Specification of dopaminergic and serotonergic neurons in the vertebrate CNS

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The early specification of dopaminergic and serotonergic neurons during vertebrate CNS development relies on signals produced by a small number of organizing centers. Recent studies have characterized these early organizing centers, the manner in which they may be established, the inductive signals they produce, and candidate signaling systems that control the later development of the dopaminergic system.

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Abbreviations

5HT	serotonin
ANR	anterior neural ridge
A/P	anteroposterior
BDNF	brain-derived neurotrophic factor
bFGF	basic FGF
DA	dopamine
D/V	dorsoventral
E	embryonic day
EGF	epidermal growth factor
FGF	fibroblast growth factor
FP	floorplate
GDNF	glial cell line derived neurotrophic factor
IL	interleukin
MHB	mid–hindbrain boundary
Shh	Sonic hedgehog
TGF	transforming growth factor
TH	tyrosine hydroxylase

Introduction

Mature neurons in the mammalian brain develop in stereotypic positions along the dorsoventral (D/V) [1], and anteroposterior (A/P) [2] axes of the neural tube. It has been proposed that signaling (organizing) centers — including the mid–hindbrain boundary ([MHB] or isthmus), the floorplate (FP), and the anterior neural ridge (ANR), which operate along the two main axes of this system — establish an epigenetic grid of Cartesian coordinates, and that neural progenitors assume distinct cell fates according to their location on this grid (see e.g. [3,4]). This review focuses on recent findings regarding the specification of two prominent neuronal cell types, dopaminergic (DA) and serotonergic (5HT) neurons, as well as the development of the MHB, one of the organizing centers that plays a critical role in specifying the majority of DA neurons and a subpopulation of 5HT neurons. In addition, factors that influence later differentiative steps for DA neurons will be discussed.

Location and function of DA and 5HT neurons

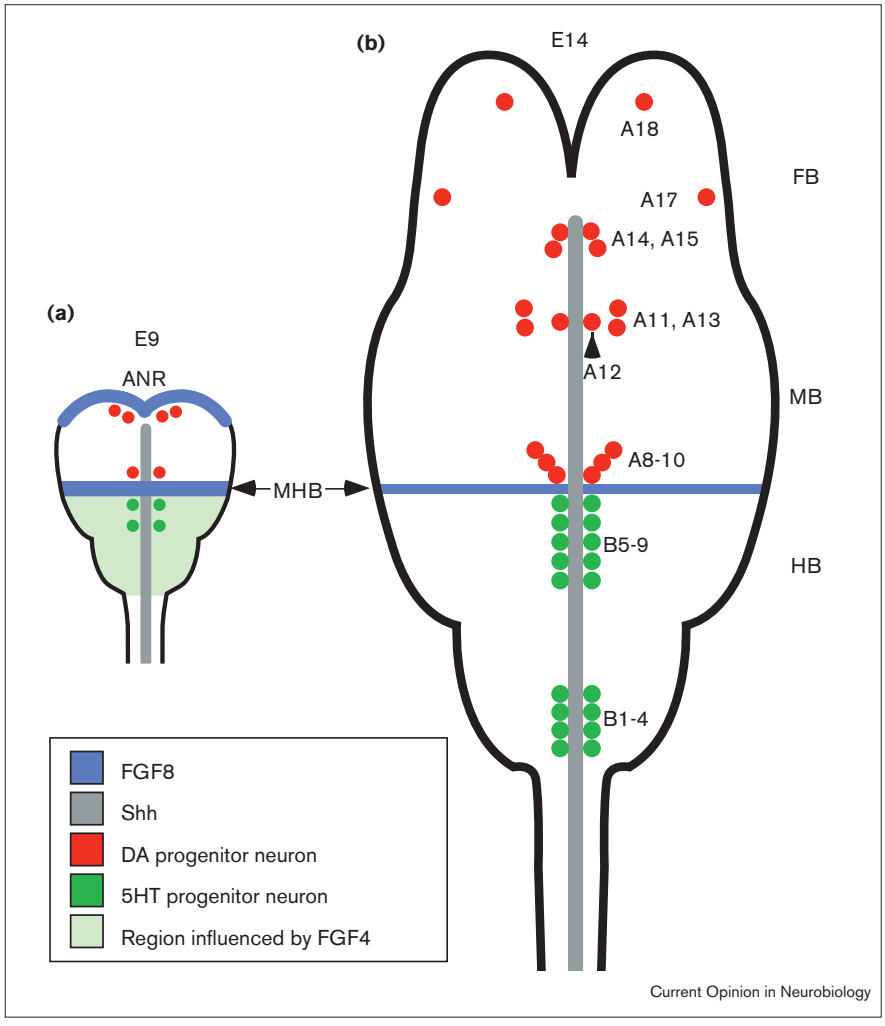
Historically, DA neurons of the adult mammal have been placed into nine distinct groups, with the most prominent groups residing in the ventral midbrain (these groups are called A8, A9, and A10), and in the diencephalon (groups A11–A15) [5,6]; the telencephalon contains two smaller groups of DA neurons, and these are restricted to the olfactory bulb (group A16) and the retina (group A17) (Figure 1) [7,8]. (Groups A1–A7 are noradrenergic.) Midbrain DA neurons, also known as neurons of the substantia nigra pars compacta and the ventral tegmental area, innervate the striatum (or the limbic system) and neocortex, respectively [8,9]. The loss of substantia nigral neurons results in the motor disorders characteristic of Parkinson's disease [10], whereas overstimulation of ventral tegmental DA neurons has been associated with schizophrenia and drug addiction. Although functionally important, the major diencephalic DA cell groups are less well characterized, but are known to be involved in the release of pituitary hormones [9].

Mammalian 5HT neurons have also been classically divided into nine cell groups (B1–B9). The more rostral 5HT groups (B5–B9) are said to reside in the midbrain and rostral hindbrain, whereas groups B1–B4 are located more caudally (Figure 1) [8]. Rostral 5HT neurons have ascending serotonergic projections and innervate virtually all areas of the brain, whereas the caudal cell groups have descending projections to the spinal cord. 5HT neurons are involved in the coordination of complex sensory and motor patterns that are associated with different behavioral states, and their abnormal function has been implicated in affective illness, schizophrenia, aggression and depression [9].

Both DA and 5HT neurons are monoaminergic, producing the neurotransmitters dopamine and serotonin through a series of enzymatic modifications of primary amino acids. The original substrate, and the majority of synthetic enzymes, differ between these two classes of neurons; however, both DA and 5-HT neurons use the enzyme aromatic amino acid decarboxylase (AADC) to hydroxylate tyrosine for the production of dopamine, and to hydroxylate tryptophan for the production of serotonin [7].

Despite the apparent anatomical diversity of DA and 5HT neurons in the adult brain, recent data have provided evidence for a much simpler topographical distribution of the progenitors of these neurons [11••]. DA neurons appear to be specified and reside rostral to the MHB, and they depend on ventral midline structures, such as the ANR or the MHB, for their development. 5HT neurons appear to be born caudal to the MHB, and they depend critically on the FP for their development. A subset of 5HT neurons also depends on the MHB for their specification.

Figure 1



Schematic drawings of DA and 5HT progenitors, and the relative position of differentiated DA and 5HT neurons at later ages. **(a)** Dorsal view of an early somite embryo depicting the relationship of DA and 5HT progenitors to the *Shh* (gray) and *Fgf8* (blue) signals. In isolated pieces of neural tissue grown for 7 days (corresponding to E9–E16 *in vivo*), DA neurons developed only in ventral tissue derived from regions just rostral to the MHB or just caudal to the ANR. DA neurons never developed in isolated pieces of caudal forebrain, rostral midbrain, or future dorsal regions. Likewise, 5HT neurons arose exclusively from isolated pieces of ventrally derived rostral and caudal hindbrain [11••]. **(b)** Schematic showing the approximate location of the multiple groups of DA and 5HT neurons in their mature positions (redrawn on the basis of position and nomenclature in [5]). It is hypothesized that all DA and 5HT neurons arise from the respective regions and progenitors illustrated in the E9 schematic; however, due to the limits of the explant culture system, and the lack of specific markers for the different groups of mature neurons, this has not been proven.

Specification along the dorsoventral axis

Midbrain and forebrain DA neurons

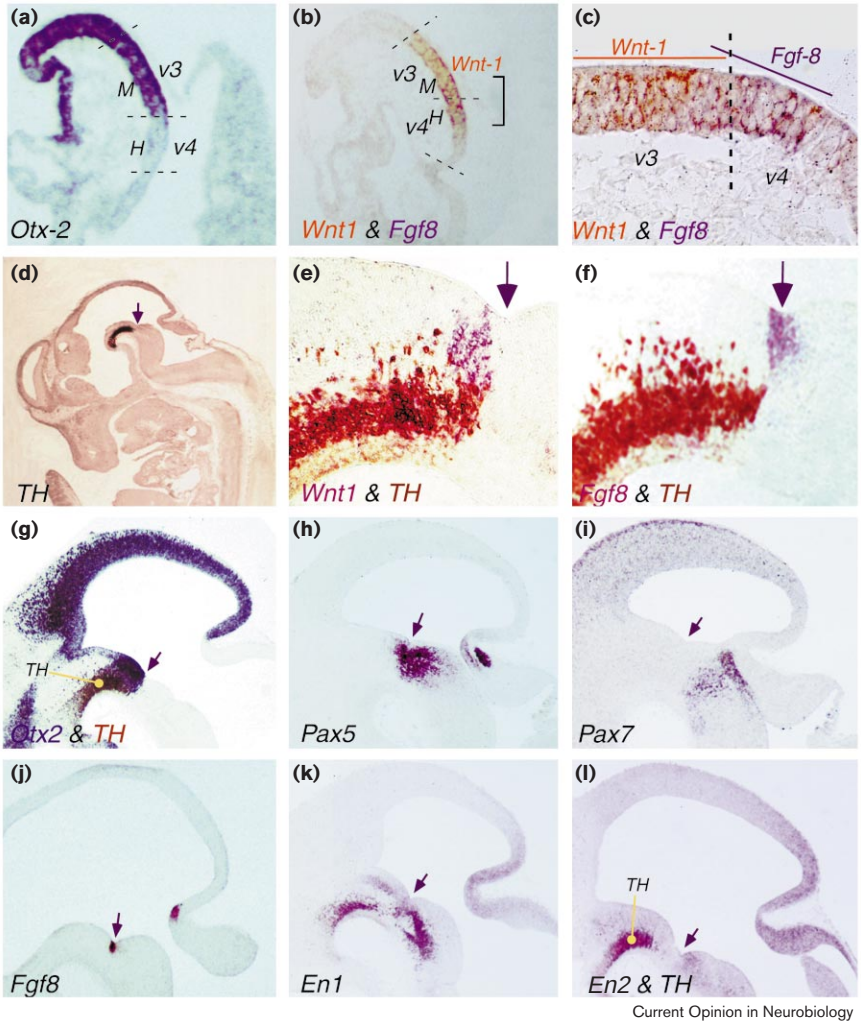
Midbrain DA neurons can be identified by the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis. They are first detected at about embryonic day 12.5 (E12.5) in the rat, near the MHB [12–16]. *In vitro* embryological studies have shown that these neurons differentiate near the FP in the ventral midline of the neural tube and can be induced ectopically in dorsal midbrain in explants, or in transgenic mice, by the FP [17]. The inductive effects of the FP can be mimicked by Sonic hedgehog (Shh) [18,19], and its effects are prevented by Shh-blocking antibodies [11••], suggesting that Shh is responsible for the FP activity. The diencephalic and telencephalic DA neurons, which have been less well studied, appear to arise later in development than midbrain DA neurons. Surprisingly, although these neurons occupy multiple locations in the adult [7,8], rat explant culture studies have shown that they may all originate from a small region in the anterior forebrain [11••] (Figures 1 and 2). Similar to their midbrain counterparts, forebrain DA neurons develop adjacent to an endogenous source of Shh in the ventral forebrain. In addition, they can be induced ectopically in dorsal forebrain locations by the FP, and their development is prevented by Shh-blocking antibodies [11••].

Thus, it appears that multiple groups of DA neurons (possibly all) along the A/P axis of the mammalian brain require Shh for their specification. Importantly, however, Shh cannot induce DA neurons in the diencephalon or hindbrain — regions that do not normally contain endogenous DA neurons [11••] — suggesting that it is able to specify the fate of DA neurons along the D/V axis, but not along the A/P axis.

Specification of 5HT neurons

The development of 5HT neurons also appears to be regulated by Shh. These neurons can be induced ectopically in the dorsal hindbrain (but not in other locations), in explant cultures, and *in vivo* by the notochord [20] or FP [21], both of which produce Shh, as well as by Shh itself. In addition, both rostral 5HT neurons, which reside near

Figure 2



Relation of MHB-associated genes to DA and 5HT progenitors and neurons at early somite and mid-gestational ages. **(a–c)** Saggital sections of 5 somite, E9 rat embryos showing *in situ* hybridization to *Otx2;* the caudal limit of *Otx2* expression marks the caudal boundary of the midbrain **(a)**, and to *Wnt1* (orange) and *Fgf8* (purple) **(b,c)**. *Wnt1* and *Fgf8* are both expressed in tissue that gives rise to midbrain DA neurons (v3), and of *Fgf8*, but not *Wnt1*, in tissue that gives rise to rostral 5HT neurons (v4) [11••]. Dashed lines depict approximate rostral and caudal borders of v3 and v4. **(d–l)** Saggital sections of E14 mid–hindbrain region marked for DA neurons by TH **(d)**, *Wnt1* and TH **(e)**, *Fgf8* and TH **(f)**, *Otx2* and TH **(g)**, *Pax5* **(h)**, *Pax7* **(i)**, *Fgf8* **(j)**, *En1* **(k)**, or *En2* and TH **(l)**. Purple arrows mark the neuroepithelial constriction.

the MHB (which may correspond to groups B5–B9) and more caudal 5HT neurons (possibly groups B1–B4) fail to develop in the presence of Shh-blocking antibodies [11••]. Thus, Shh controls the development of 5HT neurons along the D/V axis of the neural tube, but cannot induce them ectopically along the A/P axis.

Specification along the A/P axis

Location of midbrain DA and rostral 5HT neurons in the early and midgestational embryo

In E14 rat mid–hindbrain, DA and 5HT neurons occupy adjacent, non-overlapping domains and abut the ventral MHB, with midbrain DA neurons rostral to the MHB, and hindbrain 5HT neurons caudal to the MHB [21]. Consistent with the position of these neuronal groups *in vivo* at E14, DA and 5HT neurons develop in adjacent, but distinct pieces of isolated E9 mid- or hindbrain explants, respectively, *in vitro* [11••]. Thus, the progenitors for these two neuronal cells types lie on either side of (or within) the MHB as early as E9, and they differentiate in this region, at a time between E9 and E14. Using a similar, isolated explant culture system, forebrain DA neurons have been found to be specified in the very anterior regions of the neural plate, in close proximity to, or in, the ANR [11••]. The proximity of developing midbrain DA and rostral 5HT neurons to the MHB, and of forebrain DA neurons to the ANR, raised the possibility that these two organizing centers provide positional information for DA neurons along the A/P axis. Consistent with this hypothesis, Ye *et al.* [11••] found that a dissected MHB could induce ectopic expression of DA neurons in the ventral diencephalon, which is normally devoid of DA neurons but does express Shh. In addition, forebrain DA neurons failed to develop in ventral forebrain explants following removal of the ANR [11••].

FGF8 is necessary for specification of mid- and forebrain DA and 5HT neurons

Fibroblast growth factor 8 (FGF8) is a secreted molecule that is discretely expressed in both the MHB and ANR at E9. This molecule has been shown to induce an ectopic

midbrain in the chick diencephalon [22] and to be responsible for the induction of the transcription factor *BF1* in the telencephalon [23]. At E14, DA and 5HT neurons lie in proximity to the residual *Fgf8* signal; at E9, the *Fgf8* signal is highly expressed in the transverse pieces of tissue that give rise to fore- and midbrain DA neurons and to rostral, but not caudal, 5HT neurons (Figure 2) [11••]. FGF8 therefore became a candidate for the inductive signal from the MHB and ANR for DA and 5HT neurons. Ye *et al.* [11••] found that blockade of FGF8 with a soluble FGF receptor in isolated fore-, mid-, and hindbrain explants prevented the development of both mid- and forebrain DA neurons, as well as rostral, but not caudal, 5HT neurons. The dependence of midbrain DA neurons on FGF8 is supported by the phenotype of mice with a defect in the *Fgf8* gene. Animals with severely reduced levels of FGF8 have deficits in the caudal midbrain and rostral hindbrain [24•], and they lack TH-positive DA neurons [11••]. A moderate reduction in FGF8 levels in mice does not completely deplete midbrain DA neurons [24•]; however, the number of these DA neurons is significantly lower than in normal littermates [11••]. Thus, FGF8 fits the criteria for an endogenous factor that delimits the position of DA neurons along the A/P axis of the neural tube.

Two additional members of the FGF gene family, *Fgf17* and *Fgf18*, are also expressed in the MHB and the ANR [25,26] (Figure 3); however, they do not appear to compensate for a deletion in the *Fgf8* gene [24•], suggesting that either they do not play a role in specification of DA neurons or they act downstream of FGF8.

Importantly, FGF8 has been found not only to be necessary for the development of DA neurons, but also to be capable of inducing DA neurons ectopically in the ventral diencephalon [11••]. Moreover, although neither FGF8 nor Shh can induce DA neurons in the dorsal diencephalon when applied alone, they can induce DA neurons in this location when applied together. Thus, both of these molecules are critical for the specification of DA neurons, and the stereotypic location of DA neurons along the A/P and D/V axes is defined by the integration of these two signals [11••].

Specification of 5HT neurons in the rostral hindbrain

Rostral hindbrain 5HT neurons, like mid- and forebrain DA neurons have been shown to depend on both Shh and FGF8 for their development [11••]. These two molecules, however, cannot induce 5HT neurons in ectopic locations, suggesting that a third signal may be involved. A candidate for the third signal is FGF4, as it induces ectopic 5HT neurons when added to ventral midbrain explants, a tissue that contains endogenous FGF8 and Shh [11••]. Surprisingly, under these conditions, endogenous midbrain DA neurons no longer develop [11••]. These findings suggest that FGF4, in combination with Shh and FGF8, induces 5HT neurons to develop in the hindbrain, whereas its absence in midbrain tissue, which contains Shh and FGF8, allows the development of DA neurons. FGF4 is not localized to the hindbrain neural plate or mesoderm, but is highly expressed in the primitive streak, a region juxtaposed to the posterior neural plate during early development [11••,27]. It is possible that FGF4 acts to pre-pattern the future hindbrain and that the later exposure to FGF8 and Shh confers a 5HT phenotype on these progenitors. In support of this notion is the finding that when midbrain progenitors are not pre-exposed to FGF4, later exposure to FGF8 and Shh results in the adoption of the DA phenotype.

In summary, DA and 5HT neurons develop on either side of the MHB, in closely apposed, but non-overlapping domains. Both of these groups of neurons are dependent on Shh and FGF8 for their development, and these factors are sufficient to induce DA neurons, but not 5HT neurons, ectopically in the diencephalon. A third factor is required for 5HT neuron induction; this factor is, or is mimicked by, FGF4 [11••].

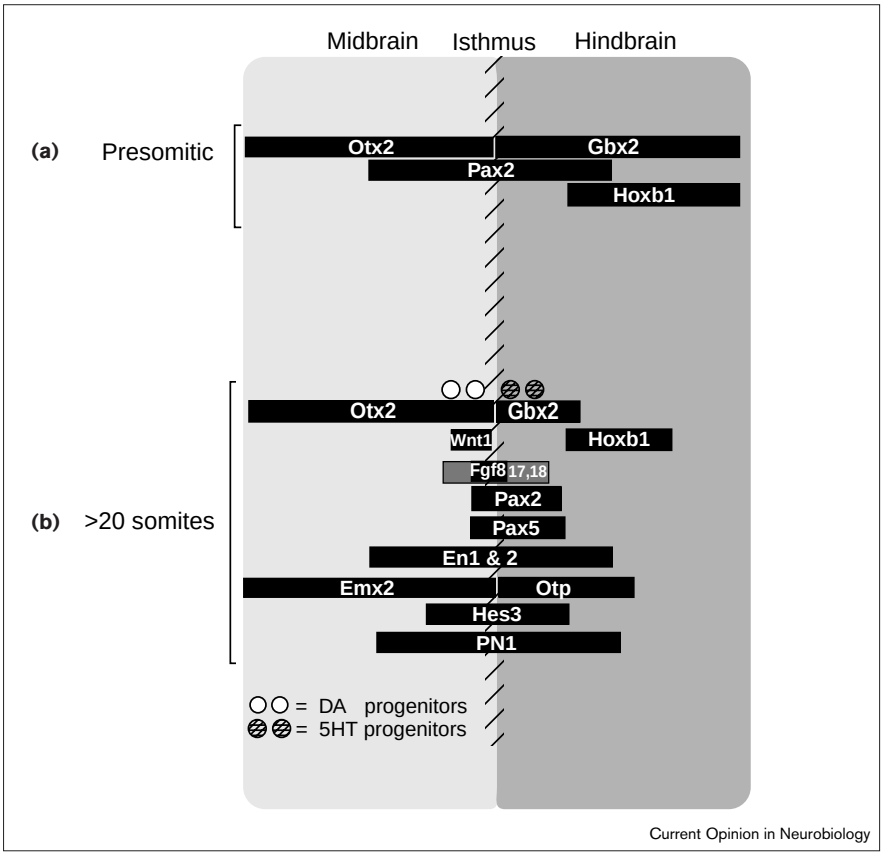
Development of the MHB

Definition of the MHB and its relationship to DA and 5HT neurons

Given the role the MHB has in the induction of DA and 5HT neurons, it is important to characterize this region and to elucidate the mechanisms by which it is established, maintained and acquires a distinctive rostral–caudal polarity. The MHB has been anatomically characterized as a neuroepithelial constriction between the midbrain and hindbrain; however, as an organizing center, the MHB is the tissue that has the ability to recreate pattern when transplanted to a different region of the neural tube. The anatomical boundaries of this functional center have not yet been determined experimentally, so investigators have used genes that either are expressed specifically or have borders that terminate in the region at early embryonic ages to define it. These genes are midbrain-specific (e.g. *Wnt1*), hindbrain-specific (e.g. *Otp*), are expressed in the junction (e.g. *En1*, *En2*, *Pax2*, *Pax5*, *Pax8*, and *Fgf8*, *Fgf17* and *Fgf18*), or cover a broad domain that terminates at the boundary between the mid- and hindbrain (e.g. *Otx2* and *Gbx2*) (Figures 2 and 3).

On the basis of the expression of multiple MHB-associated genes, it has been determined that the MHB initially covers a broad expanse within the neural plate, occupying territory in both the presumptive midbrain and presumptive hindbrain. Between the headfold stage (> 1 somite) and mid-gestation, it appears that this region gradually diminishes in size, subsequently occupying an area between the midbrain and hindbrain. At early stages (5 somites), *Fgf8*, *Wnt1*, *Otx2* are expressed in the regions of the neural plate that, when isolated, give rise to midbrain DA neurons (explant v3 from Ye *et al.* [11••]). In contrast, *Fgf8* and *Gbx2*, but not *Wnt1*, are expressed in the region that gives rise to rostral 5HT progenitors (explant v4 from Ye *et al.* [11••]). *En1* and *En2* are expressed in both

Figure 3



Schematic of the relative position of MHB-associated genes and of DA and 5HT progenitors. **(a)** Presomite stage: *Otx2*, *Gbx2*, *Hoxb1* [40•] and *Pax2* [28]. **(b)** > 20 somites: *Otp*, *Pax2*, *Emx2*, *Otx2*, *Wnt1*, *Fgf8*, *En-1* and *En2* expression [44••,77–81], *Fgf17* and *Fgf18* [25,26], *Hoxb1*, *Gbx2* [40•,82], *PN1* [83], *Hes3* [84], *Pax2* and *Pax5* [85]. Boundaries are estimated based on available data and are not necessarily precise.

v3 and v4 (Figures 2a–c and 3). By midgestation, *Fgf8*, *Wnt1*, *Otx2*, *En1* and *En2* continue to be expressed in close proximity to DA neurons (in addition, *En1* is actually expressed within DA neurons) and 5HT neurons, which now can be identified *in situ* by the expression of their mature markers TH and 5HT, respectively (Figure 2d–l).

Gene expression and regulation in the MHB

Although associated with the MHB, the role of each of these genes in the specification or refinement of the MHB was not fully understood. The *Pax2* transcription factor is the gene found to be expressed first in the MHB region, and it is detected at the headfold stage [28]. Two additional genes are activated at around the 1 somite stage, the secreted protein *Wnt1* and the homeobox transcription factor *En1* [28]. These three genes occupy overlapping but non-identical domains. *Wnt1* is initially expressed throughout the future midbrain, and its anterior expression domain coincides with the anterior extent of *Pax2*. However, at least in zebrafish [29••], the lateral boundaries of *Wnt1* exceed those of *Pax2*. *En1* expression occupies a subset of the *Pax2* expression domain, but unlike *Wnt1*, *En1* expression extends caudally across the MHB and is thus expressed in both the midbrain and the hindbrain [28].

By 3 somites, *Fgf8* is expressed and, in the zebrafish, its expression domain is initially posterior to, but does not overlap with, *Pax2* [30••]. By 5–6 somites, however, the expression of *Fgf8* is contained entirely within the *Pax2* expression domain. Around this time, additional regional genes, including *Pax-5* (3–4 somites), *En2* (3–4 somites) and *Pax-8* (7–8 somites), are induced in the caudal midbrain and rostral hindbrain, in domains which are a subset of the *En1* domain. By midgestation, the mid- and hindbrain have undergone pronounced growth, and *Pax2*, *Pax5*, *Pax 8*, *Wnt1* and *Fgf8* are retained in a restricted region in this area (Figure 2). *Wnt1* and *Fgf8* are confined to narrow strips, with *Wnt1* clearly restricted to the midbrain and *Fgf8* expression restricted to the region around the neuroepithelial constriction; it is difficult to assign either to the mid- or hindbrain (Figure 2d–l). Interestingly, transgenic mice that have an expanded MHB region (both rostrally and caudally) show ectopic *Fgf8* expression that expands only rostral to the MHB [21], suggesting that *Fgf8* may normally be restricted either to the midbrain or to a true boundary area, and thus represent the 'isthmus'.

Despite the fact that multiple genes are sequentially expressed in overlapping or adjacent domains within and around the MHB, the isolated deletion of each of these genes suggests that none of them performs a 'master regulatory' function in terms of specifying the region. Instead, as suggested by their distinct expression domains, many of

them are independently activated during development. Thus, *Wnt1* and *Fgf8* expression is activated normally in mice and zebrafish deficient in *Pax2* [29••,31]. Likewise, *Pax2*, *Wnt1*, *En1*, and *Fgf8* are induced in rodents and zebrafish that lack *Fgf8* [11••,30••], and *Fgf8* and *Pax2* are induced in mice deficient in *En1* [32]. In addition, *Pax2*, *En1* and *Fgf8* are induced in mice lacking *Wnt1* [33,34]. There are two exceptions: *En2* (and *En-3* in zebrafish), whose induction depends on the intact expression of *Pax2* and *Fgf8*; and *Pax5* and *Pax8*, whose induction depends on an intact *Pax2* signal [35•].

Although the majority of MHB-associated genes are induced independently in mutant mice and zebrafish, they are co-dependent for their stable expression: when one gene is missing, the remaining MHB markers are extinguished over time [11••,30••,32,33,35•,36]. This suggests that the MHB genes identified so far function to define, stabilize and maintain the boundaries of the MHB, but may not specify this region; however, one of them (*Fgf8*) is able to do so under experimental conditions [22].

The specific function of these genes, apart from regulating each other's expression, is not fully understood. It appears that *Fgf8* is responsible for mitogenesis [37•], as well as cell fate determination in the mid- and hindbrain [11••], that *Wnt1* is required for proliferation of cells in the midbrain [33,36], and that *En1* is required for the induction of axonal guidance cues, and possibly for cell survival, in the mid- and the hindbrain [32,37•].

Initial specification of the MHB

Even though the genes restricted to the MHB region clearly play an important role in the maintenance of this organizing center, the mechanisms responsible for the initial formation of this region remain elusive. A few possibilities are considered here.

Planar signals in the neuroectoderm

Planer signals within the neuroectoderm may initiate development of the MHB by the juxtaposition of different neural plate regions. Consistent with this is the finding that if the midbrain (and presumably the MHB) is surgically removed, it will re-form between the forebrain and the hindbrain [38]. In addition, at least in the zebrafish, it appears that the hindbrain neural plate (and possibly the MHB) is committed before it comes in contact with vertical signals [39]. The juxtaposition of the mid- and hindbrain is demarcated early (pre-headfold) by the expression of two key transcription factors families: *Otx1* and *Otx2* (mouse homologs of the *Drosophila orthodenticle* gene) in the fore- and midbrain; and *Gbx2* (a vertebrate homeobox gene related to the *Drosophila unplugged*) in the anterior hindbrain. The caudal limit of *Otx* gene expression precisely abuts the rostral limit of *Gbx2* expression [40•].

In agreement with the notion that these two families play a role in the development and/or position of the MHB, it has been reported that *Otx2*-deficient mice fail to develop neural tissue anterior to the hindbrain [41–43] and that *Gbx2*-deficient mice fail to develop hindbrain motor nuclei, cerebellum, and derivatives of rhombomeres 1–3 [40•]. Surprisingly, there is still a recognizable isthmus (or MHB) and there is discrete, although transient, expression of MHB-associated genes, including *Fgf8*, *Wnt1*, *En1*, *Pax2*, and *Gbx2* in the $Otx1^{-/-}/Otx2^{-/+}$ or $Gbx2^{-/-}$ mice [40•,44••]. However, the expression domains of these genes is altered. In the $Otx1^{-/-}/Otx2^{-/+}$ mice, *Fgf8*, *Wnt1*, *En-2* and *Gbx2* expand rostrally, whereas in *Gbx2*-deficient mice, the expression of *Fgf8*, *Wnt1* and *Otx2* expands caudally.

Overexpression studies provide additional support that these genes are directly involved in mid- or hindbrain specification. Transgenic mice that express *Otx2* in the hindbrain under the *En1* promoter in transgenic mice display upregulation of midbrain-specific genes and deletions of portions of the cerebellum, suggesting a transformation of hindbrain to midbrain (V Broccoli V, A von Holst, E Boncinelli, W Wurst, *Soc Neurosci Abstr* 1998, 24:215.3). Likewise, transgenic mice that express *Gbx2* in the midbrain from early ages causes partial deletion of the midbrain (S Millet, K Campbell, DJ Epstein, GWM Bothe, AL Joyner, *Soc Neurosci Abstr* 1998, 24:215.2).

Although these findings are consistent with involvement of *Otx2* and *Gbx2* in the development of the MHB, they do not prove that these two factors create this organizing center. First, because $Otx2^{-/-}$ mice fail to develop fore- or midbrain structures, the direct involvement of the Otx2 protein in the formation of the MHB is hard to assess [41,45]. Second, as described, *Gbx2*-deficient mice still develop a residual MHB [40•]. Thus, *Otx* and *Gbx* may not be essential for the formation of the MHB, but may be involved, at least in part, in its refinement and position.

Mesodermal induction of the MHB

It has been suggested that vertical signals from underlying mesodermal tissue may be responsible, at least in part, for the formation of the MHB. For example, *Fgf8* in the isthmus might be induced by *Fgf8* secreted by the underlying cardiac mesendodermal cells [22]. However, the fact that *Pax2* expression in the MHB precedes the expression of *Fgf8* in the cardiac mesoderm argues against this possibility.

Yet another hypothesis is that the anterior mesendoderm in the mouse (or the germ ring in the zebrafish) is a source of positional information for the MHB. Zebrafish germ ring cells, which give rise to somitic mesoderm, posterior mesoderm and endoderm, can induce an ectopic midbrain and a morphologically discernible MHB in the telencephalon [46]. Likewise, mouse anterior mesendoderm can ectopically induce the expression of En1 and En2 posterior to the hindbrain [47]. However, *En1* is still expressed in mice deficient in the secreted protein Cripto; these mice do not have an organizer or any embryonic mesoderm or endoderm [48]. Although the findings in $Cripto^{-/-}$ mice suggest

that embryonic tissues are not critical in MHB formation, it is important to note that expression of *En1*, which is used in experimental situations to identify the MHB, may be misleading because it may reflect only the presence of midbrain tissue but not specifically the MHB.

Extraembryonic tissues

The best evidence, to date, suggests that the extraembryonic endoderm provides a signal for anterior neural pattern, including specification of the MHB. First, removal of the anterior extraembryonic endoderm in the chick embryo leads to a failure to pattern anterior neural tissue, including the MHB [36]. Likewise, mice that do not express *Otx2* [49••], *Nodal* (a transforming growth factor β [TGFβ] family member) [50•], or *Smad2* (an essential component in the TGFβ signal cascade) [51••] in the extraembryonic endoderm fail to develop a neural plate rostral to the hindbrain and fail to express MHB markers, even though *Otx2*, *Nodal* and *Smad-2* are still expressed in embryonic tissues [36,49••,50•,51••]. Furthermore, unlike the full deletion of *Otx2* (where the anterior brain is missing), the selective elimination of *Otx2* in the neural plate, with intact *Otx2* in the extraembryonic endoderm, results in the normal induction of *Pax2* and *En2* (but not *Wnt1*) [49••]. Thus, the extraembryonic endoderm is necessary for activation of at least a subset of MHB-associated genes in the neural plate. As no ectopic induction of anterior brain, or MHB, by the extraembryonic endoderm had been reported, it is not yet clear whether the signal produced by the extraembyronic tissue is permissive or instructive.

Taken together, these findings suggest that signals that originate from the anterior visceral endoderm, and possibly from the primitive streak, are responsible for the anterior and posterior characteristics, respectively, on the neural plate and thus, by default, may define the position of the MHB as the border between anterior and posterior tissue. The definitive endoderm and mesoderm may subsequently refine and stabilize the MHB, in conjunction with loops of regulatory genes that act within it. The MHB would then act collaboratively with other signaling centers, such as the FP, to specify the fate and position of mature DA and 5HT neurons, as well as possibly other neuronal classes.

Factors that affect later events in DA neuron maturation

Specification or expansion of DA neurons *in vitro*

In parallel to studies in explant culture that have described the specification of DA neurons from undifferentiated progenitors, several groups have observed that TH-positive cells can be induced to appear in more mature tissues. Ling *et al.* [52••] demonstrated that interleukin 1 (IL-1) causes a significant increase in the number of TH-positive cells in dissociated tissue culture (derived from the E14.5 rat midbrain and expanded in the presence of epidermal growth factor [EGF], which form proliferating spheres or 'neurospheres'). TH-positive cells were not present in neurospheres exposed only to EGF or to EGF plus any of 16 other cytokines/growth factors, including Il-2, IL-3, IL-4, IL-6, Il-9, IL-11, tumor necrosis factor-α, colony stimulating factor, interferon–γ, stem cell factor, leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), TGFβ, erythropoietin, and glial cell line derived neurotrophic factor (GDNF).

When IL-1 was used in combination with IL-11, LIF or GDNF, or with conditioned media from embryonic striatal cells, there was a further increase in the number of TH-positive cells (resulting in up to 25% of the culture being TH-positive), enhancement of the neuronal morphology in the TH-positive cells, and the induction of additional DA neuron markers.

Although these findings raise the possibility that IL-1 participates in the control of DA cell fate, no obvious behavioral or morphological abnormalities that would be associated with deficits in the DA system have been reported in mice deficient for IL-1 [53] or its receptor [54]. Furthermore, it is unclear whether the presence of TH neurons in these neurospheres results from the survival of the DA neurons that would have been present in the tissue used to make the neurospheres or whether IL-1 induced undifferentiated progenitors to adopt a DA phenotype.

Through a series of studies, Iacovitti and colleagues [55–59] have shown that DA neurons can be induced in dissociated cultures of primary embryonic striatum, a brain region that would not normally make DA neurons, in the presence of muscle-derived factors [55]. They further showed that these striatal cultures [56–58], or neural progenitor cell lines [59], could be similarly induced to express TH in the presence of acidic FGF, in combination with DA agonists, or protein kinase C (PKC) or PKA activators. In addition, cultured neural progenitors from the embryonic basal forebrain ventricular zone can be induced to express TH in the presence of activin and FGF2 [60].

Maturation factors

A large number of factors have been implicated in enchancing the late development of DA neurons. As these factors not only may play a role in the maturation of DA neurons, but may also participate in the late specification of DA neurons, they bear mention. For example, cultured DA neurons respond to the serine protease thrombin (which is involved in blood coagulation) by changing the length, number and branching pattern of their neurites [61]. The seven transmembrane receptor for thrombin is expressed in the embryonic ventral midbrain. Similar morphological changes in DA neurons can be induced by members of the nerve growth factor protein family, such as BDNF, neurotrophin-3 and neurotrophin-4/5 [62,63], by bFGF [64], and by members of the TGF and GDNF protein families [65]. In addition, a study of mice deficient in the TGFα gene revealed that these mice have a 50% reduction in the number of DA neurons in the substantia

nigra, but a normal complement of other midbrain DA neurons [66••], indicating that TGFα may participate in the expansion or differentiation of this particular subtype of DA neuron.

Additional factors that were shown to influence the development, survival, or the maturation state of DA neurons *in vitro* and/or whose receptors are expressed in DA neurons include the pituitary adenylate cyclase-activating polypeptide (PACAP) [67], calcitonin gene-related peptide [68], endothelin [69], neurotensin [70], estrogen and progesterone [71], substance P [72], retinoic acid [73], and the opioid peptides [74]. Although little is known about the function of these factors in the development of DA neurons *in vivo*, they could participate in late specification of these neurons to particular subtypes or serve as mitogenic or survival factors for DA progenitors.

Intracellular mediators of the DA phenotype

Most of the intracellular proteins that mediate the development of DA neurons in response to inductive signals have not been identified. However, gene ablation studies in the mouse have revealed at least one essential gene in this pathway, the orphan steroid receptor Nurr1. Deletion of this protein, which is widely expressed in the embryonic and adult central nervous system from E10.5 in the mouse, prevents the development of midbrain DA neurons [75]. Although Nurr1 expression can be induced by Shh [11••], it may be responsible for only late steps in the differentiation of DA neurons. This is suggested by the finding that early commitment to the DA cell lineage, as measured by expression of the homeodomain transcription factor *Ptx3*, is still observed in the absence of Nurr1 [76••]. Moreover, Nurr1 does not appear to be sufficient for the induction of DA cell fate, because its ectopic expression in the dorsal midbrain of transgenic mice does not lead to ectopic development of DA neurons (M Hynes, A Rosenthal, unpublished data). As it marks early differentiation to the DA lineage, Ptx3 itself is a candidate mediator of the development of DA neurons. This protein is expressed starting from E11 in the mouse, almost exclusively in midbrain DA neurons. The consequence of ablating *Ptx3* has not yet been reported; however, our data suggest that like Nurr1, Ptx3, by itself, is not sufficient to induce DA neurons in the embryonic midbrain (P Burbach, M Hynes, A Rosenthal, unpublished data).

Conclusions

In summary, it appears that the secreted factors Shh and FGF8, which are expressed in the MHB, are both necessary and sufficient for the specification of both DA neurons in multiple regions anterior to the MHB and a subset of 5HT neurons. In addition, the intracellular signaling molecule Nurr1 and the secreted factor TGFβ have been shown to be necessary for the development, or maturation, of midbrain DA neurons, but not yet shown to be sufficient to cause naive progenitors to adopt a DA cell fate. Finally, DA neurons can be expanded or induced in dissociated cultures by cytokines and growth factors; however, a requirement for these factors during the normal course of the development of DA neurons has not been demonstrated. Future studies will focus largely on factors that control the multiple steps towards maturation of DA neurons and specification of the various subtypes of DA neurons as well as factors that influence the morphology, axon outgrowth and synaptogenesis of these neurons.

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