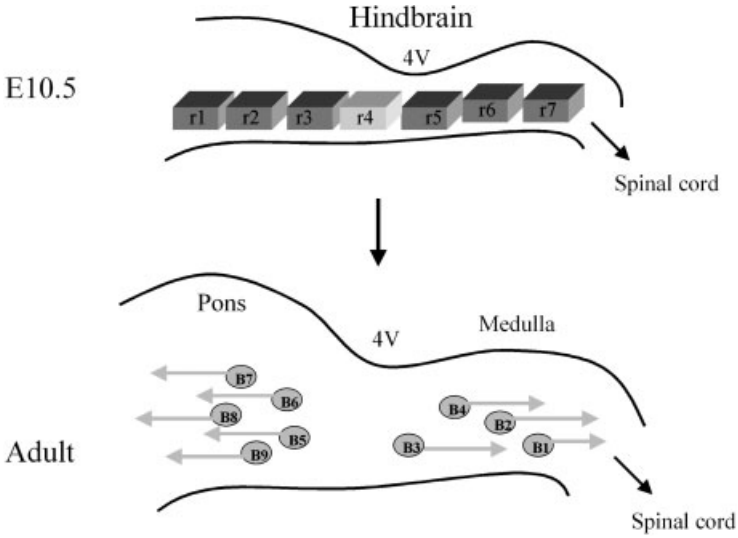


**Fig. 8.1** Schematic diagram of the organization of 5–HT neurons in the brainstem during early mouse embryonic development and at adult stage. The developing hindbrain comprises a series of repeated segments called rhombomeres. Beginning at E10.5, 5–HT neurons are generated from r1–r3 and subsequently from r5–r7 (green). No 5–HT neurons are generated from r4 (pink). In the adult brainstem, nine clusters (B1–B9) of 5–HT neurons are classified in raphe nuclei according to their distinct locations and structures. Caudal 5–HT neurons (B1–B4) project to the brainstem and the spinal cord, whereas rostral 5–HT neurons (B5–B9) project to all parts of the brain. r1 may give rise to the dorsal raphe nucleus (B7/B6), whereas r2–r3 may generate the rest of rostral 5–HT neurons (B5, B8, and B9). By contrast, caudal 5–HT neurons (B1–B4) probably originate in r5–r7. The ontogenic relationship between rhombomeres, except r1, and 5–HT neurons remains unclear. Abbreviations: r = rhombomeres; 4V = fourth ventricle.

**Table 8.1** Classification of 5–HT neurons and ontogenic relationship between B nuclei and rhombomeres.

| Location | B nuclei | Raphe nuclei | Rhombomere |
|---|---|---|---|
| Caudal | B1 | Nucleus raphes pallidus | r5–r7 |
| Caudal | B2 | Nucleus raphes obscurus | r5–r7 |
| Caudal | B3 | Nucleus raphes magnus | r5–r7 |
| | | Rostral ventrolateral medulla | |
| | | Lateral paragigantocellular reticular nucleus | |
| Rostral | B4 | Central gray of the medulla oblongata | r2–r3 |
| Rostral | B5 | Pontine median raphe nucleus | r2–r3 |
| Rostral | B6 | Pontine dorsal raphe nucleus | r1 |
| Rostral | B7 | Midbrain dorsal raphe nucleus | r1 |
| Rostral | B8 | Midbrain median raphe nucleus | r2–r3? |
| | | Caudal linear nuclei | |
| Rostral | B9 | Medial lemniscus | r2–r3? |

Through extensive projections, 5–HT exerts modulatory function in the neurotransmission of many types of neurons, and its dysregulation has been implicated in numerous psychiatric disorders, such as anxiety, aggression, and depression, and in pain modulation (Nelson and Chiavegatto, 2001; Millan, 2002).

During early embryonic development, the distinction among different groups of 5–HT neurons is much less clear. The 5–HT neurons are among the earliest-born neurons that are generated in the CNS. In mice, 5–HT neurons are generated between E10.5 and E12.5 as a stream of cells exits the cell cycle and migrates to settle down either near the midline of the ventral hindbrain or to the more lateral region that constitutes the reticular formation (Ding et al., 2003; Pattyn et al., 2004). The axons of 5–HT neurons do not cross the midline, and fuse until the late stages of embryonic development and early postnatal stages. Like the development of other neurons in the neural tube, the development of 5–HT neurons follows a well-defined spatiotemporal sequence. The generation of rostral 5–HT neurons precedes caudal 5–HT neurons along the brainstem axis. Moreover, before the generation of 5–HT neurons, visceral motor neurons (vMNs) are derived from the same domain of the ventral VZ; the vMNs are derived between E9.5 and E10.5 (Pattyn et al., 2003). A few 5–HT cells appear in the rostral-most part of the pons between E10.5 and E10.75. By E11.5, an increasing number of 5–HT neurons appear in the more caudal part of the pons and the rostral part of the medulla. At E12.5, almost all of distinct classes of 5–HT neurons are present in the brainstem (Ding et al., 2003). Anatomically, the embryonic hindbrain is composed of a series of segments called rhombomeres (r) (Lumsden and Krumlauf, 1996). Most 5–HT neurons derive from r2–r3 and r5–r7 (Pattyn et al., 2003). Rhombomere 1 gives rise to the dorsal raphe nuclei (presumptive B7 and B6 neurons), whereas r4, from which no 5–HT neurons are generated, serves as a spacer dividing rostral from caudal 5–HT neurons (Pattyn et al., 2003). The relationship among B clusters, 5–HT nuclei, and embryonic rhombomeres is listed in Table 8.1.

The generation of diverse ventral cell types, including somatic motor neurons and vMNs along the neural axis, requires floor plate- and notochord-derived signals (Tanabe and Jessell, 1996; Briscoe et al., 1999; Jessell, 2000). The induction and specification of 5–HT neurons may depend on *Sonic hedeghog* (*Shh*), which acts through a number of transcription factors (Hynes and Rosenthal, 1999; Goridis and Rohrer, 2002). Because *Shh* expression is not limited to the hindbrain, other region-specific secreted factors may also participate in the specification of 5–HT neurons. The transcription factor *Nkx2.2* can specify the ventral cell types in the developing spinal cord in response to *Shh* signaling; thus *Nkx2.2* may have an analogous role in specifying 5–HT neurons in the hindbrain (Ericson et al., 1997; Briscoe et al., 1999; Gaspar et al., 2003). However, the manner in which these signaling molecules are converted into a combinatorial transcriptional code that confers the identity of 5–HT neurons is still unknown.

## 8.2
## Transcription Factors in the Development of 5–HT Neurons

Over the past few years, an increasing number of transcription factors have been identified in 5–HT neurons and their precursors. To date, at least six transcription factors have been identified in the development of 5–HT neurons (Hendricks et al., 1999, 2003; Cheng et al., 2003; Ding et al., 2003; Craven et al., 2004; Pattyn et al., 2003, 2004) (Table 8.2). These transcription factors can be divided into several classes according to their spatiotemporal expression profile. *Nkx2.2* and *Mash1* are expressed in 5–HT progenitor cells, and their expression stops as soon as 5–HT progenitors exit the cell cycle and become postmitotic. By contrast, *Gata2* is expressed in both the VZ and postmitotic cells (Nardelli et al., 1999). Finally, *Gata3*, *Pet1*, and *Lmx1b* are mainly expressed in postmitotic 5–HT neurons. Among the six transcription factors, only *Pet1* is exclusively expressed in 5–HT neurons. Researchers have shown that the other five transcription factors are important developmental regulators of the specification of distinct neuronal types in the CNS (Guillemot et al., 1993; Ericson et al., 1997; Briscoe et al., 1999; Nardelli et al., 1999; Ding et al., 2004). These results strengthen the idea that similar mechanisms used by these transcription factors in other neuronal types may be adopted in the development of 5H-T neurons, or vice versa.

## 8.3
## Transcription Factors Expressed in 5–HT Progenitor Cells

### 8.3.1
### *Nkx2.2*

*Nkx2.2* is a homeodomain-containing transcription factor that plays an essential role in the specification of the ventral cell type in response to *Shh* signals in the spinal cord and hindbrain (Ericson et al., 1997; Briscoe et al., 1999). *Nkx2.2* is the

**Table 8.2** Summary of the phenotype of knockout mice lacking individual transcription factor in 5–HT neurons.

| Genes | Expression | 5–HT defects | KO lethality | 5–HT-specific TFs | Reference(s) |
|---|---|---|---|---|---|
| Nkx2.2 | VZ | 100% except in r1 | P0 | Lost except in r1 | Ding et al. (2003); Pattyn et al. (2003) |
| Mash1 | VZ | Almost 100% | P0 | All lost | Pattyn et al. (2004) |
| Gata2 | VZ, postmitotic | 100% | E10–12.5 | All lost | Craven et al. (2004) |
| Lmx1b | Postmitotic | 100% | P0 | All lost | Cheng et al. (2003); Ding et al. (2003) |
| Pet1 | Postmitotic | 70% | Mostly viable | Unknown | Hendricks et al. (2003) |
| Gata3 | Postmitotic | Mostly in the caudal | E11.5–13.5 | Unknown | Pattyn et al. (2004); van Doorninck et al. (1999) |

KO = knockout; TF = transcription factor; VZ = ventricular zone.

earliest transcription factor that has been shown to be required for the specification of 5–HT neurons (Briscoe et al., 1999; Cheng et al., 2003; Ding et al., 2003; Pattyn et al., 2003). In the developing hindbrain, *Nkx2.2* is restricted to the VZ (Pattyn et al., 2003). Between E9.5 and E10.5, it is coexpressed with two other transcription factors, *Nkx2.9* and *Phox2b*, that are important for the generation of vMNs. The extinction of *Phox2b* and *Nkx2.9* expression in the ventral-most domain of the hindbrain is accompanied by the cessation of vMN generation, and is a prerequisite for the initiation of the generation of 5–HT neurons from the same domain (Pattyn et al., 2003). In the absence of *Phox2b*, production of 5–HT neurons is premature, in addition to the failure of vMN generation. Thus, *Nkx2.2* may serve as one of the intrinsic factors upstream of *Phox2b* to instruct the switch of vMN progenitor cells into 5–HT neuronal progenitor cells.

In *Nkx2.2* mutants, *Nkx2.9* expression expands to the ventral-most part, and 5–HT neurons fail to be generated to a large degree. These findings are consistent with the idea that *Nkx2.2* is necessary for the specification of 5–HT neurons (Ding et al., 2003; Pattyn et al., 2003). *Nkx2.9* is unable to rescue the deficiency of *Nkx2.2* with regard to the generation of 5–HT neurons; this finding suggests that *Nkx2.2* may be endowed with a 5–HT-specific property. Intriguingly, not all 5–HT neurons depend on *Nkx2.2* for their specification. *Nkx2.2* is dispensable in r1 because 5–HT neurons derived from r1 are not affected by the *Nkx2.2* mutation (Ding et al., 2003; Pattyn et al., 2003). These data suggest that a discrete mechanism underlies the specification of 5–HT neurons in r1. Perhaps other unidentified r1–specific factors are involved.

In the ventral hindbrain, *Nkx2.2* possesses dual function with regard to the generation of motor neurons and 5–HT neurons. *Nkx2.2* is crucial for the generation of vMNs by initiating *Phox2b* expression that in turn represses 5–HT fate before the

onset of the neurogenesis of 5–HT neurons in the ventral-most part of the hindbrain. In the absence of *Phox2b*, *Nkx2.2* adopts a default pathway to promote 5–HT fate.

### 8.3.2 *Mash1*

*Mash1* is a mouse homologue of the *Drosophila* proneural genes *achaete-scute*, and a basic helix-loop-helix (bHLH) transcription factor that normally functions as either a homodimer or heterodimer (Bertrand et al., 2002). *Mash1* is one of the earliest-identified transcription factors involved in the determination of neuronal fate in vertebrates (Guillemot et al., 1993). Moreover, it has emerged as a key fate determinant for many types of neurons in the nervous system (e.g., noradrenergic neurons, 5–HT neurons, and telencephalic neurons) (Blaugrund et al., 1996; Goridis and Brunet, 1999; Fode et al., 2000; Parras et al., 2004). The function of *Mash1* in the development of central 5–HT precursor cells, however, has been explored only recently (Pattyn et al., 2004). During development, *Mash1* expression is restricted to the VZ and is coexpressed with *Nkx2.2* throughout the generation period of vMNs and 5–HT neurons (Pattyn et al., 2004). In the developing hindbrain, *Mash1* is the only known proneural bHLH transcription factor expressed in the domain of 5–HT progenitor cells.

The requirement for *Mash1* in the development of 5–HT neurons has been shown at two levels. First, in *Mash1* knockout mice, vMNs are generated normally, whereas all postmitotic transcription factors (*Lmx1b*, *Pet1*, *Gata2*, and *Gata3*) fail to be detected in the developing brainstem (Pattyn et al., 2004). Even though it is dispensable for the generation of vMNs, *Mash1* is essential for the differentiation of 5–HT progenitor cells. Second, the examination of two components of the Notch signaling pathway, *Dll* and *Hes5*, in *Mash1* mutants has revealed the loss of these two genes in the domain of 5–HT neuronal progenitor cells (Pattyn et al., 2004). Thus, *Mash1* is required for mediating the Notch signaling pathway that leads to 5–HT neurogenesis, and the absence of *Mash1* results in defective neurogenesis for 5–HT neurons.

When the *Mash1* coding sequence is replaced by another proneural gene, *Ngn2*, only about 15% of 5–HT neurons are found as compared with the wild-type control mice, despite a rescued neurogenesis as indicated by normal expression of *Dll* and *Hes5*. This finding indicates a partial block of the differentiation of 5–HT neurons (Parras et al., 2002; Pattyn et al., 2004). The observation that the function of *Mash1* in the specification of 5–HT neurons cannot be completely substituted by other bHLH factors, despite their shared similar bHLH domain, suggests that *Mash1* possesses 5–HT neuron-specific characteristics. Because the lack of *Mash1* does not lead to an alteration of *Nkx2.2* that is unable to activate *Mash1*, *Mash1* is unlikely to exert its function by regulating *Nkx2.2*. Instead, *Mash1* may act in parallel with *Nkx2.2* to specify 5–HT neurons. Other cofactors may also be required because electroporation of DNA plasmids that express either gene or both fails to induce 5–HT neurons (Pattyn et al., 2004). Therefore, although both genes are necessary, neither *Nkx2.2*

nor *Mash1* is sufficient for inducing the generation of 5–HT neurons, even in the absence of *Phox2b* (Pattyn et al., 2004).

## 8.4 Transcription Factors Expressed in the Ventricular Zone and Postmitotic 5–HT Neurons

### 8.4.1 *Gata2* and *Gata3*

*Gata2* and *Gata3* are members of the GATA family that contain zinc fingers which bind to the consensus core (A/T)GATA(A/G) (Patient and McGhee, 2002). Among six GATA family members identified in vertebrates, *Gata2* and *Gata3* are important for the development of 5–HT neurons (van Doorninck et al., 1999; Craven et al., 2004; Pattyn et al., 2004). In the developing hindbrain, the expression of *Gata2* precedes that of *Gata3* (Nardelli et al., 1999). The onset of *Gata2* expression occurs at E9.0, most notably in r4 and transiently in r2. By E10.5, *Gata2* expression has expanded to all rhombomeres and is detected in progenitor cells in the VZ and in the postmitotic cells. By contrast, *Gata3* expression is weak in the VZ and mainly occupies the region outside the ventral VZ (Nardelli et al., 1999).

*Gata2*–null mice exhibit severe anemia and die between E10 and E11 (Tsai et al., 1994). One team has analyzed the neural development in the developing hindbrain of *Gata2* mutants and revealed several defects in neurogenesis (Nardelli et al., 1999). The team, however, did not examine the development of 5–HT neurons (Nardelli et al., 1999). The early lethality of *Gata2* mutants precluded detailed analysis of 5–HT neuronal development. For this problem to be avoided, *in-vitro* explant culture of E8 ventral hindbrain was used, and the tissue was examined at the equivalent of E13.5, when all 5–HT neurons are generated. In contrast to those in the control mice, 5–HT neurons were completely missing in *Gata2* mutants, even in the presence of *Gata3* (Craven et al., 2004). Thus, *Gata2* could be a critical factor for the specification of 5–HT neurons.

*In-ovo* electroporation of *Gata2* into chick embryos also suggests that the role of *Gata2* in the development of different clusters of 5–HT neurons may differ (Craven et al., 2004). Overexpression of *Gata2* in r1 induced *Pet1* and *Lmx1b*, but not in r2–r3 and r5–r7. Therefore, *Gata2* is necessary and sufficient for the development of 5–HT neurons in r1, whereas in r2–r3 and r5–r7 it is only necessary but not sufficient. Because the capacity of *Gata2* to induce other 5–HT-specific transcription factors is restricted to r1 (Craven et al., 2004), *Gata2* may be a key factor that helps to confer r1–specific 5–HT phenotype in addition to its generic function in the specification of 5–HT neurons.

*Gata3*$^+$ cells are almost completely co-localized with 5–HT in the caudal raphe nuclei, whereas in the rostral part of hindbrain, only 46% of *Gata3*$^+$ cells are overlapped with 5–HT staining (van Doorninck et al., 1999). *Gata3* mutants die between E9.5 and E12.5 and exhibit multiple defects, including brain and spinal cord abnormalities, abnormal liver hematopoiesis, and bleeding (Pandolfi et al., 1995). Two

teams have rescued the early lethal phenotype to analyze the development of 5–HT neurons in the absence of *Gata3*. The analysis of chimeric mice composed of *Gata3*$^{-/-}$/*Gata3*$^{+/+}$ wild-type cells shows that the development of 5–HT neurons is compromised in the caudal, but not in the rostral, raphe nuclei of the hindbrain (van Doorninck et al., 1999). In line with this chimeric study, rescued *Gata3* mutant embryos by noradrenergic agonists showed an 80% loss of 5–HT$^{+}$ neurons in the most caudal part of the hindbrain, whereas a less severe loss of 5–HT in the more rostral part of the hindbrain was found (Pattyn et al., 2004).

In *Gata3* mutants rescued by a noradrenergic agonist, the expression of *Pet1*, *Lmx1b*, and *Gata2* was largely normal at E13.5 (Pattyn et al., 2004). However, a partial loss of *Gata3* was observed in the caudal part of the hindbrain of *Lmx1b* mutants (Ding et al., 2003). Although these data suggest that *Lmx1b* and *Pet1* may lie either upstream of or parallel to *Gata3*, the possibility cannot be excluded that the loss of *Gata3* in *Lmx1b* mutants could also be due to a gradual loss of 5–HT neuronal identity in general, rather than to the loss of a regulation by *Lmx1b*.

The reports of *Gata3* expression in *Gata2* mutants are conflicting. In one study, no *Gata3* expression was detected in the hindbrain of *Gata2* mutants (Nardelli et al., 1999), whereas in the others, *Gata3* expression was not affected at a similar stage (Pata et al., 1999; Craven et al., 2004). By contrast, *Gata2* expression remains unaltered in *Gata3* mutants (Craven et al., 2004). *Gata2* can activate *Gata3* when *Gata2* is overexpressed in the chick neural tube, but not vice versa. Therefore, these results suggest that *Gata3* functions either downstream or independent of *Gata2* and *Lmx1b*. Despite these studies, the epistatic relationship among these genes is still not well understood, and further work is required to resolve some of the discrepancies.

## 8.5
## Transcription Factors Expressed in Postmitotic 5–HT Neurons

### 8.5.1
### *Lmx1b*

*Lmx1b* is a member of the LIM (Lin–11 from *Caenorhabditis elegans,* Isl–1 from the rat, and Mec–3 from *C. elegans*) homeodomain (LIM-HD) transcription factor family that has been implicated in many aspects of developmental and biological processes (Curtiss and Heilig, 1998; Dawid et al., 1998; Bach, 2000). The LIM-HD motif comprises two zinc fingers that mediate protein-protein interaction and can bind the same domain or different class of protein; thus, diverse interactions are allowed among proteins (Bach, 2000; Matthews and Visvader, 2003). Many LIM-HD transcription factors are particularly important in the regulation of the specification and differentiation of the nervous system (Curtiss and Heilig, 1998).

*Lmx1b* is a mouse orthologue of the chicken *Lmx1* that is required for the limb bud development (Chen et al., 1998). Its chromosome location syntenically matches a dominantly inherited human disease called nail-patella syndrome (NPS) (Chen et

al., 1998; Dreyer et al., 1998). In the CNS, *Lmx1b* is widely expressed in a variety of neuronal types, including the dorsal spinal cord, dopaminergic neurons, and the eye (Kania et al., 2000; Pressman et al., 2000; Smidt et al., 2000; Asbreuk et al., 2002; Ding et al., 2004). *Lmx1b* is one of the earliest known transcription factors to be expressed in postmitotic 5–HT neurons (Cheng et al., 2003; Ding et al., 2003).

Bromodeoxyuridine (BrdU) tracing experiments show that most of *Lmx1b*-expressing cells are not stained for BrdU. Although most *Lmx1b*$^+$ cells are postmitotic, a few *Lmx1b*$^+$ cells are also stained for BrdU and co-localized with *Nkx2.2* (Ding et al., 2003). This observation raises the possibility that *Lmx1b* may serve as a "brake" signal to instruct 5–HT precursor cells to cease their proliferation. At E14.5, all 5–HT neurons are also stained for *Lmx1b* in the ventral hindbrain (Fig. 8.2). The domain of *Lmx1b* expression in the floor plate is dispensable for the development of 5–HT neurons (Ding et al., 2003). The expression of *Lmx1b* is persistent not only in all postmitotic 5–HT neurons through embryonic development but also in the adult brain (Z.-F. Chen, unpublished results). This suggests that *Lmx1b* may have multiple functions in different stages of 5–HT neuronal development.

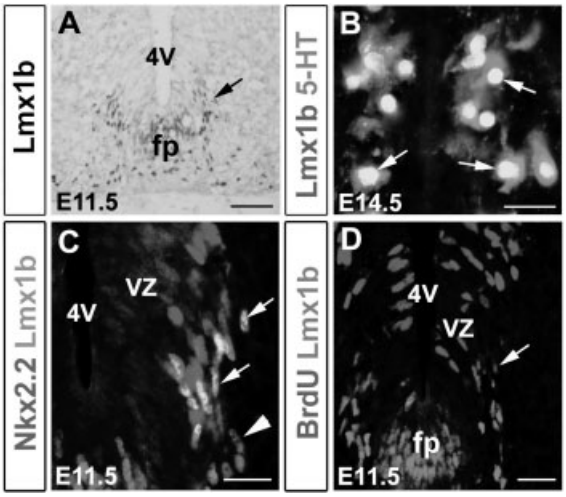


**Fig. 8.2** Double staining of *Lmx1b* and 5–HT in embryonic mouse hindbrain. (A) *Lmx1b* staining in r5 detected with immunocytochemical staining. (B) *Lmx1b* (red) and 5–HT (green) double staining. Arrows indicate double-stained cells. (C) *Nkx2.2* (red) and *Lmx1b* (green) double staining. *Nkx2.2* is mainly detected in the VZ, whereas *Lmx1b* is found in postmitotic cells. Arrows indicate double-stained cells, whereas arrowhead indicates *Lmx1b*-expressing cells only. (D) BrdU (red) and *Lmx1b* (green) double staining, indicating postmitotic expression of *Lmx1b* (arrow). Scale bars: 100 μm (A); 25 μm (B); and 50 μm (C, D). Abbreviations: fp = floor plate; 4V = fourth ventricle; VZ = ventricular zone. (This figure also appears with the color plates.)

In *Lmx1b*-null mutants, most of the 5–HT neuron-specific markers are lost from the beginning (Cheng et al., 2003; Ding et al., 2003) (Fig. 8.3). Thus, *Lmx1b* could play an essential role in the specification of 5–HT neurons. The transient expression of *Pet1* in E11.5 *Lmx1b* mutants suggests that the fate of 5–HT neurons may be partially specified (Cheng et al., 2003). The overexpression of *Lmx1b* in the ventral hindbrain of embryos by *in-utero* electroporation, however, fails to induce more 5–HT neurons (Y.-Q. Ding et al., unpublished data). Thus, the early function of *Lmx1b* appears to be necessary but not sufficient for the specification of 5–HT neurons. In contrast, co-electroporation of *Lmx1b*, *Pet1,* and *Nkx2.2* together in the chick neural tube induces ectopic 5–HT neurons (Cheng et al., 2003). Although mechanisms involving 5–HT neural development between chick and mouse may vary to a certain degree (Craven et al., 2004), the previous data suggest that multiple transcription factors may work in a coordinated fashion to specify 5–HT neurons. In *Lmx1b* mutants, 5–HT neurons are eventually lost, and this loss suggests that *Lmx1b* may also be a survival factor for more differentiated 5–HT neurons.

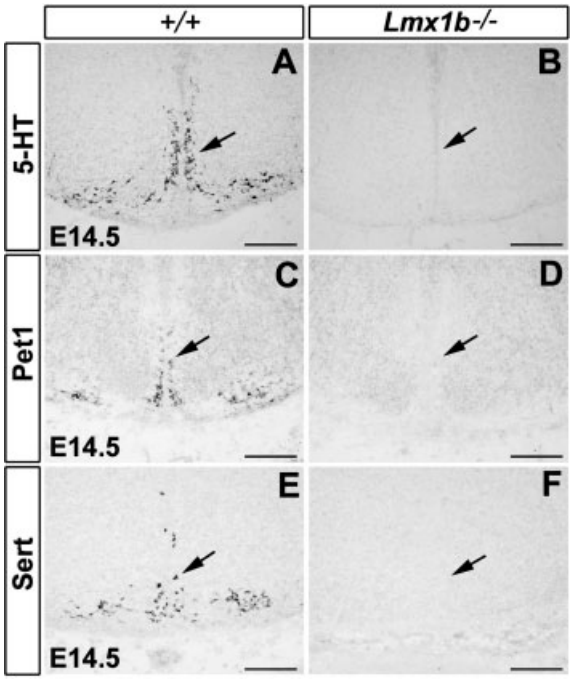


**Fig. 8.3** Loss of 5–HT-specific markers in *Lmx1b* mutant embryos. (A, B) 5–HT staining in wild-type (A) and *Lmx1b* mutant embryos (B). (C, D) *Pet1* expression in wild-type (C) and mutant embryos (D). (E, F) *Sert* expression in wild-type (E) and *Lmx1b* mutant embryos (F). Scale bars: 100 μm.

*Lmx1b* mutants die at birth as a result of multiple defects, including kidney defects (Chen et al., 1998). In order to overcome perinatal lethality problem, we have recently deleted *Lmx1b* only in 5–HT neurons by using a conditional knockout approach (Z.-F. Chen, unpublished results). The preliminary results of these studies indicate that some 5–HT neurons are initially generated and progressively lost in *Lmx1b*-conditional knockout mice. These findings indicate a role for *Lmx1b* in maintaining the survival of 5–HT neurons. However, it has not been determined whether the loss of 5–HT neurons in the absence of *Lmx1b* could be attributed to abnormal apoptosis.

In addition to binding to proteins that interact with DNA, the LIM-HD is capable of binding to proteins that regulate the cytoskeleton, thereby mediating the morphogenesis of neurons (Bach, 2000). In the dorsal spinal cord, *Lmx1b* is critical for the migration of the dorsal horn neurons (Ding et al., 2004). *Lmx1b* most likely has a role in the morphogenesis of 5–HT neurons during development. The persistent expression of *Lmx1b* in fully differentiated 5–HT neurons suggests that *Lmx1b* may be required for maintaining the mature phenotype or synaptic activity of 5–HT neurons in the CNS. A temporal deletion of *Lmx1b* in the raphe nuclei at a postnatal stage may help to answer this question.

The downstream targets of *Lmx1b* remain unknown. In *Lmx1b* mutants, several 5–HT-specific differentiation markers such as the serotonin transporter (SERT) fail to be expressed. However, this lack of expression is more likely due to a general blockade of the differentiation program of 5–HT neurons than to a loss of direct regulation of these terminal differentiation genes by *Lmx1b*. How the loss of *Lmx1b* results in a disruption of a genetic program for 5–HT neuronal development remains to be elucidated. In the developing dorsal horn, *Lmx1b* orchestrates expression of multiple downstream transcription factors (Ding et al., 2004). Similarly, *Lmx1b* may have an analogous role in the development of 5–HT neurons.

### 8.5.2 *Pet1*

*Pet1 (*pheochromocytoma 12 ETS [E26 transformation-specific]) is a member of the ETS (E26 transformation-specific) family that consists of more than 40 members in a variety of organisms (Graves and Petersen, 1998; Wasylyk et al., 1993). The ETS transcription factors contain a DNA-binding domain of about 85 amino acids that bind to a core consensus GGAA/T (Graves and Petersen, 1998; Sharrocks, 2001; Oikawa and Yamada, 2003). Many ETS family members exhibit cell type-specific expression patterns and are required for the proliferation and differentiation of diverse cell types such as hematopoietic cells and vascular endothelial cells (Oikawa and Yamada, 2003). ETS factors are also involved in the determination and specification of neuronal connectivity during neural development (Arber et al., 2000). In the CNS, ETS proteins are components of the signal transduction pathway and are able to activate downstream effector genes (Koo and Pfaff, 2002). For example, two ETS family members, PEA3 and ER81, are expressed in proprioceptive neurons in the dorsal root ganglia and their central targets, and are important for the establishment of neuronal connectivity between primary afferents and their central targets in the spinal cord (Arber et al., 2000; Livet et al., 2002).

*Pet1* was originally cloned from the adrenal chromaffin-derived phaeochromocytoma (PC12) cell line and has been found in rat, mouse, and human brains (the *fev* gene) (Fyodorov et al., 1998; Pfaar et al., 2002; Maurer et al., 2004). A *Pet1*–specific binding sequence has been identified in upstream regions of several 5–HT specific genes that are required for 5–HT synthesis, binding, or transportation: 5–HT1a receptor, serotonin transporter (Sert), tryptophan hydroxylase (TPH) gene, aromatic L-amino acid decarboxylase gene (AADC) (Hendricks et al., 1999). Co-transfection of the reporter gene that contains several *Pet1* binding sites with *Pet1*–binding domain showed a *Pet1*–dependent transcriptional activity by the reporter gene (Hendricks et al., 1999). Thus, *Pet1* appears to modulate the transcription activities of several effector genes that define the differentiated 5–HT neuronal phenotype, probably through a synergic interaction with other cofactors, which appears to be a common mechanism for many ETS factors (Oikawa and Yamada, 2003). Mechanistically, *Pet1* most likely functions as a transcriptional activator, even though its transactivation activity of downstream targets is relatively weak (Hendricks et al., 1999). On the other hand, the human homologue of *Pet1*, *Fev*, has been shown to act as a transcriptional repressor via its alanine-rich carboxy-terminal domain (Maurer et al., 2003). Whether *Pet1* contains similar repressor activity, however, is unclear.

Unlike other transcription factors, *Pet1* is found exclusively in postmitotic 5–HT neurons. Such a highly restricted expression pattern in the CNS for a transcription factor is not common, because the repeated use of the same transcription factor in distinct types of cells is a recurring theme in the nervous system. Recently, a 1.8–kb genomic fragment immediately upstream of the *Pet1* coding region has been shown to be able to direct the expression of the *LacZ* gene, which recapitulates expression of *Pet1* (Scott and Deneris, 2005). The 1.8–kb *lacZ* transgene's failure to show its activity in some of 5–HT neurons in *Pet1*–null background indicates that *Pet1* expression itself is required for maintaining the full activity of this enhancer in 5–HT neurons (Scott and Deneris, 2005). Nonetheless, the characterization of a 5–HT-specific 1.8–kb enhancer paves the way for the identification of transcription factors acting upstream of *Pet1*. Determining whether *Lmx1b*, *Gata2*, and *Gata3* interact with this element will be interesting. The unique expression pattern of *Pet1* is reminiscent of that of the homeodomain-containing transcription factor *Pitx3*, which is exclusively found in the midbrain dopaminergic neurons where *Lmx1b* expression is also found (Smidt et al., 2000; Burbach et al., 2003). Also, putative *Pitx3* binding sites are found in the promoter of tyrosine hydroxylase (TH), a enzyme which is critical for dopamine biosynthesis, and *Pitx3* can activate the TH gene through a high-affinity binding site (Lebel et al., 2001). Both *Pet1* and *Pitx3* appear to act in late steps of neuronal differentiation of two different neurotransmitter neurons. Such striking similarities between *Pitx3* and *Pet1* suggest that, in addition to shared transcription factors, tissue-specific factors might also have a key role in conferring the neurotransmitter-specific identity. *Pet1* might have been uniquely recruited by 5–HT neurons to promote 5–HT-specific characteristics during evolution.

In *Pet1* knockout mice, about 70% of 5–HT cells fail to differentiate (Hendricks et al., 2003). The differentiation capacity of the remaining 5–HT neurons is compromised because the neurons lack TPH, which may require *Pet1* for its activation

(Hendricks et al., 2003). In the absence of *Pet1,* the surviving 5–HT neurons may be attributed to some compensation effects contributed by unknown cofactors, which is in marked contrast with *Lmx1b* knockout mice in which all 5–HT neurons fail to differentiate (Ding et al., 2003). One notable observation in *Pet1* knockout mice is that *Lmx1b* expression appears normal up until the late stage of embryonic development (Z.-F. Chen, unpublished data). The developmental role of *Pet1* has not been examined in detail, and the step at which the development of 5–HT neurons is blocked remains unclear. *Pet1* is more likely to act at late steps of differentiation of 5–HT neurons.

Strikingly, *Pet1* knockout mice survive to adulthood. Recently, we have generated *Lmx1b* conditional knockout mice using the loxP-cre strategy. *Lmx1b* conditional knockout mice virtually lack 5–HT neurons after they are born. However, despite their smaller size during the first month compared with their wild-type littermates, *Lmx1b* conditional knockout mice are all viable (Z.-F. Chen, unpublished results). Therefore, 5–HT neurons are at least dispensable for prenatal development, whereas they are required for early postnatal development. These results are probably surprising given the well-documented role of 5–HT in a variety of developmental processes (Whitaker-Azmitia et al., 1996; Azmitia, 2001). Whether a compensation mechanism might have come into play in the absence of 5–HT remains unclear.

*Pet1* knockout mice exhibit aggressive behaviors. Moreover, *Pet1* knockout mice show increased anxiety-like behaviors (Hendricks et al., 2003). These two abnormal phenotypes are reminiscent of those of some 5–HT receptor knockouts (Gaspar et al., 2003; Gingrich et al., 2003). Given the multiple roles of 5–HT in numerous psychiatric disorders, these animals will also likely exhibit other behavioral deficiencies. One issue that remains to be addressed is how *Pet1* contributes to the development of abnormal behaviors. This may be an indirect effect by influencing the development of the 5–HT system, or *Pet1* may consolidate 5–HT phenotypes during adulthood directly. Regardless of the underlying mechanism, the importance of *Pet1* in the development and maintenance of mature neural morphology as well as normal behaviors has been established. This opens up an exciting possibility that an abnormal transcription regulation of the 5–HT system may also contribute to the etiology of some psychiatric diseases in humans.

## 8.6 The Relationship between *Lmx1b* and *Pet1*

*Pet1* binds to the *cis*-regulatory elements of several 5–HT effector genes, and thus probably lies at the end of the transcriptional cascade underlying the terminal differentiation and maturation of 5–HT neurons (Hendricks et al., 1999). By contrast, potential downstream targets of *Lmx1b* are unknown. *Lmx1b* may have an analogous regulatory role to that in the dorsal horn, where it controls multiple transcription factors (Ding et al., 2004), although the possibility that *Lmx1b* might regulate some 5–HT differentiation genes directly by binding to their *cis*-regulatory elements cannot be excluded. At all levels of the hindbrain, *Lmx1b* expression precedes that of

*Pet1*; thus, *Lmx1b* may act one or two steps earlier than *Pet1* in the genetic cascade. However, *Lmx1b* is unnecessary for the initiation of *Pet1* expression because *Pet1* is transiently expressed in *Lmx1b* mutants at E11.5 (Cheng et al., 2003). Ectopic expression of *Lmx1b* or *Pet1* consistently fails to initiate the expression of either gene. Because *Pet1* is lost after E11.5 in the absence of *Lmx1b*, *Lmx1b* may be required for maintaining expression of *Pet1*. Alternatively, the loss of *Pet1* may not be due to a lack of a regulatory relationship between *Pet1* and *Lmx1b*. Instead, it may reflect a loss of 5–HT identity in *Lmx1b*-null neurons in general or a switch of neuronal phenotype. Nevertheless, no evidence of up-regulation of any other neuronal markers is found in *Lmx1b*-null neurons (Z.-F. Chen, unpublished results), and this lack of evidence does not support any switch of neuronal type. *Lmx1b* expression is not affected in *Pet1* mutants during early development, and thus the two genes probably do not act in parallel (Z.-F. Chen, unpublished results). Taken together, the available evidence suggests that *Pet1* and *Lmx1b* function neither in a simple linear cascade nor in parallel, as was previously proposed (Cheng et al., 2003; Ding et al., 2003). *Lmx1b* may be required to maintain *Pet1* expression, but not vice versa. Given that many LIM-HDs function through protein-protein interaction, *Lmx1b* may cooperate with other LIM-interacting factor(s) to initiate *Pet1* expression.

## 8.7 Conclusions

Gene-targeting approaches have begun to unravel the components of a transcriptional program that dictates the specification and differentiation of 5–HT neurons during development. Figure 8.4 illustrates a tentative 5–HT-specific transcriptional cascade, although it is far from complete. In the VZ, *Gata2* and *Mash1* are important regulators of the development of 5–HT neurons in r1–r3 and r5–r7, whereas *Nkx2.2* is essential for 5–HT progenitor cells that originate only in r2–r3 and r5–r7. In the postmitotic neurons, *Gata2* appears to act upstream of *Lmx1b*, *Pet1*, and *Gata3*. *Gata3* may cross-regulate *Gata2*. *Pet1* functions probably in the last steps of the cascade that leads to the activation of terminal differentiation program of 5–HT neurons. The maintenance of *Pet1* depends on *Lmx1b*; so does *Gata3* in the caudal part of the brainstem (Fig. 8.4).

These studies have provided a strong basis for future studies, and accumulating evidence suggests that several principles exist regarding the transcriptional control of the formation of 5–HT neurons. First, transcription factors that are expressed in both 5–HT progenitor cells and postmitotic cells constitute an integral part of a genetic program that governs the specification of 5–HT progenitor cells. Second, a transcription factor may have pleiotropic functions manifested at different stages with regard to different aspects of 5–HT neuronal development. Third, although the loss of a single transcription factor could result in a loss of all 5–HT-specific markers, only combinatorial expression of a myriad of transcription factors is both necessary and sufficient to activate the generation of 5–HT neurons. Finally, the cellular diversity and complexity of 5–HT neurons may be attributed to region-specific transcription factors.

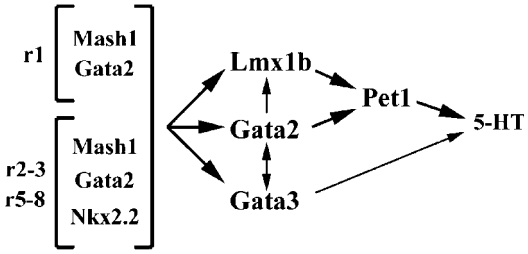


**Fig. 8.4** Summary of transcriptional cascade controlling the development of 5–HT neurons. In r1, *Mash1* and *Gata2* are essential for the specification of 5–HT neurons, whereas in r2–r3 and r5–r7, *Nkx2.2* is also required. The relationship among *Mash1, Gata2*, and *Nkx2.2* has not yet been determined. In postmitotic progenitor cells, *Gata2* acts upstream of *Lmx1b*, *Pet1*, and *Gata3* at least in cells that originated in r1. In other rhombomeres, *Gata2* may require additional unidentified transcription factor (not shown) to activate *Lmx1b*. *Gata2* and *Gata3* are able to regulate each other. The maintenance of *Pet1* and *Gata3* expression depends on *Lmx1b*. The initiation of *Pet1* expression may require *Gata2* and other unidentified transcription factors (not shown). In addition to *Pet1*, other unidentified transcription factors may also activate some of the 5–HT terminal differentiation genes.

Among all neurotransmitter systems in the CNS, the 5–HT system is probably the best understood in terms of underlying molecular mechanisms, due mainly to the findings of genetic studies of a variety of mutant mice. Despite the rapidity of progress during the past few years, however, our understanding of the molecular machinery that functions during the development of 5–HT neurons is, at best, in its infancy. We are far from completely understanding how the combinatorial and sequential action of a myriad of transcription factors is translated into a mature 5–HT neuronal phenotype. Among the many challenges, one of particular importance and immediate significance is an elucidation of the action mechanisms of these transcription factors, in particular with respect to discrete cellular processes during early specification and differentiation of 5–HT neurons. Dissection of the genetic hierarchy of these transcription factors and assessment of their cross-regulation in the execution of a developmental program and in the acquisition of 5–HT phenotype are important. Because transcription factors often assume roles in many aspects of the neuronal phenotype, in addition to their developmental functions (Goridis and Brunet, 1999), another challenge will be to determine whether they might contribute to elaboration and maturation of 5–HT functional circuitry, such as axonal growth and synaptic activities. Region-specific factors or cofactors also need to be identified so that the complexity and heterogeneity of 5–HT neurons can be understood. Finally, temporal- and spatial-specific gene ablation strategies should be used to unravel the distinct roles of transcription factors at different stages of 5–HT neuronal development and maturation. These temporal and spatially knockout mice should eventually aid in our understanding of the transcription control of the psychiatric abnormalities involved in the 5–HT system.

## Abbreviations

| | |
|---|---|
| 5–HT | 5–hydroxytryptamine |
| AADC | aromatic L-amino acid decarboxylase gene |
| bHLH | basic helix-loop-helix |
| BrdU | bromodeoxyuridine |
| CNS | central nervous system |
| LIM-HD | LIM homeodomain |
| NPS | nail-patella syndrome |
| SERT | serotonin transporter |
| TH | tyrosine hydroxylase |
| TPH | tryptophan hydroxylase |
| vMN | visceral motor neuron |
| VZ | ventricular zone |

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