Development of raphe serotonin neurons from specification to guidance

Vera Kiyasova\textsuperscript{1,2,3} and Patricia Gaspar\textsuperscript{1,2,3}
\textsuperscript{1}INSERM, UMR-S 839, 17 rue du Fer à Moulin, 75005 Paris, France
\textsuperscript{2}Université Pierre & Marie Curie, Paris, France
\textsuperscript{3}Institut du Fer à Moulin, Paris, France

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Abstract

The main features of the development of the serotonin (5-HT) raphe neurons have been known for many years but more recent molecular studies, using mouse genetics, have since unveiled several intriguing aspects of the specification of the raphe serotonergic system. These studies indicated that, although all 5-HT neurons in the raphe follow the same general program for their specification, there are also clear regional differences in the way that these neurons are specified and are guided towards different brain targets. Here we overview recent progress made in the understanding of the developmental programming of serotonergic neurons in the mouse raphe, emphasizing data showing how heterogeneous subsets of 5-HT neurons may be generated. Serotonergic progenitors are produced in the brainstem in different rhombomeres under the influence of a set of secreted factors, sonic hedgehog and fibroblast growth factors, which determine their position in the neural tube. Two main transcriptional gene networks are involved in the specification of 5-HT identity, with Lmx1b and Pet1 transcription factors as main players. A differential requirement for Pet1 was, however, revealed, which underlies an anatomical and functional diversity. Transcriptional programs controlling 5-HT identity could also impact axon guidance mechanisms directing 5-HT neurons to their targets. Although no direct links have yet been established, a large set of molecular determinants have already been shown to be involved in the growth, axon guidance and targeting of 5-HT raphe neurons, particularly within the forebrain. Alterations in the molecular mechanisms involved in 5-HT development are likely to have significant roles in mood disease predisposition.

Introduction

The central serotonergic systems are key modulators of numerous physiological functions, from motor and neurovegetative control to emotional behaviors (Lucki, 1998). Serotonin (5-HT) neurotransmission is effective early during development (Wallace and Lauder 1983) and has numerous developmental effects (Gaspar et al., 2003) that may underlie a number of psychiatric disorders such as depression, anxiety, and autism (Gross & Hen, 2004; Scott & Deneris, 2005). Indeed, a large number of studies showed that genetic variations in 5-HT-related genes could contribute to the risk of developing mood-related and impulsive disorders (Murphy et al., 2008; Bevilacqua et al., 2010; Enoch et al., 2010; Takahashi et al., 2011; Waider et al., 2011). A precise knowledge of how 5-HT neurons develop and how they reach their appropriate brain targets is therefore warranted.

The main features of the development of the 5-HT raphe neurons have been known for many years (Lidov & Molliver, 1982; Wallace and Lauder 1983) but more recent molecular studies, in particular using genetic models in mice, have since unveiled some intriguing aspects of the specification of the raphe serotonergic system, underlining that, although all 5-HT neurons in the raphe follow the same general rules for their specification, there are also some clear regional differences among these neurons. These molecular studies support the long-held view of a heterogeneity among the raphe neurons, based on a variety of evidence taken from morphological, neurochemical and electrophysiological studies of the 5-HT neurons (reviewed in Lowry & Hale, 2010; Gaspar & Lilesaar, 2011; Calizio et al., 2011).

Substantial progress in the field of developmental neuroscience in the last 15 years has uncovered a large set of extracellular signals and transcriptional regulators that control the development and maturation of different types of neurons. We now know that the final phenotype of a neuron comprises generic neuronal features that are shared by all neurons, features that are common to several neuronal types and features that are specific to one class of neuron, such as neurotransmitter expression and/or specific axonal pathways. These features are determined by the place of birth of the neuron, the gradient of morphogens to which it is exposed and the cascade of transcription factors regulating its specification and terminal differentiation (Goridis & Rohrer, 2002; Cordes, 2005; Scott & Deneris, 2005; Flames & Hobert, 2011). Numerous studies on the early development of hindbrain 5-HT neurons show that, from very early stages, 5-HT raphe cells appear to be subdivided into specific subsets, projecting to different parts of the brain, and may require different transcriptional and environmental factors for their development.

We will overview recent progress made in understanding the development of serotonergic neurons, summarizing the available evidence that indicates a genetically programmed heterogeneity of the central 5-HT neurons. We will first consider how serotonergic progenitors are produced in the brainstem and the secreted factors...
determining their position within the neural tube. We will then describe the main transcriptional cascades involved in their specification, which may contribute to their genetic diversity. Finally, we will review the available studies that identified molecular signals involved in the wiring of the 5-HT raphe neurons. Several recent studies have uncovered molecules that are important for axon growth or guidance of 5-HT axons to their targets.

Where from: early induction of the central serotonin neurons

The central serotonergic neurons form a relatively small population of neurons, some 20–30 000 neurons in the rat central nervous system (Jacobs & Azmitia, 1992) described as the B1–B9 cell groups (Dahlström & Fuxe, 1964). Developmental studies in rodents allowed the subdivision of the raphe population into two main clusters: a caudal cluster in the medulla, corresponding to the raphe pallidus, obscurus and pontis (B1–B5), and a rostral cluster in the pons containing the dorsal raphe and median raphe (B6–B9) (Lidov & Molliver, 1982) (Figure 1). All of these neurons share a common serotonergic phenotype that includes the enzymes necessary for synthesis [aromatic amino acids decarboxylase and tryptophan hydroxylase (TPH2)] and degradation (monoamine oxidase B), the specific 5-HT plasma membrane transporter (SERT), the vesicular monoamine transporter, and several autoreceptors, among which are the 5-HT1A, 5-HT1B and 5-HT2B receptors (Lemos et al., 2006; Banas et al., 2011; Calizo et al., 2011). Interestingly, many of these genes contributing to the 5-HT phenotype, such as TPH2, SERT or the 5-HT receptors, show polymorphisms that correspond to functional changes, at either the level of the expressed proteins or the biological activity of these proteins (Lesch et al., 2011). Genetic studies support the notion that single or combined nucleotides of these genes are predisposing factors to several neuropsychiatric disorders such as anxiety or depression (reviewed in Haavik et al., 2008; Holmes, 2008; Waider et al., 2011; Homberg & Lesch, 2011). This underlines the importance of understanding how 5-HT phenotypes and raphe circuit formation are regulated during development.

In mice, the central serotonergic neurons appear in two waves of differentiation at embryonic day (E)10 and E11 (Briscoe et al., 1999; Pattyn et al., 2003). The rostral cluster arises the day before the caudal cluster. The 5-HT progenitors reside in the embryonic hindbrain as bilateral clusters on each side of the midline. They migrate away from the ventricle by soma translocation mediated by dynamin (Hawthorne et al., 2010). The movement of serotonergic neurons occurs in waves according to the date of birth, explaining the typical sigmoid shape of the rostral raphe nuclei.

During embryogenesis, all the cells in the neural tube acquire their regional identity by virtue of the position that they occupy along two primary neural axes: dorsoventral and anterior-posterior (Cordes et al., 2005; Fuccillo et al., 2006; Ribes & Briscoe, 2009). For the serotonergic neuronal population, clear limits along these axes and the signals regulating them were identified. The rostral molecular border for the 5-HT neurons is the mid-hindbrain boundary (MHB), an organizing centre between the midbrain and hindbrain that expresses fibroblast growth factor 8 (Fgf8) and directs the patterning of the adjacent territories during embryogenesis (Wurst & Bally-Cuif, 2001). The importance of Fgf8 signaling for the serotonergic population was demonstrated by multiple studies in various species. Brodski et al. (2003) analysed the size and placement of 5-HT vs. dopamine neurons in En1+/Cre2 mutants, in which the MHB was moved caudally. They showed that the serotonergic neurons were displaced caudal to the new boundary, and the rostral territories were converted into dopamine neurons. There was a corresponding reduction in the rostrocaudal extent of the 5-HT cell groups. Conversely, when the MHB was shifted rostrally, the locus of production of the serotonergic neurons moved in the rostral direction (Brodski et al., 2003). Similar results were observed in zebrafish by Teraoka et al. (2004), who demonstrated that acerebellar−/− zebrafish embryos lacking functional Fgf8 show a significant reduction of hindbrain 5-HT neurons.

Dorsalventral positioning of the precursors is provided by another morphogen, Sonic hedgehog (Shh), which is produced by the notochord and floor plate (midline of the neural tube). The Shh signaling involves two receptors: the 12-pass membrane hedgehog receptor patched (PTC1) (Marigo et al., 1996; Goodrich et al., 1997) and the seven-pass G-protein-coupled receptor Smoothened (Stone et al., 1996; Ingham & McMahon, 2001). Ultimately, Smoothened activity regulates the activity of the zinc-finger-containing transcription factor, Gli (Matise & Joynner, 1999). The role of Shh in developing 5-HT neurons was demonstrated by Hynes et al. (2000) when analyzing a transgenic mouse line with a constitutively active form of Smoothened. The 5-HT precursors in these mice were dorsalized and serotonergic neurons were misplaced in the cerebellum, indicating a role of Shh in the dorsalventral control of the 5-HT phenotype (Hynes et al., 2000). Further studies showed that Shh action is possible through the activation of the Gli2 zinc-finger transcription factor. In Gli2+/− mice, 5-HT neurons are born, but there is a 50% decrease of the 5-HT raphe population (Matise et al., 1998). Analysis of Gli mutants in zebrafish by Teraoka et al. (2004) demonstrated that a decrease in Shh activity similarly led to the reduction of hindbrain serotonergic population.

Interestingly, the impact of the described morphogens is not the same in the rostral and the caudal 5-HT clusters. The rostral serotonergic group appears earlier (E10.5) and anatomically, it is in close proximity to the MHB. As shown in explant experiments by Ye et al. (1998), the rostral group of 5-HT neurons can only be induced by the coordinated expression of two morphogens, Fgf8 and Shh, whereas the caudal cluster that appears 24 h later is less dependent on Fgf8. The gradient of Fgf8 is low caudally, whereas that of Fgf4, secreted by the paraxial mesoderm, is high. Thus, induction of the caudal 5-HT neurons appears to be regulated by the cooperation of Fgf4 and Shh (Ye et al., 1998).

Moreover, during embryogenesis, the hindbrain is transiently subdivided into several compartments called the rhombomeres. Rhombomeres 1, 2 and 3 give rise to the rostral cluster of raphe neurons, and rhombomeres 5–7 give rise to the caudal raphe cluster (Figure 1). The gap between them is rhombomere 4 where only motor neurons are generated (Pattyn et al., 2003). Recent molecular fate mapping reports elucidated the link between the rhombomeric origin of 5-HT neurons and their anatomical localization in the adult raphe nuclei. Jensen et al. (2008) demonstrated that the dorsal raphe nuclei (B7 and B6) derive entirely from rhombomere 1, whereas the median raphe nuclei (B8 and B9) comprise a mixture of precursors from rhombomeres 1 to 3 (Fig. 1). This leads to the suggestion that the dorsal raphe 5-HT neurons are particularly dependent on signaling from the MHB, whereas those of the median raphe are less sensitive to Fgf8 for their induction.

Becoming a serotonin neuron

Genetic models have clarified the transcriptional cascades that regulate the region-specific generation and differentiation of 5-HT neurons. Many of these mechanisms have been reviewed previously...
suggests that high levels of evidence from both loss-of-function and gain-of-function studies by 1999). The generation of 5-HT neurons in rhombomere 1 is provided by Pet1 and Insm1 for the terminal differentiation. et al. to switch on the serotonergic neuronal fate through the repression of make up the code for the specification of 5-HT neurons in Pet1 for acquiring their identity. requirements of 5-HT neurons in Pet1 for acquiring their identity. First cascade: Shh – Nkx(s) + Foxa2 – Gata(s) – Lmx1b Shh signaling in the hindbrain during induction leads to the activation of two main transcription factors: the forkhead transcription factor Foxa2 and the homeodomain transcription factor Nks2.2. The expression of Nks2.2 appears to be sufficient to induce the differentiation of both ventral motor neurons (Pattyn et al., 2003) and a significant proportion of 5-HT neurons (Craven et al., 2004). The majority of 5-HT neurons are lost in Nks2.2–/– mice, with the exception of a small rostral cluster in rhombomere 1 (Briscoe et al., 1999). The generation of 5-HT neurons in rhombomere 1 is provided by Nks6.1 (Vallstedt et al., 2001) and Foxa2 (Jacob et al., 2007). The evidence from both loss-of-function and gain-of-function studies suggests that high levels of Nks6.1 are needed together with Nks2.2 to make up the code for the specification of 5-HT neurons in rhombomere 1 (Craven et al., 2004). The role of Foxa2, in turn, is to switch on the serotonergic neuronal fate through the repression of ventral motor neuron generation in the rostral rhombomeres (Jacob et al., 2007).

To initiate the specification of 5-HT neurons, the Nkx genes switch on the downstream actors of the pathway, the zinc-finger transcription factors Gata2 and Gata3. Both Gata2 and Gata3 are expressed broadly in the midbrain. The analysis of mice mutant for these genes was complicated by the fact that Gata2–/– embryos die between E9.5 and E10.5 of pan-haematopoietic defect (Tsai et al., 1994) and Gata3–/– mice die by E11–E12 with massive internal bleeding and gross abnormalities of the nervous system (Pandolfi et al., 1995). Most of the data on the functions of Gata2 and Gata3 were thus obtained from the analysis of embryonic mouse explants or electroporation of morpholinos in chick. Explants from Gata2–/– mice embryos showed a complete absence of serotonergic neurons, although Gata3 expression was maintained in rhombomere 1. Conversely, in Gata3–/– embryos, the rostral subpopulation of 5-HT neurons differentiated normally (Craven et al., 2004). The absence of Gata3 affected the cytoarchitecture of the caudal 5-HT neurons. Chimeric Gata3–/– mice lack 80% of the caudal 5-HT neurons. Thus, Gata3 is a transcription factor with a clear cluster-specific role (Doorninck et al., 1999).

The action of the two Gata genes is followed by the activation of a Lim homeodomain transcription factor, Lmx1b, in both the rostral and caudal domains of developing hindbrain. Lmx1b also regulates the development of midbrain dopaminergic neurons (Smidt et al., 2000). The loss of Lmx1b affects the entire population of serotonergic neurons, including the R1 rostral subgroup. The expression of the upstream factors (Shh and Nks2.2) is unchanged, indicating that the precursors of future 5-HT neurons are born but do not acquire their neurochemical phenotype (Ding et al., 2003). Cheng et al. (2003) demonstrated that, in Lmx1b–/– mice, no vesicular monoamine transporter immunoreactivity was detected in the hindbrain, indicating that Lmx1b regulates transporter expression in all aminergic neurons, including 5-HT neurons. Moreover, the combination of three transcription factors (Nks2.2+Lmx1b+Pet1) was necessary and sufficient to induce the generation of 5-HT neurons ectopically (Cheng et al., 2003). Subsequent analysis of conditional Lmx1b–/– mice (Lmx1b was specifically invalidated in Pet1-expressing raphe neurons) emphasized the notion that SERT and TPH2 are also strongly regulated by Lmx1b, although this may be indirect via an effect on Pet1 (Zhao et al., 2006).
Interestingly, Lmx1b expression is modulated by neuronal activity. Recent work of Demarque & Spitzer (2010) demonstrated that the expression of Lmx1b in the hindbrain of developing Xenopus laevis can be modulated by spontaneous calcium spike activity. The study showed that decreasing neuronal activity increased the number of neurons expressing Lmx1b. Functionally, this led to changes in the number of serotonergic neurons in the raphe and in larval swimming behavior. Although further studies are needed to determine if physiologically relevant stimuli, such as stress or temperature, can trigger activity-dependent 5-HT specification, this work provided a plausible mechanistic explanation for a large set of previous observations showing that stress or malnutrition during gestation can modify the development of 5-HT neurons (Peters, 1986; Hayashi et al., 1998; Serfati et al., 2008).

Second cascade: Ascl/Mash1 – Gata3 – Lmx1b – Insm1

In parallel with the Shh- and Nkx-induced regulation, the classic proneural helix–loop–helix gene Ascl1/Mash1 plays an important role in both the generation of postmitotic precursors in the hindbrain and the cell type specification of the future 5-HT neurons. The analysis of Ascl1/Mash1−/− embryos by E11.5 by Pattyn et al. (2003; 2004) showed the absence of serotonergic neurons at all rostrocaudal levels in the hindbrain. When expressed in the chick spinal cord after electroporation, Ascl1/Mash1 was capable of switching on Gata3 expression (Tiveron et al., 2003). However, when over-expressed alone or even in combination with Nkx2.2 in chick, Ascl1/Mash1 failed to activate other 5-HT markers such as Lmx1b and Pet1. This led the authors to re-examine Gata3−/− mice rescued by the administration of noradrenergic agonists (Lim et al., 2000) throughout the phase of 5-HT neuron generation. They observed a drastic reduction in the number of 5-HT-positive neurons in the caudal-most region of the raphe, whereas, unexpectedly, the expression of Pet1 and Lmx1b was unchanged. This indicated that Gata3 is required downstream of Ascl1/Mash1 for 5-HT differentiation in a pathway that appeared to be parallel to that of Pet1 and Lmx1b (Pattyn et al., 2004).

Recent studies by Cheng et al. (2007) and by Jacob et al. (2009) introduced new actors into this pathway, the iroquois homeodomain factor Irx1a in zebrafish and the zinc-finger gene, Insm1 in mice. A severe reduction of 5-HT expression was noted in Insm1−/− embryos at all axial levels of the brainstem. Furthermore, the expression of Insm1 was strikingly downregulated in Ascl1/Mash1−/− embryos specifically in the lateral proportion of the upper hindbrain cells, which corresponds to 5-HT precursor groups. The analysis of the Insm1−/− mice phenotype showed a marked reduction in the number of 5-HT neurons with no changes in the progenitor pool, but with abnormal expression of the postmitotic markers of the 5-HT fate (Lmx1b, Gata2 and Pet1). Interestingly, the decrease of neurotransmitter level through all of the nuclei was more pronounced than that of the Pet1 transcription factor. This led the authors to analyze all of the players of the serotonergic biosynthetic pathway showing an unsuspected regulation of Tph2 by Insm1.

Overall, these data identified the Ascl1/Mash1 and Insm1 genes as part of the gene regulatory networks that control 5-HT identity (Jacob et al., 2009).

Pet1 and heterogeneity of serotonin projections

To date, the two transcriptional pathways described above both lead to the activation of a common transcription factor in the brainstem, Pet1. Pet1 (plasmacytoma expressed transcription factor 1) was described by Hendricks et al. (1999) as the only gene whose expression is limited to the hindbrain 5-HT neurons. Its expression in the rostral and caudal clusters precedes the appearance of 5-HT by approximately 12 h. Transcriptionally active Pet1-binding sites were found in or near the promoter regions of genes responsible for the mature serotonergic phenotype (Tph, amino acid decarboxylase, serotonin transporter, and 5-HT1A) (Hendricks et al., 1999) and, indeed, constitutive Pet1 knockout mice showed a profound down-regulation of these genes in the raphe (Hendricks et al., 2003). However, Pet1−/− mice also revealed a genetic heterogeneity among the raphe serotonergic neurons. Although the majority of raphe neurons failed to differentiate as 5-HT neurons and remained in an arrested state of differentiation not expressing other neurotransmitter markers, such as glutamate or GABA, a sizeable fraction of the 5-HT neurons, approximately 20–30% differentiated in each of the raphe cell groups (Hendricks et al., 2003; Kiyasova et al., 2011).

Moreover, further genetic study of the LacZ expression driven by the 5-HT-specific regulatory region of Pet1 in Pet1−/− mice revealed that Pet1 autoregulation is essential for a subset but not all of the raphe serotonergic neurons (Scott et al., 2005). Additionally, studies in zebrafish provided evidence that the central serotonergic neurons can be subdivided into at least two populations, one dependent upon and the other independent of pet1 (Lillesaar et al., 2007). In the zebrafish central nervous system, serotonergic neurons are not restricted to the brainstem, and several distinct clusters are described, in the epiphysis, hypothalamus and pretecal area of the diencephalon, in addition to the anterior and posterior raphe nuclei (Kaslin & Panula, 2001). The synthesis of 5-HT in these different cell populations is controlled by three different Tph enzymes, all with a unique temporal and spatial expression pattern. However, only one, pet1, was found in the zebrafish, with an expression that was limited to the anterior and posterior raphe. Furthermore, although tph2 is expressed in both the diencephalic and hindbrain serotonergic neurons, pet1 was expressed at detectable levels only in the hindbrain population (Lillesaar et al., 2007). Transgenic zebrafish expressing green fluorescent protein under the control of pet1 confirmed these observations, showing that not all of the 5-HT innervation of the brain was labelled (Lillesaar et al., 2010). Overall, these observations demonstrated a heterogeneity among the 5-HT nuclei.

Because in Pet1−/− mice the remaining 5-HT neurons were uniformly distributed in the different raphe neurons, it was unclear whether this outlined a separate population of 5-HT raphe neurons. However, a systematic analysis of the 5-HT innervation pattern in the forebrain and hindbrain showed that the residual 5-HT axons were outlining highly selective anatomical targets with a characteristic synaptic differentiation (Kiyasova et al., 2011). In accordance with the initial description of these mutants, we observed a drastic decrease in the number of 5-HT neurons in the entire raphe nuclei. However, we found that the 5-HT innervation provided by the residual Pet1-resistant neurons was concentrated in several brain areas, and specific nuclei that were involved in stress responses. For instance, a dense innervation to the basolateral amygdala, paraventricular nucleus of the hypothalamus, and the intralaminar thalamic nuclei, nuclei of tractus solitarius and nucleus ambiguus was noted, whereas neighbouring areas were devoid of fibers. To determine whether this striking distribution was caused by developmental growth abnormalities, we analyzed the growth characteristics of the Pet1-resistant raphe neurons and the topography of their projections. These characteristics were unchanged, whereas the Pet1-dependent raphe neurons appeared to lose not only their 5-HT phenotype, but also their normal projections to the hippocampus (Kiyasova et al., 2011). Similarly
raphe projections to the somatosensory cortex were found to be reduced (Liu et al., 2010). Biochemical and pharmacological analyses showed that the residual 5-HT axon terminals were functional and maintained normal release properties in vitro and in vivo. Unexpectedly, the ultrastructural analysis of three areas containing 5-HT terminals in the Pet1−/− mice showed that the serotonergic terminal boutons formed asymmetric synapses with a high frequency (70%). This observation was in contrast with the general idea of 5-HT systems acting as modulators with primarily non-synaptic release sites (Beaudet & Descarries, 1981). At the same time, it provided additional morphological evidence supporting the notion of a genetically determined subset of 5-HT neurons with the intrinsic capacity to form synaptic junctions. Indeed, it is known that, according to the brain region, 20–50% of 5-HT terminals form synapses (Descarries et al., 2010). The functional consequence of this uneven distribution of 5-HT innervation was a decreased anxiety behavior of Pet1−/− mice in novelty exploration and increased fear responses to conditioned aversive cues (Kiyasova et al., 2011).

Interestingly, a recently developed genetic tool to study the role of Pet1 during adult life also showed an unequal requirement of Pet1 for the maintenance of a 5-HT phenotype. The tamoxifen-induced Cre recombination allowed for a selective deletion of Pet1. After treatment with tamoxifen, 6–8-week-old Pet1loxP/loxP ePet1CreERT2 mice showed a >70% reduction in Pet1 mRNA expression in the B5–B9 raphe nuclei, resulting in a 50% decrease in Tph2 expression in the rostral clusters and a 25% reduction in the forebrain levels of B5–B9 raphe nuclei, resulting in a 50% decrease in Tph2 expression (Beaudet & Descarries, 1981). At the same time, it provided additional morphological evidence supporting the notion of a genetically determined subset of 5-HT neurons with the intrinsic capacity to form synaptic junctions. Indeed, it is known that, according to the brain region, 20–50% of 5-HT terminals form synapses (Descarries et al., 2010). The functional consequence of this uneven distribution of 5-HT innervation was a decreased anxiety behavior of Pet1−/− mice in novelty exploration and increased fear responses to conditioned aversive cues (Kiyasova et al., 2011).

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In conclusion, some differences in the developmental programming of 5-HT identity lead to a heterogeneous population of serotonergic neurons in the raphe. This is due to the contribution of a gene network with variable influences across the dorsoventral and rostrocaudal extents of the hindbrain. This can be summarized in the following way: (Fig. 1). Induction is controlled by Shh and Fgf8 in the rostral cluster (rhombomeres 1–3), whereas Shh and Fgf4 operate in rhombomeres 5–7 (caudal cluster). Precursor development and early specification are further regulated by: Nkx2.2, Nkx6.1, Gata2 and Ascl1/Mash1 in rhombomere 1; Nkx2.2, Gata2 and Ascl1/Mash1 in rhombomeres 2 and 3; and Nkx2.2, Gata3, Ascl1/Mash1 in the caudal rhombomeres 5–7. The terminal differentiation of 5-HT and maintenance are regulated in both clusters by Lmx1b. Additional control of Tph2 is provided by Insm1, which is regulated by Ascl1/Mash1 in rhombomeres 2–7. Finally, the expression of a complete neurochemical serotonergic phenotype in 70% of developing serotonergic neurons is controlled by Pet1, whereas the remaining 30% acquire their properties via a combination of as yet unidentified transcription factors. The role of each of these genes and the specific phenotype observed after invalidation or over-expression are summarized in Table 1.

As described above, the hindbrain serotonergic system is thought to comprise several functional units from a developmental point of view. This notion has been strengthened recently by several studies providing evidence of the differential expression of genes in the rostral and caudal 5-HT clusters and between the rhombomeres (Jensen et al., 2008; Jacob et al., 2009; Wylie et al., 2010; Kiyasova et al., 2011). In the study of Wylie et al. (2010), neurons expressing the green fluorescent protein under the control of Pet1 were used for whole-genome microarray profiling of rostral and caudal 5-HT neurons at E12.5. This study identified several hundred transcripts that were differentially enriched in the developing rostral and caudal 5-HT neurons; some encoding homeodomain transcription factors, axon guidance molecules, or intracellular signaling pathways that may underlie different developmental and functional properties of these neurons. For instance, the differential expression of numerous homeodomain genes in the rostral 5-HT neurons and Hox genes in the caudal group suggest that different intrinsic transcriptional cascades may operate in these 5-HT neurons. Perhaps not surprisingly, but validating the screen, the study indicated a highly enriched expression of Engrailed in the anterior subset of rostral 5-HT neurons in rhombome 1. Similarly, multiple Hox family genes and proteins were detected essentially in the caudal cluster of 5-HT neurons (Wylie et al., 2010).

Where to: axonal pathfinding of raphe neurons

Raphe nuclei 5-HT neurons are known to provide diffuse projections to almost all of the forebrain areas and spinal cord. Some of the recent studies mentioned above provide evidence supporting the notion that, despite the very diffuse projections, there may be a genetic encoding of 5-HT wiring circuits resulting in different functional connectivity.

Table 1. The summary of loss-of-function and gain-of-function studies evaluating fate determinants of 5-HT neuron development

<table>
<thead>
<tr>
<th>Gene</th>
<th>Loss of function</th>
<th>Gain of function</th>
<th>References</th>
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<tbody>
<tr>
<td>Shh</td>
<td>Loss of rostral and caudal 5-HT neurons</td>
<td>5-HT neurons in ectopic locations</td>
<td>Hynes et al. (2000) and Ye et al. (1998)</td>
</tr>
<tr>
<td>Fgf8</td>
<td>Loss of the rostral cluster of 5-HT neurons</td>
<td>Not determined (N.D.)</td>
<td>Ye et al. (1998)</td>
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<tr>
<td>Nkx2.2</td>
<td>Loss of caudal group of 5-HT neurons</td>
<td></td>
<td>Briscoe et al. (1999), Craven et al. (2004) and Pattyn et al. (2003)</td>
</tr>
<tr>
<td>Nkx6.1</td>
<td>Loss of rhombomere 1 5-HT neurons, ectopic generation of 5-HT neurons in rhombomere 4</td>
<td>Ectopic 5-HT neurons when Nkx2.2 and Nkx 6.1 are co-transfected</td>
<td>Extension of the area of 5-HT neurons Jacob et al. (2007)</td>
</tr>
<tr>
<td>Foxa2</td>
<td>Respecification of raphe 5-HT neurons into motoneurons</td>
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<td></td>
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<tr>
<td>Gata2</td>
<td>Loss of rostral 5-HT neurons</td>
<td>Ectopic 5-HT neurons</td>
<td>Craven et al. (2004), Pattyn et al. (2004) and Doorninck et al. (1999)</td>
</tr>
<tr>
<td>Gata3</td>
<td>Reduced causal 5-HT neurons</td>
<td>Ectopic 5-HT neurons when coexpressed with Gata2 or Ascl1/Mash1</td>
<td></td>
</tr>
<tr>
<td>Ascl1/Mash1</td>
<td>Reduced number of 5-HT neurons at all rostrocaudal levels</td>
<td>Ectopic 5-HT neurons with Gata3</td>
<td>Pattyn et al. (2004)</td>
</tr>
<tr>
<td>Insm1</td>
<td>Incomplete 5-HT phenotype (activity of TPH2 is reduced)</td>
<td>N.D.</td>
<td>Jacob et al. (2009)</td>
</tr>
<tr>
<td>Lmx1b</td>
<td>Loss of all 5-HT neurons</td>
<td>Ectopic 5-HT neurons with Nkx2.2 and Pet1</td>
<td>Cheng et al. (2003), Ding et al. (2003), Zhao et al. (2006) Cheng et al. (2003), Hendricks et al. (2003) and Kiyasova et al. (2011)</td>
</tr>
<tr>
<td>Pet1</td>
<td>Loss of 70% of 5-HT neurons</td>
<td>Ectopic 5-HT neurons with Nkx2.2 and Lmx1b</td>
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Based on anatomical tracing studies, which have been essentially performed in rats, it is known that the rostral raphe nuclei give rise to the ascending axonal projections to the midbrain and forebrain, whereas the axons of the caudal nuclei descend to the spinal cord. The rostral projections become visible soon after the 5-HT immunoreactivity is revealed in the brainstem. They grow as a fascicle within the medial forebrain bundle and reach the diencephalon by E15 in rats (Aitken & Törk, 1988). They then enter the telencephalon 48 h later, passing preferentially through the diagonal band of Broca, the septal area and into the cerebral cortex (Lidov & Molliver, 1982). Within the cerebral cortex, the fibers separate into two fascicles; one superficial (within the marginal zone) and the other, deep to the cortical plate (D’Amato et al., 1987). The establishment of the major axonal pathways within the serotonergic system can be subdivided into three main steps: (i) the organization of 5-HT neurons within the raphe nuclei and the initial orientation of the axons along the anterior–posterior axis; (ii) the guidance of axons along several main tracts; and (iii) the terminal innervation of the target area. The molecular mechanisms that control these different developmental steps were largely unknown until recently; however, genetic screens and analysis of mutant mice have begun to elucidate several of these mechanisms as summarized below (Fig. 2, Table 2).

The organization and polarity of the raphe 5-HT neurons were recently found to be controlled by proteins of the Wnt family. These are evolutionarily conserved guidance cues that regulate the direction of the ascending sensory and descending motor axons in the spinal cord (Lyuksyutova et al., 2003). One of the Wnt signaling pathways is a planar cell polarity pathway. It contains a series of factors that were initially discovered and described in flies, but that have vertebrate orthologs. Frizzled, a family of seven-pass transmembrane, (Cels3) receptors, Van Gogh-like (Vangl) and Disheveled are among the factors found to regulate tissue morphogenesis and directed cell migration (Wang & Nathans, 2007; Simons & Mlodzik, 2008). Some of these core components are expressed in the raphe 5-HT neurons (Fenstermaker et al., 2010). Recent analysis of Frizzled3−/− and Vangl2−/− mice showed that, at E12.5, ascending and descending 5-HT axons displayed marked mistargeting of their projections. 5-HT axons of the rostral cluster neurons projected posteriorly and laterally; instead of going strictly anteriorly, they erroneously descended into rhombomere 4. The descending axons from the caudal cluster of 5-HT neurons project posteriorly and laterally instead of going strictly posteriorly. (B) 5-HT axons from the dorsal raphe nuclei project to different cortical areas (red), and median raphe project to the hippocampus (Hip) (blue). In WT mouse brains, some 5-HT axons cross the midline and some remain ipsilaterally. When Slit1;Slit2 are invalidated, 5-HT fibers enter the telencephalon normally, but some abnormally recross the midline in the basal telencephalon (red dashed line) (midline crossing defects). In GAP43+/− mice, terminal target innervation is disrupted. 5-HT axons fail to innervate the dorsal neocortex and hippocampus. Celsr, cadherin EGF LAG seven-pass G-type receptor; Fzd, Frizzled; R1–R7, rhombomere 1–7.

Fig. 2. Schematic representations of main axon pathfinding defects in the serotonergic system. (A) Defects in anterior–posterior guidance of 5-HT axons in developing hindbrain. In wild-type (WT), 5-HT neurons in the rostral cluster give rise to ascending axons and in the caudal cluster to descending. In hindbrain of Fzd3−/−; Vangl2−/− and Celsr3−/− mice, axons of the rostral cluster of 5-HT neurons project posteriorly and laterally instead of going strictly anteriorly. Moreover, in the absence of Fzd3 and Vangl2, the descending axons from the caudal cluster of 5-HT neurons project posteriorly and laterally instead of going strictly anteriorly.
neurons were shorter than their wild-type counterparts, grew together in tight bundles and projected randomly along the anterior–posterior axis. The only known ligands for planar cell polarity signaling are Wnt proteins, some of which are expressed in the developing hindbrain as shown by in situ hybridization. There is a rostrocaudal gradient of Wnt5a expression at the midline, where ascending serotonergic neurons are projecting anteriorly (Figure 2A). To determine whether Wnt ligands direct the 5-HT axons, ‘open-book’ explant cultures of the hindbrain were analyzed and Wnt-coated beads were introduced along the midline to disrupt the endogenous Wnt gradients. This led to the strong attraction of ascending 5-HT axons to the midline, and a change in the positioning of neurons. This misguidance phenotype was similar to that observed in planar cell polarity mutants, suggesting an attractive role of Wnt5a for the ascending 5-HT axons. Overall, this study reports that Wnt signaling is required for two different aspects of monoaminergic circuit organization: control of anterior–posterior guidance of the axons and the correct cell body orientation within the brainstem raphe nuclei. This action is provided through the planar cell polarity pathway (Fenstermaker et al., 2010).

Once properly oriented, 5-HT axons are guided along the midline to the forebrain as a result of the coordinated interaction of Slit proteins with their Robo receptors. Bagri et al. (2002) demonstrated that both Slit1 and Slit2 played an essential role for the long distance connectivity of 5-HT axons. They analyzed the distribution of these fibers in the embryos of Slit2−/− and Slit1::Slit2−/− mice and demonstrated an abnormal trajectory of 5-HT-positive fibers within the medial forebrain bundle. In Slit2−/− mice, 5-HT fibers were displaced ventrally when crossing the diencephalon. In Slit1::Slit2−/− mice, 5-HT fibers entered the telencephalon normally, but a significant percentage abnormally crossed the midline in the basal telencephalon. These defects were readily apparent at E14.5, suggesting that the loss of Slit function affects the development of the serotonergic system as it courses rostrally into the forebrain (Bagri et al., 2002).

On reaching the forebrain, 5-HT axons continue elongating and grow into different structures. Possibly because of their length, 5-HT axons are particularly sensitive to the loss of several molecules that are implicated in axon elongation. This is the case with the growth-associated protein 43 (GAP43) and microtubule-associated protein ‘stable tubule only polypeptide’ (STOP). GAP43 is a phosphoprotein, widely expressed in early development, which has a role in axonal pathfinding, neurotransmitter release and synaptic plasticity. A persistent and severe disruption in the pattern of ingrowth of 5-HT axons was found in GAP43−/− mice from postnatal day 0. 5-HT axons failed to innervate the dorsal neocortex and hippocampus in the absence of GAP43. However, interestingly, the density of innervation in the striatum or amygdala was normal (Donovan et al., 2002). This specificity may be conferred by extracellular and intracellular signals. Neurons from GAP43−/− mice, for instance, are unable to respond to the neural cell adhesion molecules, such as NCAM, L1 and N-cadherin, which normally induce neurite outgrowth (Meiri et al., 1998), whereas integrin-mediated responses are unaffected, suggesting that GAP43 is required for some types of axonal navigation mediated by cell-to-cell contact. Based on these data, Donovan et al. (2002) hypothesized that the hippocampus and frontal, parietal and occipital cortex require contact-mediated axonal guidance for normal 5-HT innervation, whereas the striatum and amygdala do not.

Similarly to GAP43, the microtubule-associated protein STOP plays a role in the growth of 5-HT axons. The STOP protein is required for the stabilization of microtubules and is important for the axon elongation, neuronal morphogenesis and maintenance (Guillaud et al., 1998). The STOP−/− mice show significant alterations in brain levels of monoamines (Bouvrais-Veret et al., 2008). The invalidation of the Stop gene led to a marked imbalance of 5-HT tone in brainstem vs. forebrain areas. Decreased serotonergic innervation was observed in the cingulate cortex, hippocampus and basal ganglia, whereas, in the brainstem, 5-HT tissue levels were higher in knockouts than in controls (Fournet et al., 2010). As these data were obtained in adult mice, the developmental mechanisms of this abnormal innervation are not yet known. However, it is possible that the absence of the STOP protein disrupts microtubule stability and disturbs 5-HT axon elongation during development.

Axon targeting defects have been observed in a recent study of Katori et al. (2009). The authors showed that the terminal distribution of 5-HT fibers is regulated by protocadherin (Pdchx). Pdchx transcripts are strongly expressed in the raphe nuclei from embryonic stages to adulthood. Its deletion led to abnormally distributed serotoninergic fibers in many brain regions. In Pdchx−/− mice, 5-HT fibers reach most of their targets normally at birth (postnatal day 0). However, the arborization of serotonergic axons in the hippocampus, substantia nigra and olfactory bulb is abnormal. 5-HT fibers were

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<td>Fzd3−/− mice</td>
<td>Ascending raphe axons project laterally and posteriorly, descending project randomly</td>
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concentrated in the stratum lacunosum-moleculare of the hippocampus and remained very sparse in the dentate gyrus. In the substantia nigra and geniculate nuclei, the axons were concentrated in the peripheral regions and were sparse in the central parts of the nuclei. The study did not however explain the precise mechanisms of the Pdchz protein control on serotonergic axon outgrowth and pathfinding because of the lack of information on their subcellular localization and adhesion properties (Katori et al., 2009).

Conclusion
Overall, the data summarized in this review emphasize the developmental mechanisms underlying the heterogeneity of the central 5-HT system. Although these neurons share a common transcriptional control, slight differences in the combination of factors controlling 5-HT identity or axon targeting could contribute to generate heterogeneity within the raphe. For the moment, it has allowed to distinguish the rostral and caudal groups of the raphe. Furthermore, based on fate map studies, we now know that the largest proportion of the dorsal raphe 5-HT neurons originates from rhombomere 1. However, it is not yet known which gene networks are involved in defining the identity of small subgroups within the rostral and caudal clusters and within the dorsal and median raphe. Evidence from Pet1−/− mice strongly suggests that a different developmental programming of 5-HT neurons could occur within a given raphe nuclear division. The basis for this remains to be established.

Concerning pathfinding, despite recent identification of some guidance molecules involved in pathfinding of 5-HT neurons, we are still far from understanding what guides the subpopulations of 5-HT neurons within the dorsal and median raphe to their specific targets. For the moment, molecular differences have been found between the rostral and caudal clusters of raphe neurons. Some of these molecules, such as engrailed or Hox genes, could underlie the differences in projections of these two groups of neurons. However, the link between the transcriptional regulation and guidance phenotype still needs to be elucidated.

The results of such studies will be of particular importance to understand the dysfunctions of 5-HT systems in a number of psychiatric disorders. This may ultimately lead to the identification of targets. For the moment, molecular differences have been found between the rostral and caudal clusters of raphe neurons. Some of these molecules, such as engrailed or Hox genes, could underlie the differences in projections of these two groups of neurons. However, the link between the transcriptional regulation and guidance phenotype still needs to be elucidated.

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Abbreviations
E, embryonic day; Fgf8, fibroblast growth factor 8; GAP43, growth-associated protein 43; MHB, mid-hindbrain boundary; Pdchz, protocadherin z; Shh, Sonic hedgehog; STOP, stable tubule only polypeptide; Tph, tryptophan hydroxylase; Vangl, Van Gogh-like; 5-HT, serotonin.

References


Development of raphe serotonin neurons


