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Review

Development of dopaminergic neurons in the mammalian brain

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Abstract. Dopaminergic neurons in the mammalian brain have received substantial attention in the past given their fundamental role in several body functions and behaviours. The largest dopaminergic population is found in two nuclei of the ventral midbrain. Cells of the substantia nigra pars compacta are involved in the control of voluntary movements and postural reflexes, and their degeneration in the adult brain leads to Parkinson's disease. Cells of the ventral tegmental area modulate rewarding and cognitive behaviours, and their dysfunction is involved in the pathogenesis of addictive disorders and schizophrenia. Because of their clinical relevance, the embryonic development and maintenance of the midbrain dopaminergic cell groups in the adult have been intensively studied in recent years. In the present review, we provide an overview of the mechanisms and factors involved in the development of dopaminergic neurons in the mammalian brain, with a special emphasis on the midbrain dopaminergic population.

Key words. Development; dopamine; neuron; midbrain; forebrain; substantia nigra; ventral tegmental area.

Introduction

Localization and function of dopaminergic neurons in the adult mammalian brain

Dopamine (DA) is one of the catecholaminergic neurotransmitters of the vertebrate central nervous system (CNS), where it is synthesized in a common biosynthetic pathway as a precursor to noradrenaline and adrenaline. Tyrosine hydroxylase (Th) is the first and rate-limiting enzyme in this pathway that converts the essential amino acid tyrosine to L-dihydroxy-phenylalanine (L-DOPA). L-DOPA is then decarboxylated by the enzyme L-aromatic amino acid decarboxylase (Aadc/Ddc) to produce dopamine. Expression of Th has therefore been widely used as a molecular marker for DA-synthesizing (or dopaminergic, DA) neurons. Much attention has been paid in recent years to the population of DA neurons in the mammalian brain given their pivotal role in the control and modulation of motor and endocrine functions, and affective and cognitive behaviours. The cell bodies of these neurons are found in stereotypic positions within the mammalian brain, as listed below from caudal to rostral (fig. 1):

1) DA neurons of the ventral midbrain (mesencephalon) are arranged in two distinct nuclei: the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA); and in the retrorubral field (RrF). Historically, these DA cell groups were designated A8 (RrF), A9 (SNc) and A10 (VTA) [1–3]. In the rat brain, the midbrain dopaminergic (mDA) cell group is made up of around 45,000 neurons, whereas in the human brain, it consists of approximately 590,000 neurons in the first four decades of life [4].
2) Another population of DA neurons is found in the diencephalon and comprises the A11–A15 groups consisting of approximately 1000 cells. The largest groups are the DA neurons of the posterior hypothal-

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amus (A11) and of the zona incerta in the ventral thalamus (A13) [5; reviewed in ref. 6]. Some authors consider the A15 group to be part of the telencephalic DA population [for example ref. 7, 8].

3) Finally, a small DA cell population is found in the telencephalon, comprising the A16 group of olfactory bulb periglomerular interneurons and the A17 group of retina amacrine interneurons.

Besides occupying stereotypic positions in the brain, all DA cell groups described above have characteristic projection areas (fig. 1) [1, 3, 9]. Thus, the neurons of the SNc (A9 group) innervate the dorsolateral striatum and caudate putamen forming the so-called nigrostriatal pathway. Neurons of the VTA (A10) and RrF (A8) project to the ventral striatum (nucleus accumbens, amygdala and olfactory tubercle) as part of the so-called mesolimbic system and establish additional ascending connections to the prefrontal cortex (the so-called mesocortical system). The DA cells of the diencephalic A11 group (posterior hypothalamus) send major descending projections to the autonomic areas of the lower brain stem and spinal cord whose function is not well understood. The incertohypothalamic DA neurons from the A13 group (zona incerta) project diffusely to different areas of the hypothalamus and to the amygdala, whereas the neurons of the A12 (arcuate nucleus) and A14 (para- and periventricular hypothalamic nucleus) groups innervate the median eminence of the hypothalamus and the pituitary gland as part of the tuberohypophysial/tuberoinfundibular system. The connectivity of the A15 cell group (lateral and ventral hypothalamus) is not yet established. The telencephalic A16 and A17 DA cell groups, in contrast, make locally restricted connections as periglomerular and amacrine interneurons, respectively.

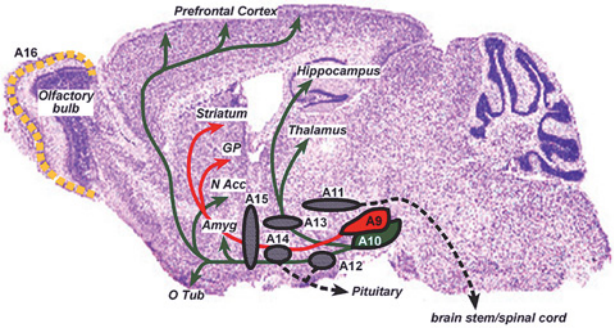

Figure 1. Positions and axonal projections of the different DA cell groups in a sagittal view of the adult rodent brain. The mesencephalic A9 (SNc, red) and A10 (VTA, green) groups project to the dorsolateral striatum (caudate nucleus and putamen) and globus pallidus (GP), or to the ventromedial striatum (nucleus accumbens, N Acc), amygdala (Amyg), olfactory tubercle (O Tub) and prefrontal cortex, respectively. Additional connections from the A10 (VTA) group are established with thalamic and hippocampal regions. The approximate location of the diencephalic A11–A15 groups of the hypothalamus/ventral thalamus (grey dotted fields) and their descending projections into the brain stem/spinal cord are indicated by dashed lines as they do not colocalize within the same section. Innervation of the anterior pituitary by the tuberohypophysial/tuberoinfundibular system is indicated by a dashed line and arrow. The telencephalic A16 group consists of periglomerular interneurons within the olfactory bulb (yellow dashed line). The A8 (retrorubral field) and A17 (retina amacrine interneurons) DA groups as well as the local hypothalamic projections of the diencephalic groups have been omitted from the picture.

Each DA cell group is integrated in the control and/or modulation of specific brain functions according to its distinct projection fields. The DA neurons of the SN innervating the striatum (nigrostriatal system) are integrated in a complex network including other subthalamic and cortical areas that control voluntary movement and body posture. The mesocortical and mesolimbic systems, on the other hand, are involved in the modulation and control of cognitive and emotional/rewarding behaviours. The incertohypothalamic and tuberoinfundibular/-hypophysial systems of the diencephalic DA cell groups function in neuroendocrine control, such as regulation of gonadotropin-releasing hormone (GnRH) and prolactin secretion [10; reviewed in ref. 6].

Dysfunction and/or degeneration of DA neurons results in human disease

Because of their important regulatory and modulating functions in the brain, different severe human diseases are associated with the specific loss of DA neurons or the dysfunction of DA pathways in the brain. Hyperprolactinaemia (the excess of prolactin secretion by the pituitary lactotrophs), for example, is often found to be the cause of female infertility [11] [reviewed in ref. 6]. DA released from the neurons of the tuberoinfundibular system downregulates prolactin secretion by the lactotrophs, and a loss of this inhibitory DA regulation is thought to promote hyperprolactinaemia at least in part [6]. Degeneration of the DA neurons in the SNc and concomitant loss of DA innervation of the striatum leads to the characteristic symptoms of Parkinson's disease (PD), namely bradykinesia/hypokinesia, resting tremor and rigidity [12–14]. PD is one of the most common neurodegenerative disorders worldwide affecting about four million people [14], thus highlighting the importance of a normally functioning nigrostriatal system in the human brain. Dysregulation of DA synaptic transmission in the mesolimbic system has been linked to the development of drug addiction (increased DA transmission) [15–17] and depression (decreased DA transmission) [18], whereas an exaggerated forebrain (mesocorticolimbic) DA transmission is thought to contribute to the psychotic symptoms of schizophrenia (delusions and hallucinations) [19]. Correspondingly, many psychotic drugs increase the extracellular DA content, and the antipsychotic action of different psychopharmacological agents such as neuroleptics re-

sides in a blockade of DA transmission on the receptor/receiving side [13]. In contrast, many antidepressants also act on the DA system by increasing DA transmission within the mesocorticolimbic system [18]. Drug abuse and depression meanwhile constitute two of the major public health problems in industrialized countries. The worldwide incidence of schizophrenia remains constant at about 1%. It thus becomes evident that the mDA neurons play the most prominent role in the brain, as all four disorders (PD, addiction, depression and schizophrenia) are linked to a degeneration or dysfunction of the ventral mDA cell groups of the SNc and VTA. This neuronal population has therefore also long been in the focus of clinical interest. Current treatments of these disorders, however, are still symptomatic, and prophylactic/preventive approaches are not yet available. Therapeutic strategies for PD, for instance, include drug treatment (administration of L-DOPA, DA receptor agonists, anticholinergic and -glutamatergic drugs) and stereotaxic surgery (pallidotomy/thalamotomy or deep brain stimulation) [12, 20, 13]. A restorative/regenerative strategy for PD, consisting in the replenishment of the degenerating mDA neurons and their striatal projections by healthy neurons that were generated either *in vitro* or *in vivo*, has also received much attention. This has been accomplished so far by grafting DA-producing cells of fetal origin into the striatum of PD patients. The technical and ethical problems encountered in these trials, however, have prevented a wider implementation of this technique [21]. More recently, grafting of in-vitro-differentiated stem cells has been proposed as the better option for regenerative therapies [22]. To this end, a full understanding of the molecular and cell-biological cues directing the differentiation programme of an uncommitted stem cell into a mature mDA neuron is required. The mechanisms underlying mDA neuron development have therefore been the subject of intensive research in recent years.

Several excellent reviews have appeared covering the development of mDA neurons [23–29]. We will therefore restrict ourselves to the most important findings in this field concerning the factors and mechanisms controlling mDA neuron development, with a special emphasis on the most recent advances and publications.

Development of the midbrain DA neurons

Time course of mDA neuron development

The precise time point of origin of the first postmitotic mDA neurons is still a matter of debate. Using a special fixation procedure, Di Porzio et al. [30] reported the first appearance of Th-positive cells in the ventral midbrain at embryonic day (E) 9.0–9.5 of mouse development [24]. It has meanwhile become widely accepted, however, that the first mDA neurons are born around day E10.5 of mouse development [27, 28]. At this time point, these cells are characterized by expression of the nuclear receptor family member Nr4a2 (also known as Nurr1) and should be rather considered as postmitotic mDA precursors, since these are not yet fully differentiated mDA neurons [31]. In a strict sense, as soon as these precursors start expressing the rate-limiting enzyme for DA synthesis, Th, they can be considered capable of synthesizing DA and thus being truly DA neurons. This happens 1 day later in embryonic development at E11.5 in the mouse [31]. mDA neurogenesis peaks at around day E12.5 of mouse development and declines thereafter [32, 33]. Using a combined technique of [^{3}H]thymidine autoradiography for birth-dating and Th immunostaining for identification of the DA cells, the different mDA cell groups have been reported to be generated at slightly different time points [32]. The neurons of the SNc and RrF are generated first between E10 and E13 (peaking at E11 and E12), whereas the neurons of the VTA are generated approximately 1 day later between E10 and E14 (peaking at E12 and E13). Furthermore, based on their final position in these areas, there is apparently a rostrolateral (early) to caudomedial (late) gradient in neurogenesis of the mDA neurons [32]. The time course of mDA neuron development is conserved between mouse and rat, taking into consideration that mouse development precedes that of rats by approximately 1 to 2 days. As soon as the first Th-expressing neurons can be detected in the ventral midbrain, these cells already extend some neurites (fibres) in the direction of their migratory pathways and their projection areas in the forebrain [34]. In analogy to the developing rat embryo [35], the first dopaminergic fibres can be assumed to reach their target area in the striatum 1 day after Th immunoreactivity can first be detected in the mouse ventral midbrain (i.e. at E12.5), although a precise temporal mapping of these events has not been performed in the mouse embryo. Migration of the DA neurons to their final positions in the ventral mesencephalon and innervation of their target fields takes place during the subsequent days of embryonic development and the first postnatal weeks. In the rat, this aspect of mDA neuron development which can also be considered as 'maturation' of the mDA system appears to be finished around the third postnatal week [35]. At this time point, the mDA system has acquired its adult morphology and functionality.

What are the progenitors for the Nr4a2-expressing mDA precursors? The only presently available marker for this progenitor population is the retinaldehyde dehydrogenase Aldh1a1 (also known as Raldh1). Its expression is first detected at E9.5 in the cephalic flexure of the mouse embryo and persists throughout development into postnatal stages [31]. At these later stages, *Aldh1a1* is expressed in postmitotic Th-positive neurons of the SNc [31, 36]. A functional implication of this gene in mDA neuron development *in vivo*, however, is still missing, as no loss-of-

function data are available. Retinaldehyde dehydrogenases (Raldhs) catalyze the oxidation of retinaldehyde into retinoic acid (RA). Participation of RA signalling in mDA neuron development is still controversial. Biochemical analyses have revealed a high RA-synthesizing activity in embryonic and adult mouse striatum most likely derived from Aldh1a1-expressing dopaminergic terminals in this region [37]. Studies of an RA-responsive reporter transgenic mouse line on an Aldh1a2 (the prominent Raldh activity during early mouse embryogenesis) null mutant background, in contrast, did not reveal any reporter activity in the ventral midbrain of transgenic embryos [38]. RA signalling, however, is very likely involved in DA function and homeostasis of the adult brain, as compound mouse mutants for the two RA nuclear receptor families, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), show reduced levels of D1 and D2 dopamine receptor expression in the ventral striatum and impaired locomotion and responses to cocaine [39].

One important aspect in the time course of mDA neuron development, as in many other developing neuronal populations, is ontogenic cell death [reviewed in ref. 40]. A natural cell death event has been reported to occur in the SN in both rats [41] and mice [42]. Although the precise onset of this event during ontogenesis was not established, it happens largely postnatally in a biphasic manner [42, 43]. The first peak occurs just after birth and the second minor one in the second postnatal week. Based on different histological and immunochemical analyses, this natural cell death event is of apoptotic nature (programmed cell death) [40, 43]. Furthermore, specific overexpression of regulatory components of the intrinsic apoptotic pathway such as Bcl-2 within catecholaminergic neurons leads to a suppression of natural cell death in the SNc during development, followed by an increase in the adult number of DA neurons in the SNc [42]. Consistent with the classic neurotrophic factor concept, this natural mDA cell death is assumed to be regulated by interaction of the mDA neuron terminals with their target, the striatum [reviewed in refs. 40, 44]. One potent target-derived neurotrophic factor for mDA neurons *in vivo* is the glial-cell-line-derived neurotrophic factor (GDNF) [45, 46]. Other members of the GDNF family such as neurturin (NRTN) also act as potent local (ventral midbrain) and target-derived (striatum) neurotrophic factors for developing and adult mDA neurons [47]. A cell-autonomous (i.e. within the producing cells themselves) requirement of a member of the neurotrophin family, brain-derived neurotrophic factor (BDNF), for the proper maintenance of SNc DA neurons in postnatal mice has recently been shown using a transgenic approach [48]. Because of the early postnatal lethality of the GDNF and neurotrophin family null mutant mice, understanding their precise role in mDA neuron development and survival has remained a difficult task that can be solved by conditional mutagenesis. However, it is becoming increasingly clear that multiple survival-promoting factors most likely acting simultaneously and/or sequentially are required for the correct establishment and maintenance of the mDA neuronal population during development and in adulthood [reviewed in ref. 44].

The precise spatial origin of the mDA neuronal population during development is not yet clear

As mentioned before, Aldh1a1-expressing mDA progenitors are first detected in the cephalic flexure (corresponding to the ventral mesencephalon) of the E9.5 mouse embryo [31]. Two days later, at E11.5, the first Th-positive mDA neurons are detected in the same region [31]. The first Th-immunoreactive cells have been reported to arise in the mediobasal part [the floor plate (FP)] of the mesencephalon [34]. Birth-dating experiments to follow the migratory pathway of these cells revealed that they were born in the ventricular zone (VZ) from where they migrated radially out towards the pial surface of the ventral midbrain [34]. Once they reached the marginal zone of the mesencephalon, the Th-expressing neurons moved laterally parallel to the pial surface to populate their final destinations in the SN and VTA [34]. This biphasic migratory path of mDA neurons is also called the 'inverted-fountain model' and may be paralleled by a rostrocaudal movement or displacement of these neurons [32]. In the light of more recent findings, the 'inverted-fountain model' has been questioned in favour of the 'corona model' [49, 50]. According to the latter model, mDA precursors are born in the subventricular zone (SVZ) of the ventral midbrain and migrate radially towards the pial surface. Neurons generated from more lateral positions of the neural tube migrate perpendicularly to form the SNc, whereas the VTA neurons originate from the medial part (FP) of the ventral midbrain [50].

Another unclear issue is the phylogenetic origin of the mammalian mDA neuronal population [reviewed in refs. 4, 51]. While the DA cell groups of the retina, olfactory bulb and hypothalamus are found at appropriate positions in all vertebrate species studied so far, the mDA neurons of the SN and VTA appear to be a more recent acquisition of the avian and mammalian brain [51, 52]. Ancestral chordates like *Ciona intestinalis* and amphioxus and lower vertebrates (teleost fish and amphibians) lack any DA neurons in what can be considered their midbrain field [53–55]. However, these organisms possess DA cells in their hypothalamic (diencephalic) field, which, based on their ontogeny, projections and functions, can be considered homologous to the mammalian mDA neurons, especially to those of the SN [54–57]. Importantly, cartilaginous fish have mDA neurons [51]. Interestingly,

even during early neural development of the mouse embryo, the first *Th*-expressing cells of the 'midbrain'-DA system are detected in a territory corresponding to the caudal diencephalon (prosomeres 1 and 2)/rostral midbrain [8]. Thus, the mDA neuronal population may have been acquired secondarily during vertebrate evolution at the same time as the basal ganglia and the cortical areas innervated by these neurons enlarged [4], and the first mDA precursors emerging in the mammalian brain may therefore recapitulate their phylogenetic origin during their ontogeny.

The position of the mid-/hindbrain boundary determines the size and location of the mDA cell population

Even though the position in the ventral midbrain from which the mDA neurons of the SNc and VTA originate is not yet clear, these cells do develop in close vicinity to two important signalling centres of the mouse embryo (fig. 2). These are the ventral midline of the neural tube or FP, and the boundary between the embryonic mid- and hindbrain (the mid-/hindbrain boundary, MHB, or isthmus). The cells comprising the FP secrete the protein Sonic hedgehog (Shh) along almost the entire length of the neural tube except its most anterior part belonging to the ventral telencephalon. Shh has been shown to control the induction of most ventral cell types of the hindbrain and spinal cord by a relay mechanism involving other homeodomain transcription factors [reviewed in ref. 58]. It is therefore considered to be the key signalling molecule conveying cell identity along the dorsoventral (DV) axis of the neural tube. The MHB is established in molecular terms at early stages of neural development by the opposing expression domains of two transcriptional repressors: Otx2 in the presumptive fore- and midbrain, and Gbx2 in the presumptive hindbrain and spinal cord. Expression of different genes belonging to the Pax and Engrailed (En) families of homeodomain transcription factors and to the secreted members of the Wnt and fibroblast growth factor (Fgf) families, Wnt1 and Fgf8, is later initiated in a precise spatiotemporal order at or across this boundary [reviewed in refs. 59–62]. Transplantation studies revealed that isthmic tissue can induce an ectopic cerebellum and midbrain when grafted into the caudal forebrain [63, 64], and this effect can be mimicked by implantation of Fgf8b-soaked beads [65, 66]. The MHB is therefore also called the isthmic organizer (IsO), and Fgf8b is the key signalling molecule of this organizing centre with patterning activity on its own along the anteroposterior (AP) axis of the neural tube. As will be described below, both Shh and Fgf8 were implicated as two of the secreted factors required for specification of the DA phenotype in the ventral midbrain and forebrain [67]. More recent data have demonstrated that the position of the MHB along the neuraxis indeed controls the location and size of the mDA and rostral hindbrain serotonergic (rh-5HT) neuronal populations [68]. The latter population normally arises immediately caudal to the MHB in the ventral hindbrain, while mDA neurons are specified rostral to the MHB (fig. 2). Shifting the MHB caudally by ectopically expressing *Otx2* in the rostral hindbrain ($En1^{+/Otx2}$ knock-in mice) enlarges the mDA neuronal population to the new posterior border of *Otx2* expression at the expense of the rh-5HT neurons [68]. Shifting the MHB rostrally into the forebrain by reducing the Otx dosage in the brain ($Otx1^{-/-}$; $Otx2^{+/-}$ mice) repositions the mDA population at the anterior border of the new MHB so that they are now ectopically located in the forebrain, and increases the size of the rh-5HT population which

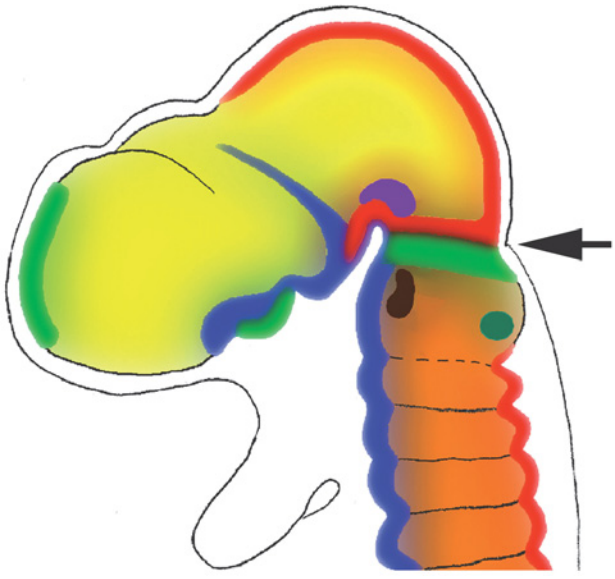

Figure 2. mDA neurons develop close to two important signalling centres of the mouse embryo. Sagittal view of an E10.5 mouse embryo head; anterior is to the left. Sonic hedgehog (Shh, blue) is secreted from the FP of the spinal cord and hindbrain, and from the floor/basal plate of the midbrain and caudal forebrain, thus establishing a ventrodorsal gradient that confers dorsoventral positional information. The mid-/hindbrain boundary (MHB) (black arrow) or isthmic organizer is established at the expression border of Otx2 (yellow) in the fore- and midbrain and Gbx2 (orange) in the hindbrain and spinal cord. Expression of the secreted factors fibroblast growth factor 8 (Fgf8, green) and Wnt1 (red) is confined to the caudal border of the MHB in the rostral hindbrain, ventral telencephalon and anterior neural ridge (Fgf8), and to the rostral border of the MHB in the caudal midbrain, dorsal midline of the midbrain and caudal diencephalon, ventral midline of the midbrain (cephalic flexure) and dorsal midline of the hindbrain and spinal cord (Wnt1), respectively. Signals provided by the MHB confer anteroposterior positional information. The mDA precursors (violet) are induced within the cephalic flexure (ventral midbrain) by a combination of Fgf8, Shh and Wnt1 signals. Two additional neuronal populations develop close to the MHB in the ventral hindbrain (rh-5HT neurons of the raphe nuclei, brown) or dorsal hindbrain (NA neurons of the Locus coeruleus, turquoise).

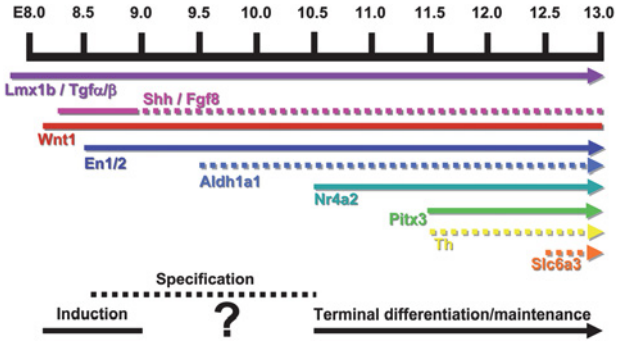

Figure 3. Time-scale of the early development of mDA neurons and factors that have been implicated in their induction and differentiation/maintenance. The scale at the top of the figure illustrates the first five days of mouse neural development, starting at E8.0 when neurulation initiates shortly after gastrulation, and ending at E13.0, when the majority of mDA neurons have been generated (see text for references). The colour-coded bars below indicate the onset of expression of the corresponding secreted or transcription factor, enzyme or transporter protein according to the time-scale at the top, and the time interval during which the corresponding protein is assumed to act in mDA development. Solid bars indicate that the corresponding molecule is necessary for the normal development of mDA neurons during that time interval, and arrows indicate that the corresponding protein is expressed beyond midgestation into later fetal and postnatal stages and may also be required at these later stages. Dotted bars indicate that a direct requirement of the corresponding protein for mDA development has not yet been demonstrated (Aldh1a1), or that the mDA neuronal population still develops in a normal fashion even in the absence of the corresponding protein (Th and Slc6a3). The time intervals during which induction of mDA progenitors, specification of the mDA cell fate in precursors, and terminal differentiation/maintenance of postmitotic mDA neurons in the mouse ventral midbrain are suggested to take place are depicted at the bottom of the figure. The question mark points to our still very rudimentary knowledge of the processes and factors linking early inductive signals to the molecular network controlling the differentiation and maintenance of mDA neurons in the mammalian brain. Abbreviations: Tgf, transforming growth factor; Shh, Sonic hedgehog; Fgf, fibroblast growth factor; En, engrailed; Aldh1a1, aldehyde dehydrogenase 1 family, member a1; Nr4a2 (Nurr1), nuclear receptor subfamily 4, group a, member 2; Th, tyrosine hydroxylase; Slc6a3 (dopamine transporter), solute carrier family 6, member 3.

now extends rostrally to the posterior border of the new MHB [68]. This finding reinforced the idea that signals coming from the MHB together with signals from the FP confer positional cues for the specification of the mDA neuronal population in the ventral midbrain. Later work extended this idea by showing that the Otx dosage itself controls the DV and AP patterning of the midbrain [69–71]. It does so by controlling the DV extent of the *Shh* domain [which in the midbrain under normal conditions is expanded dorsally to include not only the FP but also the basal plate (BP) of the neural tube] and of members of the Shh signalling cascade as well as the DV positioning of certain members of the Shh-responsive class II homeobox transcription factors, such as Nkx2-2 and Nkx6-1, in the ventral midbrain [69, 70]. Furthermore, and as was expected from previous results, the Otx dosage is critically involved in the precise AP positioning of the MHB, especially of the expression domains of some genes transcribed at the MHB such as *Fgf8* and *Wnt1* [68–70, 72, 73]. These latter results, on the other hand, have somewhat compromised previous findings about Fgf8 and Shh as being key components of the mDA cell fate specification pathway. Instead, they suggest that specification of the mDA cell fate is a rather intricate process based on the interaction of several regulatory genetic networks in one and the same cell. One may envision this process as taking place at precisely one grid point of a Cartesian coordinate system set up by these diverse interacting networks within the AP and DV axes of the neural tube. This grid point thus has unique characteristics in composition, space and time and determines how, where and when an mDA neuron will be generated. In the subsequent paragraphs, we will describe each of the known cell-intrinsic and extrinsic factors involved in the development of mDA neurons separately, as our current knowledge about the integrated mechanisms acting during early induction and specification of mDA cell fate is far from being complete (fig. 3).

Secreted factors controlling induction, specification and maintenance of the mDA cell fate:

Shh. One of the first factors shown to be able to induce ventral cell types including mDA neurons is the secreted protein Shh. The notion that this protein is the principal ventralizing activity of the neural tube, however, arose from previous experiments using grafts of FP/notochord tissue onto ectopic dorsal locations, removal of the endogenous FP/notochord, or coculture of ventral neural plate explants with exogenous notochord and FP [74, 75]. Grafting of FP/notochord tissue onto ectopic dorsal locations of the neural tube or coculture of neural plate explants (from which the underlying mesoderm/notochord and FP were previously removed) together with exogenous FP/notochord tissue led to the induction of ventral cell types in the explants or at ectopic dorsal locations of the neural tube [74, 75]. Removal of the underlying notochord and of FP tissue, in turn, led to the loss of most ventral cell types and a dorsalization of the neural tube [74]. Since Shh is first expressed in the axial mesoderm (notochord and prechordal mesoderm) and later induced in the overlying ventral midline of the neural plate (the FP cells) [76], it became an obvious candidate to mediate these ventralizing effects. These studies were further refined to show in both mouse and chick that coculture of explants from the presumptive midbrain and exogenous FP tissue leads to the induction of DA neurons in the explant region adjacent to the exogenous FP, and that this effect can be mimicked by purified Shh protein [77–79]. Analyses of

transgenic mice ectopically expressing the bioactive N-terminal part of the Shh protein (*Shh-N* TG mice) or one of the intracellular mediators of the Shh signal, the zinc finger transcription factor Gli1 (*Gli1* TG mice), across the MHB in mostly dorsal parts of the neural tube showed that in both mouse mutants, mDA neurons were ectopically induced in the dorsal midbrain [80]. These findings reinforced the idea that Shh or one of its downstream mediators is sufficient to induce the mDA neuronal fate at dorsal locations in the midbrain. Notably, mDA neurons were not induced in the dorsal hindbrain of these transgenic mice, indicating that other factors must confer AP positional information to their progenitors. Final evidence for a pivotal role of Shh in the induction of the mDA cell fate came from a set of experiments performed with in-vitro-cultured regional explants of early rat embryos [67]. Ventral or dorsal forebrain explants that normally do not generate mDA neurons were cultured in the presence of Fgf8-coated beads alone or together with an Shh-blocking antibody (ventral explants), or in the presence of either Fgf8-coated beads or soluble Shh protein alone or together (dorsal explants) [67]. Ectopic mDA neurons were readily induced in ventral explants (possessing an endogenous Shh source) in the presence of Fgf8-coated beads, but this effect was abolished by the addition of the Shh-blocking antibody. Conversely, ectopic mDA cells were induced in dorsal explants (devoid of an endogenous Shh source) only in the presence of both Fgf8 and Shh together. As will be described in the next paragraph, these experiments also suggested that in addition to Shh, Fgf8 appears to play an important role in the induction of the mDA neuronal fate, probably by conferring the appropriate AP positional information.

Fgf8. The experiments performed by Ye et al. [67] also revealed a requirement for Fgf8 for the induction of ectopic mDA neurons in forebrain explants. In the same study [67], the authors reported that ectopic Th-expressing neurons can be induced in ventral forebrain explants by co-culturing them with isthmic tissue (expressing *Fgf8*), and that the appearance of mDA neurons in ventral midbrain explants can be abolished by a soluble blocking receptor for Fgf8. Altogether, and as mentioned above, these results indicated that a gradient of secreted Fgf8 protein may be providing positional information for the specification of mDA neurons along the AP axis of the neural tube. Fgf8 had not previously been implicated in the development of mDA neurons because of the severe gastrulation defects of $Fgf8^{-/-}$ null mutants leading to their early death (around E9.5) [81], and the progressive tissue loss around the MHB (including the ventral midbrain) in conditional *Fgf8* null mutants [82]. In addition, conditional Fgf receptor (FgfR) mutants did not reveal a requirement for Fgf signal transduction for the development of mDA neurons. Conditional *FgfR1* (one of the three *FgfRs* and the one expressed most prominently in neural tissue [83]) mutants in the MHB region ($En1^{+/Cre}$; $FgfR1^{flox/flox}$ mice) showed a somewhat disorganized but otherwise unaffected mDA population [84]. As indicated above, the role of Fgf8 and Shh in the induction and specification of mDA cell fate has been challenged by findings in mice and other species [85]. First, the presence or absence, precise position and extension of the *Shh* and *Fgf8* domains in the mid- and hindbrain of mutant mice does not always correlate with changes in the generation, location and size of the mDA neuronal population [69, 70]. Second, a thorough analysis of various zebrafish mutants for the Hedgehog (Hh), Fgf8 and Nodal [transforming growth factor-β (TGF-β)] pathways has not revealed a strict requirement of Hh and Fgf8 signalling for the generation of the diencephalic DA populations in the bony fish [86]. Teleost fish lack the mesencephalic DA system but their diencephalic DA system may be considered in part homologous to the mammalian mDA population of the SN [57, 87]. In the zebrafish Hh pathway and Fgf8 mutants, the diencephalic DA populations were generated in initially normal numbers and at unaltered locations, whereas they failed to develop in the zebrafish Nodal pathway mutants [86]. Nodal belongs to the family of TGF-β factors and as will be described in the next paragraph, TGFs have indeed been implicated in the development of mDA neurons in both chicken and mice.

Transforming growth factors. Counting of Th-expressing cells in the ventral midbrain of postnatal and adult $TGF\text{-}\alpha^{-/-}$ knock-out mice revealed a reduction to almost half of the normal number of DA neurons in the SNc of these mutant mice, whereas the cell number in the VTA remained unchanged [88]. This reduction in Th-positive neurons from the SNc of $TGF\text{-}\alpha^{-/-}$ mice was already evident at birth, suggesting that the loss of these cells occurred during development of the mDA system and was probably not only due to a trophic effect of TGF-α on mDA precursors. More recent data have uncovered a requirement for TGF-β for the induction of mDA cell fate during early neural development in chicken embryos and the survival of these neurons at later embryonic stages [89]. Addition of a general TGF-β-neutralizing antibody [90, 91] to chicken embryos at a stage before the mDA phenotype had been specified caused a reduction by approximately 60% in the number of mDA neurons generated 5 days later [89]. The effect of neutralizing TGF-βs was specific for the mDA population, as Th-positive neurons in the diencephalon and in the hindbrain (locus coeruleus noradrenergic neurons) were not affected by this treatment [89]. Addition of the TGF-β-neutralizing antibody to chicken embryos at intermediate stages of development, i.e. after specification of the mDA cell fate in precursors but before the mDA system had acquired its mature Th-positive phenotype, did not cause a significant

reduction in the number of Th-expressing neurons. Even later application of this antibody at a stage when Th-expressing cells had already been generated in the chick embryo, however, lead to a significant loss of mDA neurons [89]. This result suggested that TGF-βs may also act as survival-promoting factors for mDA neurons *in vivo*. In the same paper, the authors show that two TGF-β isoforms (TGF-β2 and TGF-β3) and the TGF-β receptor II (TβR-II) are expressed in the ventral midbrain of rat embryos, and that application of TGF-β3 to rat ventral mesencephalic cell cultures increased the number of Th-positive cells generated in these cultures [89]. The precise role of TGF-βs for the *in vivo* development of mDA neurons in mammals, however, has not yet been established.

One drawback of the secreted factors described above is that they do not appear to provide signals exclusively directed at induction, specification and/or maintenance of the mDA phenotype, since they are expressed in much broader or other areas of the neural tube than just the cephalic flexure of the ventral midbrain. Shh and TGF-βs, for example, are expressed in the notochord and FP along almost the entire length of the neural tube, thus providing a rather 'general' ventralizing signal [76, 92]. Fgf8, on the other hand, is expressed within the rostral hindbrain in a ring encircling the neural tube at the MHB and thus may provide a rather 'symmetrical' signal along the AP axis that could affect the generation of neuronal populations on both sides of the MHB (as was indeed suggested by experiments performed by Rosenthal and colleagues). Keeping with the idea of a Cartesian grid conferring positional information along the AP and DV axes of the neural tube, one would postulate the existence of an 'asymmetrical' signal identifying the ventral midbrain versus ventral hindbrain for the induction of the mDA neuronal fate. This signal, of course, may still interact with or act along with the other factors described above.

Wnt. A good candidate to provide such an 'asymmetrical' signal in the developing midbrain is the secreted glycoprotein Wnt1. *Wnt1* is first expressed in a rather broad domain comprising the presumptive midbrain and later refined to a narrow stripe along the dorsal midline (roof plate) of the midbrain and caudal diencephalon, in a ring at the anterior border of the MHB within the caudal midbrain, and most importantly, in two stripes at both sides of the medial FP within the cephalic flexure (ventral midbrain) [93–96; reviewed in ref. 59]. This latter expression domain makes Wnt1 a very likely candidate to convey at least some of the positional information for the generation of the mDA neuronal population in the ventral midbrain. Despite this striking expression pattern, only recently were Wnt proteins (so far comprising 19 members in vertebrates [97]) shown to promote the mDA phenotype in cultured cells [98]. In this study, conditioned medium from Wnt1-expressing cells increased proliferation and neurogenesis of ventral mesencephalic precursor cells in general, and promoted the generation of Th-expressing cells from Nr4a2/Nurr1-positive precursors in culture, albeit to a lesser extent than Wnt5a. Wnt5a is another member of the Wnt family expressed in the FP and BP of the midbrain [96, 98], overlapping with the region where and at a time when mDA neurons are generated. In the study by Castelo-Branco et al. [98], conditioned medium from Wnt5a-expressing cells did not increase the proliferation of ventral mesencephalic precursors and was more potent than Wnt1 in promoting the generation of Th-positive cells from Nr4a2/Nurr1-expressing precursors. These results were confirmed by using purified Wnt5a protein [99]. The DA-phenotype-promoting activity of the Wnt-conditioned medium was abolished by addition of a Wnt inhibitor, thus indicating specificity of this effect [98]. Since Wnt1 and Wnt5a are considered to belong to two different functional classes of the Wnt family according to the intracellular signalling pathways they activate [96, reviewed in ref. 100], it was of interest to establish which Wnt signalling pathway is responsible for the increment of Th-positive neurons in ventral mesencephalic precursor cultures. At least three different Wnt signalling pathways are known [reviewed in refs. 100–102]: i) the Wnt/β-catenin pathway, also called the canonical Wnt pathway, acts through stabilization of cytoplasmic β-catenin by inhibiting a multiprotein complex including glycogen synthase kinase-3β (GSK-3β). In the absence of a Wnt signal, this multiprotein complex constitutively phosphorylates β-catenin, thereby targeting it for ubiquitylation and proteasomal degradation. In the presence of a Wnt signal, GSK-3β is inhibited and unphosphorylated (stabilized) β-catenin enters the nucleus to initiate transcription of target genes by interacting with LEF/TCF transcription factors. ii) The Wnt/calcium pathway, which has not yet been fully unravelled but includes intracellular calcium release and subsequent activation of calcium-dependent kinases such as protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CamKII). iii) The planar cell polarity (PCP) pathway, which presumably includes signalling via Rho/Rac and c-Jun N-terminal kinase (JNK), but is the least understood pathway. Subsequent work showed that either inhibition of GSK-3β or overexpression of β-catenin in ventral mesencephalic precursor cell cultures increased the number of Nr4a2/Nurr1-positive precursors that differentiated into Th-expressing cells, thus having the same effect as treatment with Wnt1 or Wnt5a [103]. These results indicated that the canonical pathway conveys the Wnt signals for generation of DA neurons *in vitro*.

At present, no other secreted factors are known to be involved in the early induction, specification or differentiation of the mDA neuronal fate. Considerable data, however, have accumulated about trophic factors promoting

the maintenance (survival) of mDA neurons once they have been generated and acquired their full DA phenotype. These factors will not be considered here as they have recently been reviewed elsewhere [44].

All the secreted factors described above bind to specific receptors on the cell surface and initiate an intracellular signalling cascade that ultimately leads to changes in gene activity or expression within the nucleus of the receiving cell. They can therefore be considered as the extracellular 'signalling' part inducing or maintaining a cell-intrinsic genetic programme that in turn controls all aspects of cell fate specification and differentiation. Key components of the cell-intrinsic genetic programme are the nuclear transcription factors. In the next section, transcription factors currently known to be implicated in the control of mDA cell fate specification and neuronal differentiation will be presented.

Transcription factors and nuclear receptors controlling induction, specification and maintenance of mDA cell fate

Nr4a2 (Nurr1). One of the first nuclear effectors that was implicated in the proper development of mDA neurons is the orphan nuclear receptor Nr4a2 (also known as Nurr1) [104–107; reviewed in ref. 108]. The *Nr4a2*/*Nurr1* gene was first identified in 1992 [109] and later described as an immediate early gene that is upregulated under a variety of conditions [110–113; see also references in ref. 108]. *Nr4a2*/*Nurr1* encodes an 'orphan' (as no cognate ligand binds to it) nuclear receptor belonging to the conserved superfamily of ligand-activated transcription factors including the oestrogen, thyroid hormone, vitamin D and retinoid receptors [114, 115]. Despite intensive research, a ligand binding to Nr4a2/Nurr1 has not yet been identified. Instead, recent structural data revealed that the ligand binding domain of Nr4a2/Nurr1 is occluded by hydrophobic amino acid side chains, thus impeding normal binding of possible ligands [116]. Nr4a2/Nurr1 may therefore act as a ligand-independent nuclear receptor, either as a monomer or homodimer, or may heterodimerize with the RXR nuclear receptors [117]. The latter report showed that endogenous (but not yet identified) ligands for RXR are present in the rodent ventral midbrain and are able to activate Nr4a2/Nurr1-RXR heterodimers *in vivo* [117]. One potential RXR ligand purified from ventral midbrain tissue was identified as the polyunsaturated fatty acid docosahexaenoic acid [117, 118]. Furthermore, activation of Nr4a2/Nurr1-RXR heterodimers by cognate RXR ligands promoted survival of mDA neurons in culture [117]. However, Nr4a2/Nurr1 may have an earlier RXR-independent function during development of the mDA system by inducing cell cycle arrest of mDA precursors and their differentiation into mature mDA neurons [119]. The mDA differentiation-promoting activity of Nr4a2/Nurr1 appears to rely crucially on a direct protein-protein interaction with the cyclin-dependent kinase (CDK) inhibitor $p57^{Kip2}$ [120]. This interaction is especially noteworthy since *p57*^{Kip2} seems to be a direct target gene of Nr4a2/Nurr1-mediated transcriptional control and is also expressed in differentiating mDA precursors [120]. Furthermore, *p57*^{Kip2} knock-out embryos phenocopy the *Nr4a2*/*Nurr1* null mutant [120], further indicating a common pathway in mDA development. Nevertheless, the dependency of Nr4a2/Nurr1 mDA-survival-promoting activity on retinoid signalling may be particularly interesting in view of previous results indicating a requirement of retinoid signalling for proper maintenance of the DA system in the adult brain [see above and ref. 39].

Nr4a2/*Nurr1* is strongly expressed in the ventral midbrain from E10.5 on but also in other areas of the fore- and hindbrain of the developing mouse embryo [31, 104, 121]. In the postnatal and adult brain, *Nr4a2*/*Nurr1* expression is still largely confined to the CNS including the DA system [121–124]. Notably, while it is strongly expressed in the mDA neurons of the A8–A10 groups and in DA cells of the posterior hypothalamus (A11 group) and olfactory bulb (A16 group), it has a weaker expression restricted to only a few Th-positive cells in the paraventricular and periventricular hypothalamic nuclei and the arcuate nucleus (A12, A14–15 groups) [124]. Transcription of *Nr4a2*/*Nurr1*, however, appears to initiate only at later postnatal stages in the A12 and A14–16 DA groups, as one report shows *Nr4a2*/*Nurr1* expression only in the mDA and A11 diencephalic cell populations of newborn mice [125]. *Nr4a2*/*Nurr1* is not expressed in the A13 diencephalic DA neurons [124, 125]. Interestingly, only the mDA cell groups show a clear phenotype in *Nr4a2*/*Nurr1*$^{-/-}$ null mutants. The mDA progenitors expressing Aldh1a1 arise normally in these embryos at E9.5–10.5 [31], and mDA neurons are normally born in these mutants at early stages as judged by the expression of another mDA-specific marker, the homeobox gene *Pitx3*, of Engrailed (En) 1 and 2 and of *Lmx1b* (see below) [31, 105, 126]. During later stages of embryonic development, however, the mDA neurons are lost, since none of these markers can be detected at late gestational stages or at birth of the *Nr4a2*/*Nurr1*$^{-/-}$ mutants [31, 105, 127]. Furthermore, expression of genes encoding the proteins required for proper DA synthesis and neurotransmission, such as *Th*, *Aadc*/*Ddc*, vesicular monoamine transporter 2 (*Vmat2*/*Slc18a2*) and the dopamine transporter (*Dat*/*Slc6a3*), is either never initiated (*Th*, *Vmat2*/*Slc18a2*, *Dat*/*Slc6a3*) or lost until birth (*Aadc*/*Ddc*) in the mDA neurons of *Nr4a2*/*Nurr1*$^{-/-}$ null mutants [104–106, 128]. Notably, expression of *Th* is not affected in the diencephalic and olfactory bulb DA neurons (which also express *Nr4a2*/*Nurr1* in the wild type) and in other catecholaminergic populations of the hind-

brain/brain stem of *Nr4a2*/*Nurr1*$^{-/-}$ mutants [104, 106], indicating a specific requirement of Nr4a2/Nurr1 exclusively in the mDA neuronal population for induction and maintenance of these genes. These findings correlated with a complete absence of DA in the striatum of homozygote *Nr4a2*/*Nurr1*$^{-/-}$ and a significant reduction of striatal DA content in heterozygote *Nr4a2*/*Nurr1*$^{+/-}$ mutants, whereas levels of other monoamines (such as norepinephrine and serotonin) were not changed in the mutant brains [104–106]. Although the mDA progenitors and their normally Nr4a2/Nurr1-expressing postmitotic progeny initially appear in the *Nr4a2*/*Nurr1*$^{-/-}$ null mutants, they are very likely to die at later stages of development as judged by an increase in apoptotic cells in the ventral midbrain of the mutant mouse embryos shortly before or at birth [31, 105]. Wallen et al. [31] also reported a failure to innervate the striatal target areas by the mutant mDA cells using a retrograde tracing technique, whereas another report [127] suggested that the nigrostriatal innervation is preserved in the *Nr4a2*/*Nurr1*$^{-/-}$ mutant mice based on an anterograde labelling paradigm. The discrepancies between these two reports with regard to nigrostriatal target innervation could be due to these technical differences [see also ref. 108]. Noteworthy, expression of one essential coreceptor for trophic factors such as GDNF, the tyrosine kinase *Ret*, is also not detected in *Nr4a2*/*Nurr1*$^{-/-}$ null mutant mouse embryos from the earliest time point on [104, 129]. This is of interest since GDNF has been implicated in several studies as one pivotal trophic factor required for the postnatal survival of mDA neurons [see refs. 27, 40, 44 and references therein]. Thus, these results already suggested that Nurr1/Nr4a2 may exert some transcriptional control on the corresponding genes. Several groups then showed that Nurr1/Nr4a2 can indeed bind to specific recognition sites and directly transactivate the *Th* promoter in cultured cells [130–132]. Other factors, however, may cooperate in Nurr1/Nr4a2-mediated transactivation of the *Th* promoter as it was context and cell line dependent [130, 131]. Importantly, transcriptional activation of *Th* by Nurr1/Nr4a2 appeared to occur in an RXR-independent manner [130]. Since similar analyses for the other genes whose expression is not induced in the *Nr4a2*/*Nurr1*$^{-/-}$ mutants (*Vmat2*/*Slc18a2*, *Dat*/*Slc6a3* and *Ret*) have not been reported so far, the only conclusive evidence is that *Th* may be a direct target gene for Nurr1/Nr4a2 but exclusively in developing mDA neurons.

Altogether, analysis of the *Nr4a2*/*Nurr1*$^{-/-}$ null mutants indicated that the orphan nuclear receptor Nr4a2/Nurr1 is not strictly required for the specification and initial birth of mDA precursors but that it is necessary for their proper differentiation and survival at later developmental stages. The latter requirement of Nr4a2/Nurr1 may rely on two functions: (i) Nr4a2/Nurr1 may directly control transcription of some of the genes required for DA biosynthesis and neurotransmission, such as *Th*, *Vmat2*/*Slc18a2* and *Dat*/*Slc6a3*, and failure to produce these proteins in mDA precursors leads to their elimination by apoptosis; (ii) Nr4a2/Nurr1 may control transcription of genes such as *Ret*, which participate in essential signalling pathways required for the maintenance and survival of mDA neurons in the postnatal/adult brain. Disruption of these pathways due to lack of one of its key components may equally lead to the premature death of mDA neurons.

Lmx1b. Another transcription factor which is expressed from early stages on in a rather broad fashion within the developing mouse embryo is the member of the LIM-homeodomain family Lmx1b [28, 126, 133]. *Lmx1b* is expressed in the ventral midbrain and caudal diencephalon of midgestational embryos in a region encompassing the area where mDA neurons develop [126, 133]. However, *Lmx1b* is also transcribed in several other ventral and dorsal domains of the developing CNS at these stages [133]. Strikingly, *Lmx1b* shows a much more restricted expression pattern in the adult rodent brain including the SN and VTA in the midbrain tegmentum [126, 133]. The *Lmx1b* gene was initially not implicated in CNS development as the knock-out suggested a pivotal role of this transcription factor in limb and kidney development [134]. A closer look at the *Lmx1b*$^{-/-}$ mutant mouse embryos, however, showed a lack of Pitx3 expression but not of the other DA markers *Th* and *Nr4a2*/*Nurr1* within the cephalic flexure [126]. Conversely, *Lmx1b* was normally expressed in the *Nr4a2*/*Nurr1*$^{-/-}$ null mutants, but *Th* expression was not detectable in the *Lmx1b*$^{-/-}$ mutant after E16 [126]. The latter finding suggested that *Th*-expressing neurons were generated but lost during later embryonic development in the *Lmx1b*$^{-/-}$ mutants, probably because these neurons did not survive. The absence of Pitx3 expression in the *Lmx1b*$^{-/-}$ mutants was interpreted to suggest that Lmx1b may be a transcriptional activator of the *Pitx3* gene, thus defining a second pathway in mDA cell fate specification which acts independently of the *Nr4a2*/*Nurr1*-dependent neurotransmitter specifying pathway described above [126]. However, development of the midbrain appears to be generally disturbed in the *Lmx1b* knock-out [126], and the defects observed for the mDA neuronal population in this mutant could therefore also be a secondary consequence of the disturbed midbrain patterning. An interesting twist to this story is the fact that *Lmx1b* has been shown to be required for induction and/or maintenance of *Wnt1* expression in the chicken MHB region, but not vice versa [135, 136]. Therefore, the action of Lmx1b on *Pitx3* may be mediated by the secreted factor Wnt1 in a second independent pathway during mDA development (as discussed above) [see also ref. 27].

Pitx3. The paired-like homeodomain transcription factor Pitx3 (formerly called Ptx3) was independently cloned by

two laboratories: one had used a screen for novel bicoid-related homeobox genes in the rat forebrain [137], the other isolated it based on its homology to another member of the Pitx family, Pitx2 (Rieg) [138; reviewed in refs. 28, 50]. Although *Pitx3* is also expressed outside the CNS in the eye lens, tongue, and head muscles and mesenchyme [50, 137–139], it has a very striking expression pattern within the CNS. In the neural tube, *Pitx3* is exclusively transcribed in the cephalic flexure of the mouse embryo from E11.5 on [137]. *Pitx3* expression is maintained throughout development, and in the adult rodent and human brain it is confined to the DA neurons of the SNc and VTA [137, 139, 140]. Whether Pitx3 is expressed by all or only by a subset of the mDA neurons is still a matter of debate. In a paper by Smidt et al. [140], *Pitx3* mRNA and protein were shown to colocalize with *Th* mRNA and protein in all mDA cells of the adult mouse brain. On the other hand, van den Munckhof et al. [141] reported that Pitx3 is expressed by about half of the Th-positive neurons in the VTA and in most of the Th-positive neurons in the ventral tier of the SNc, whereas the dorsal tier of the SNc does not contain Pitx3-expressing cells. A very recent publication may solve this problem [142]. Using a knock-in approach, an enhanced green fluorescent protein (eGFP) reporter was targeted into the *Pitx3* locus so that all coding sequences were replaced, thus creating a reporter mouse expressing eGFP under the control of *Pitx3* regulatory elements and at the same time being a null mutant for Pitx3 protein [139]. Since the heterozygote *Pitx3*$^{eGFP/+}$ animals were viable and normal [139], a fate-mapping analysis was performed on the corresponding embryos. Interestingly, the eGFP (Pitx3)-positive cells (located ventrolaterally) initially segregated from the Th-expressing cells (located dorsomedially) in the most rostral level of the E12.5 mesencephalon (the earliest time point eGFP fluorescence could be visualized), whereas both cell populations partially overlapped at more caudal levels [142]. These different eGFP- and Th-positive domains disappeared as embryonic development proceeded past E14.5, and the authors reported a complete overlap between the eGFP signal and Th expression in the adult midbrain of transgenic mice [139, 142]. These results suggest that the mDA lineage may comprise two ontogenetically different subpopulations, and that Pitx3 is distinctly required in one subpopulation for its proper development.

The most conclusive evidence for a specific role of Pitx3 in mDA neuron development, however, came from the analysis of two mutant mouse lines [140–144]. The *aphakia* (*ak*) mouse is a naturally occurring mutant with defective early eye lens development [145]. Subsequent mapping of the *ak* locus revealed two deletions close to and within the mouse *Pitx3* gene, including 5′ regulatory sequences, the untranslated exon 1 and part of intron 1 [146, 147]. Since no expression of *Pitx3* mRNA and protein could be detected in homozygote *ak*/*ak* mice, this mouse mutant was considered a *Pitx3* null mutant [140, 141, 143, 146]. More recent evidence using a *Pitx3* knock-out mouse (see above), however, suggests that the *ak*/*ak* mouse is a *Pitx3* hypomorph [142]. The first defects in the mDA system were apparent at E12.5 in the *ak*/*ak* mouse embryos. At this time point, the rostrolateral Th-positive domain was missing in the homozygote mutants [140]. This failure became even more evident as embryonic development proceeded. In newborn *ak*/*ak* pups, the Th-expressing DA neurons of the SNc were nearly absent, and Th-positive cells in the VTA were additionally reduced by about 50% in adult *ak*/*ak* mice [140, 141, 143, 144]. Other genes involved in DA synthesis, storage, release and reuptake [such as *Aadc*/*Ddc*, *Vmat2*/*Slc18a2*, DA receptor 2 (*D2r*/*Drd2*) and *Dat*/*Slc6a3*], and in DA neuron development (such as *Nr4a2*/*Nurr1*, *Lmx1b*, *En1*/*2* and *Ret*) were still expressed in the remaining Th-positive neurons of the VTA and SNc in homozygote *ak*/*ak* mutants, indicating that Pitx3 is not necessary for the induction of all these genes [140, 143]. The loss of Th-expressing DA neurons in the SNc and VTA of adult *ak*/*ak* mice correlated with a decreased cell density and increased apoptosis in these areas [140, 141, 143, 144], suggesting that Pitx3 is required for their survival and maintenance in adulthood. As would be expected from the particular expression pattern of *Pitx3* in the brain, neither the diencephalic DA nor other catecholaminergic neuronal populations were affected in the adult *ak*/*ak* brains [143, 144]. Concomitant with the loss of SNc DA neurons, nigrostriatal innervation was lacking and the striatal DA content was drastically reduced in adult *ak*/*ak* mice [140, 141, 143, 144]. Two groups reported a behavioural phenotype in adult *ak*/*ak* mice: van den Munckhof et al. [141] measured a reduced spontaneous locomotor activity in these mice, in agreement with lower overall activity levels in these mice described by Smidt et al. [140]. Notably, only the motor output and not the motor control were affected in the mutants, suggesting that other pathways must be compensating the early loss of the nigrostriatal system in these mice.

The reason for the selective loss of SNc DA neurons in the *ak*/*ak* mice despite the fact that *Pitx3* appears to be expressed by all mDA neurons [139, 140, 142] was still unknown from the previous work. A very recent report using a different *Pitx3* mutant mouse line may shed light on this issue. As has been described above, Zhao et al. [139] generated a *Pitx3* null mutant by knocking out all *Pitx3* coding sequences and at the same time knocking in an eGFP reporter, thus allowing a precise fate-mapping of *Pitx3*-expressing cells in both normal and mutant mice. Notably, besides discovering two ontogenetically distinct subpopulations of mDA cells initially expressing either Pitx3 or Th at midgestational stages, the authors provide a new model for Pitx3 function in mDA development

based on fate-mapping of these two different subpopulations in homozygous *Pitx3*$^{eGFP/eGFP}$ null mice [142]. At E12.5, the number of cells that initiate Th expression in the null mutants is already reduced to almost half of the wild-type numbers, although the number of cells expressing eGFP does not differ between the homozygote mutants and heterozygote controls at this stage. At E14.5, the loss of Th-positive cells in the SNc primordium of the null mutants became even more evident, but the number of eGFP-positive/Th-negative cells was increased in the mutants as compared to the controls in this region. The reduction of Th-expressing DA neurons in the SNc of adult *Pitx3*$^{eGFP/eGFP}$ null mice was even more pronounced and now mirrored by the loss of eGFP-expressing cells in the same area. In addition, the number of eGFP-positive cells appeared to be reduced in the adult *Pitx3*-deficient VTA [142]. The reduction in eGFP-expressing cells in the homozygous mutants correlated with an increased apoptosis of these cells and a diminished cell density in the SNc of newborn pups. The authors deduced from these data that Pitx3 is required specifically in the rostral ventrolateral (SNc) cell population (which express first Pitx3 and later Th) for the initiation and/or maintenance of *Th* transcription, and that failure to induce or downregulation of Th expression eventually leads to the death of these cells. Thus, Pitx3 appears to be required for terminal differentiation of the SNc DA neurons by directly regulating *Th* expression in these cells, but it is not necessary for the generation and/or survival of their progenitors. The more caudal, ventromedial cells of the prospective VTA, in contrast, express Th before initiating transcription of *Pitx3* and therefore are not dependent upon Pitx3 function. To underline their *in vivo* findings, Maxwell et al. [142] performed additional *in vitro* studies by overexpressing Pitx3 in embryonic stem (ES) cells. Using a differentiation protocol, they could show that Pitx3 increases the proportion of DA (Th^+, Dat/$Slc6a3^+$) cells coexpressing Pitx3/eGFP without affecting the total number of cells in the cultures, indicating that Pitx3 has no proliferative but a DA phenotype-inducing effect under these conditions. These results contradict previous findings showing that overexpression of Pitx3 in an adult hippocampal neural precursor cell line did not have any effect on proliferation, differentiation or acquisition of a Th-positive phenotype by these cells [130]. However, analysis of the *Pitx3*$^{eGFP/eGFP}$ null mice suggests that Pitx3, in contrast to Nr4a2/Nurr1, probably has to cooperate with other local factors in order to induce *Th* transcription. The ability of Pitx3 to activate the *Th* gene has been shown *in vitro* by promoter analyses [148, 149].

Summarizing all findings, Pitx3 appears to be required for proper differentiation or maturation of a subset of mDA neurons, namely those that will form the SNc. One of the targets for Pitx3 may be the *Th* gene itself, and failure to induce Th in the differentiating SNc DA neurons eventually leads to their death. Alternatively, Pitx3 may control other yet unknown genes which are also required for proper development of the SNc DA neurons.

En1/2. The *Engrailed* (*En*) gene belongs to the family of homeobox genes originally described in the fruitfly as a so-called selector gene, since mutation of this gene leads to a homeotic transformation of the posterior wing compartment of the fruitfly into an anterior compartment [reviewed in refs. 59, 61, 150]. Subsequently, different orthologues were cloned from many vertebrate species, among them the two mouse orthologues *En1* and *En2*. Their involvement in patterning of the early mouse embryo was shown based on the analysis of mutant mice [reviewed in refs. 59, 150]. The two mouse *En* genes are expressed during early neural development across the MHB in the caudal midbrain and rostral hindbrain, and thus in a region where mDA neurons arise. Furthermore, both *En1* and *En2* continue to be expressed within the SN and VTA of the postnatal and adult mouse brain [93, 150, 151]. It was therefore hypothesized that *En1/2* may also be involved in their induction, differentiation and/or maintenance. To test this hypothesis, single- and double-mutant *En1*$^{-/-}$; *En2*$^{-/-}$ mice were analysed for expression of Th [151–153]. While mDA neurons appeared in the SN and VTA of the single *En1*$^{-/-}$ or *En2*$^{-/-}$ mutants in a comparable way to the wild-type situation, they were completely absent in the *En1*$^{-/-}$; *En2*$^{-/-}$ double-mutant mice [151]. Furthermore, the presence of mDA neurons in compound heterozygote/homozygote mice appeared to depend on *En1* expression in a gene dosage-dependent manner. Thus, the SN and VTA were substantially reduced in *En1*$^{-/-}$; *En2*$^{+/-}$ mice but appeared normal in *En1*$^{+/-}$; *En2*$^{-/-}$ mice. Notably, Th-expressing cells are generated in the ventral midbrain of the *En1*$^{-/-}$; *En2*$^{-/-}$ double-mutant embryos at E11, albeit in reduced numbers [151]. Three days later in embryonic development, at E14, these cells had disappeared. The time course of mDA neuron loss in the *En1*$^{-/-}$; *En2*$^{-/-}$ double-mutant embryos correlates with the increasing expression of *En1/2* in these cells in wild-type embryos [151, 154]. This result indicated that both En proteins together (although they can partly compensate for each other) are required for the proper development of mDA neurons at later stages, i.e. for survival and maintenance of these cells, but not for their induction or initial differentiation. Since no apoptotic cells could be detected in the ventral midbrain of E12/13 *En1*$^{-/-}$; *En2*$^{-/-}$ double-mutant embryos by conventional methods [151], the reason for their disappearance remained unclear from this work. Subsequent *in vitro* studies from the same group using a cell-mixing approach and RNA interference (RNAi) on wild-type and mutant ventral mesencephalic precursor cultures revealed that the En proteins are required cell autonomously (i.e. within the mDA neurons themselves) for survival of these neurons by preventing

their programmed cell death (apoptosis) [154]. In an attempt to identify En1/2 target genes that may play a role in their maintenance function, Thuret et al. [155] isolated three genes in a PCR-based differential display screen. These genes, although displaying a restricted expression pattern in the ventral mesencephalon and mDA population, were not regulated by En1/2 *in vivo*. Therefore, the precise mechanism of En1/2 action in mDA neuron survival and maintenance still remains elusive.

Terminal differentiation and maturation of mDA neurons

So far, we have been describing the transcription factors known to be involved in the induction of a DA neurotransmitter phenotype in the overall mDA population (Nr4a2/Nurr1) or in only a subset of these cells (Pitx3), in the acquisition of an mDA phenotype other than neurotransmitter identity and/or in survival and maintenance of mDA neurons (Lmx1b and En1/2) (fig. 3). Terminal differentiation and maturation of an mDA neuron, however, also includes initiation of the expression of all enzymes, transporters and (autoregulatory) receptors required for the proper synthesis, storage, release and reuptake of DA by these cells [such as Th, Aadc/Ddc, Vmat2/Slc18a2, DA receptor 2 (D2r/Drd2) and Dat/Slc6a3], as well as the proper innervation of their target fields in the forebrain, and establishment of regulatory input circuitries. Due to space limitations, we cannot go into details of these processes in the present review, and the reader is referred to several recently published and excellent reviews [27, 28, 156, 157].

In brief, the complete knock-out of the *Th* gene (which abolishes synthesis of all catecholamines) by conventional mutagenesis revealed an essential requirement of catecholamines for mouse fetal development [158, 159]. Most of the knock-out embryos died at midgestational stages [158] or shortly after birth [159] probably due to cardiovascular failure, but they could be completely rescued by L-DOPA administration to the pregnant females [158]. Since these experiments did not reveal the specific and exclusive requirement of DA, transgenic mice were generated in which *Th* expression was restored in noradrenergic (NA) and adrenergic but not in DA cells [160, 161]. DA-deficient mice were born at expected frequencies and were initially indistinguishable from their wild-type littermates. However, after the second postnatal week, they became hypoactive and growth retarded, and all died by 4 weeks of age. During the last 2 weeks, DA-deficient mice did not eat or drink. Again, they could be rescued by L-DOPA administration [161]. Notably, the mDA neuronal population, their projections and their striatal target area all appeared normal in these mutants, indicating that normal expression of Th is not required for proper development of the mDA system [161]. Nevertheless, due to the severe depletion of DA in the brain of these mice and as would be expected, they showed multiple behavioural abnormalities at juvenile stages [160–163]. In line with these findings, analyses of knock-out mice for all the other genes such as *Vmat2*/*Slc18a2*, DA receptor 2 (*D2r*/*Drd2*) and *Dat*/*Slc6a3* revealed their requirement for a correct DA homeostasis in the brain and, consequently, for the maintenance of appropriate behaviours, but not for the proper development of the mDA neuronal population and their projections [see refs. 28, 157, and references therein].

Normal axonal pathfinding and establishment of the right connections with their target fields in the forebrain are another important aspect of proper mDA neuron development. To date, three mouse mutants have been reported to present defects in the trajectory of ascending mDA projections [7, 164, 165]. One is the natural $Pax6^{sey/Pax6^{sey}}$ null mutant mouse, in which an abnormal positioning of the SNc and VTA neurons in the ventral midbrain close to the diencephalic boundary and a dorsal deflection of the mDA axon bundles were reported [7]. Interestingly, most of the mDA axons reached their targets in the striatum and nucleus accumbens normally in the $Pax6^{sey/Pax6^{sey}}$ mutant embryos. The reason for their dorsal deflection at the mes-/diencephalic boundary was traced back to ectopic expression of the chemoattractive/chemorepellent molecule Netrin-1 in the caudal diencephalon. This defect, however, appeared to be common to all ascending fibre pathways in the $Pax6^{sey/Pax6^{sey}}$ mice, and it was hypothesized that it may be due to the chemorepellent action of Netrin-1 on these fibres [7]. The second mutant presenting with an aberrant mDA axonal trajectory is the $Nkx2\text{-}1^{-/-}$ knock-out [164]. In $Nkx2\text{-}1^{-/-}$ mutant embryos, the majority of ascending mDA axons crossed the ventral midline of the caudal hypothalamus and innervated the contralateral striatum. Notably, the development and positioning of the mDA cell group was not affected in this mutant. The reason for the midline crossing of the DA axons in the $Nkx2\text{-}1^{-/-}$ embryos was discussed as being either a consequence of anatomical defects in the mutants (missing neuroepithelium of the third ventricle in the ventromedial hypothalamus would eliminate a physical barrier for midline crossing) or of reduced or lost expression of two diffusible axon-repellent factors, semaphorin 3A and slit2, in this region of the mutant [164, 166]. Finally, in mice lacking both *slit1* and *slit2*, many but not all ascending mDA axons are directed ventrally from the medial forebrain bundle to the medial basal hypothalamus [165]. Slits act as chemorepulsive factors in axon guidance, and slit1 and slit2 are highly expressed in the neuroepithelium of the third ventricle [165]. Although it remains unclear whether the aberrantly descending axons in the $slit1^{-/-}$; $slit2^{-/-}$ double-mutants cross the midline and project to the contralateral striatum, normal expression of slits appears to be required for the

maintenance of the dorsal position of the ascending DA axonal pathway. These studies show that normal axonal trajectory of the DA system appears to depend on the normal expression of different axonal guidance cues in the mid- and forebrain.

Development of the forebrain DA populations

In contrast to the comparable wealth of data that have accumulated about the development of the mDA neuronal population, much less is known about the ontogeny of the forebrain (diencephalic and olfactory bulb) DA cell groups in mammals. These cell groups have been suggested to be initially specified by the same signals controlling induction of the mDA neuronal population, namely Shh and Fgf8, based on their proximity to signalling centres expressing these two molecules: the anterior neural ridge (ANR) at the anterior end of the telencephalon, from which the olfactory bulbs will later develop, and the zona limitans intrathalamica (ZLI), which delimits the ventral from the dorsal thalamus in the diencephalic field [67; reviewed in ref. 60]. However, not much more evidence has accumulated to sustain these findings, and as has been discussed before, reports from zebrafish mutants and more recently from a thorough analysis of *Th* mRNA expression in the early mouse embryo have argued against this interpretation [8, 86]. In the next two paragraphs, we will briefly present the current knowledge of factors required for the development of the diencephalic and/or telencephalic DA neurons.

The diencephalic DA cell groups (Pax6 and Dlx). First evidence for a possible role of the paired- and homeodomain-containing transcription factor Pax6 in forebrain DA neuron development came from a precise mapping of Pax6 expression in forebrain catecholaminergic (Th-expressing) cell populations of the developing mouse embryo [7]. As shown by the authors, Pax6 is transiently expressed at midgestational/early postnatal stages in a diencephalic A13 DA subpopulation (zona incerta of the ventral thalamus), in the paraventricular hypothalamic A14 group and in the A15 neurons of the supraoptic nucleus. In addition, Pax6 is also expressed in the telencephalic A16 group of the olfactory bulbs (see below). Subsequent analysis of a naturally occurring mouse *Pax6* null mutant, the small-eye (*sey*) mutant, revealed that all Pax6-expressing diencephalic DA cell groups were normally induced but abnormally positioned and highly segregated in $Pax6^{sey/}Pax6^{sey}$ mutant embryos [7]. The most notable defect in this mutant, however, was an aberrant dorsal deflection of the ascending mDA axons described above. Therefore, the authors concluded that Pax6 is not required for the normal generation of the diencephalic DA cell groups but for their normal adhesive properties.

Diencephalic DA cells, at least those of the A13 group in the medial zona incerta, also express the distalless-related homeobox transcription factors of the Dlx family [167]. Expression of Th was completely abolished in the A13 cluster of $Dlx1^{-/-}$; $Dlx2^{-/-}$ double-mutant mouse embryos, concomitant with a loss of Pax6 and Isl1 expression in the same cells [167]. These findings led to the proposal of two parallel but independent pathways acting in the progenitors of this DA cell group, one controlled by Dlx proteins and the other one by Pax6. While Pax6 appears to be required for expression of the LIM-homeodomain transcription factor Lhx1 in the postmitotic progeny, normal activation of *Pax6*, *Isl1* and *Th* in postmitotic neurons requires the continuous presence of Dlx factors in these cells. Since the authors did not investigate the fate of the other diencephalic DA groups in $Dlx1^{-/-}$; $Dlx2^{-/-}$ double-mutants, whether Dlx factors are generally required for the development of this forebrain DA population remains to be established.

The telencephalic DA cells (Pax6). In contrast to the diencephalic DA cell groups, no DA periglomerular interneurons of the telencephalic A16 group could be detected in older $Pax6^{sey/}Pax6^{sey}$ mutant embryos and neonatal pups [7]. DA periglomerular interneurons are born very late at the end of gestation (around E18 in the mouse), and express Pax6 from their birth on [168]. Notably, this DA population is already drastically reduced in $Pax6^{sey/+}$ heterozygote mice, indicating a specific and obligatory requirement of Pax6 for their development [7]. Olfactory bulb periglomerular interneurons continue to be generated throughout adult life and provide therefore a good model for adult neurogenesis [169]. In line with a strict requirement of Pax6 expression in the DA precursors of the A16 group during embryonic development, Pax6 expression was recently shown to be crucial for the generation of neuronal progenitors in the rostral migratory stream (the source of new neurons for the adult olfactory bulb) and for the acquisition of a DA phenotype by their postmitotic progeny [170, 171]. Whether Pax6 is the only fate determinant for these neurons remains to be established.

Concluding remarks

Despite considerable advances in recent years in our understanding of the development of DA neurons in the vertebrate brain and the molecular cues controlling this process, several aspects still remain to be clarified. First, the precise spatial origin of the mDA neuronal subpopulations at birth remains to be established. It is not yet clear

whether some of these subpopulations originate at more rostral and others at more caudal positions in the ventral midbrain. Furthermore, these mDA neuronal subpopulations may initially express different complements of fate determinants and therefore be specified along distinct pathways. In view of the selective degeneration of SNc DA neurons in PD, understanding these differences may also be of high clinical relevance. Tightly linked to the question of their spatial origin is their phylogenetic origin. As mentioned before, lower vertebrates lack an mDA neuronal population, and this cell group appears to be a more recent acquisition during the evolution of birds and mammals. Answering these questions requires a precise ontogenetic fate-mapping of the mDA population across different phyla. Second, the evidence presented in this review reveals that although mechanisms and factors controlling the induction of an mDA-competent field at early neural development on the one hand, and processes and factors controlling the differentiation, maturation and maintenance of postmitotic mDA neurons at later stages of development on the other hand are beginning to be elucidated, we are still far from understanding the identity of the factors and the mechanisms controlling the specification of an mDA neuronal fate in their precursors. Future work will therefore concentrate on unravelling all those processes linking early inductive signals to the molecular network controlling the differentiation and maintenance of mDA neurons in the vertebrate brain. Third, the mammalian mDA neuronal population obviously has been a focus of research given its pivotal role in normal brain functioning and its clinical relevance in disease. Understanding the mechanisms governing the induction, specification, differentiation and maintenance of the other DA cell populations will therefore have to be stressed in the future in order to gain a full picture of DA neuron development and homeostasis in the vertebrate brain. Noteworthy in this respect is a recent publication showing that the adult rat mesencephalic and diencephalic DA cell groups, despite sharing the same neurotransmitter phenotype, were not more closely related to each other in their molecular composition than are the DA and NA neuronal populations [172]. The DA cell groups of the adult rat SN and VTA, in contrast to the diencephalic ones and although they also differ in their cell morphology, target innervation, electrophysiological properties and disease susceptibility, shared more than 99% of their transcripts. Again, these findings suggest that the rodent ventral mDA population shares a common phylogenetic and ontogenetic origin, while the other DA populations of the rodent brain may have diverged at an earlier stage. Fourth, two zebrafish mutations have been reported to affect the normal development of catecholaminergic neurons: the *too few* (*tof*) mutant, which lacks hypothalamic DA and 5HT neurons, and *foggy*, lacking all diencephalic, telencephalic and retinal DA neurons [56]. *Tof* encodes a forebrain-specific zinc finger transcriptional repressor, which is homologous to the mammalian Fezl (forebrain embryonic zinc finger-like protein) [173]. *Foggy*, in turn, encodes a protein related to the yeast transcription elongation factor Spt5 [174]. Whether the mammalian orthologues of these two proteins serve equal functions in DA neuron development remains to be investigated.

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