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Review

Midbrain dopaminergic neurons: A review of the molecular circuitry that regulates their development

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ABSTRACT

Dopaminergic (DA) neurons of the ventral midbrain (VM) play vital roles in the regulation of voluntary movement, emotion and reward. They are divided into the A8, A9 and A10 subgroups. The development of the A9 group of DA neurons is an area of intense investigation to aid the generation of these neurons from stem cell sources for cell transplantation approaches to Parkinson's disease (PD). This review discusses the molecular processes that are involved in the identity, specification, maturation, target innervation and survival of VM DA neurons during development. The complex molecular interactions of a number of genetic pathways are outlined, as well as recent advances in the mechanisms that regulate subset identity within the VM DA neuronal pool. A thorough understanding of the cellular and molecular mechanisms involved in the development of VM DA neurons will greatly facilitate the use of cell replacement therapy for the treatment of PD.

Introduction

In the adult CNS, almost 75% of all dopaminergic neurons reside in the ventral midbrain (VM), with 400,000–600,000 found in the human VM and 20,000–30,000 in the mouse VM (Blum, 1998; German et al., 1983; Pakkenberg et al., 1991). During embryonic development, these DA neurons are generated in the floor plate region of the mesencephalon (Ono et al., 2007), and give rise to three distinct clusters of VM DA neurons which ultimately develop into anatomically and functionally distinct entities termed the A8, A9 and A10 groups. The A9 cluster gives rise to the substantia nigra pars compacta (SNc), whose neurons project to the dorsal striatum via the nigrostriatal pathway. These neurons and their striatal projections are required for the control of voluntary movement, and the loss of these neurons is the pathological hallmark of Parkinson's disease (PD), which is a neurodegenerative disorder characterised by impaired motor function (Lees et al., 2009; Toulouse and Sullivan, 2008). The other groups of DA neurons, the A10 and A8 clusters, develop into the ventral tegmental area (VTA) and the retrorubal field (RRF), respectively, whose neurons innervate the ventral striatum and the prefrontal cortex via the mesocorticolimbic system, and are involved in the regulation of emotion and reward (Tzschentke and Schmidt, 2000). Altered/defective neurotransmission of the mesocorticolimbic DA system has been associated with the development of schizophrenia, drug addiction and depression (Meyer-Lindenberg et al., 2002; Robinson and Berridge, 1993).

Interestingly, the A9 group of SNc DA neurons, which undergo progressive degeneration in PD, are particularly vulnerable to cell death in comparison to the other VM DA neuronal populations (Alavian et al., 2008; Betarbet et al., 2000; Farrer, 2006; McNaught et al., 2004). The anatomical, functional and apparent sensitivity differences between these three populations of VM DA neurons likely results from subtle developmental differences during their ontogeny. However, little is known regarding the molecular mechanisms that regulate the phenotypic and functional diversities between these VM DA neuronal populations. Given the involvement of A9 DA neurons in PD, an intensive research effort over the last five decades has focused on identifying the molecules and mechanisms that regulate their development. This information is vital to advance efforts to generate SNc DA neurons from stem cells for application in cell replacement therapy for PD. Through the mutation of specific genes, and the subsequent analysis of VM DA neurogenesis and development, a number of molecular pathways have been shown to play key roles in the

Abbreviations: A/P, anterior–posterior; BDNF, brain-derived neurotrophic factor; DA, dopaminergic/dopamine; DAT, dopamine transporter; D/V, dorso-ventral; E, embryonic day; *En1/2*, *Engrailed-1/2*; FGF8, fibroblast growth factor 8; Fzd, frizzled; GDNF, glial cell line-derived neurotrophic factor; MFB, medial forebrain bundle; NPs, neural progenitor(s)/precursor(s); NSCs, neuroepithelial/neural stem cells; P, postnatal day; PD, Parkinson's disease; RRF, retrorubal field; Shh, sonic hedgehog; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VM, ventral midbrain/mesencephalon; VTA, ventral tegmental area; VZ, ventricular zone.

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development of VM DA neurons. This review discusses the 'normal' developmental programme that regulates VM DA neurogenesis, including the cellular and molecular determinants involved in their regional specification, induction, differentiation and maturation.

Early patterning of the ventral mesencephalon

The first key steps in VM DA generation are the early patterning events which lead to the formation of the VM region. During gastrulation, the dorsal ectoderm is restricted towards a neural fate in response to signals arising from the Speamann organiser (Harland, 2000; Hemmati-Brivanlou and Melton, 1997; Liu and Niswander, 2005). The resulting neural plate is then subdivided into restricted domains and subsequently closes to form the neural tube, which is specified by graded signals along the anterior–posterior (A/P) and dorso-ventral (D/V) axes (Puelles, 2001; Simon et al., 1995; Ulloa and Briscoe, 2007). The development of the VM region relies on appropriate A/P and D/V patterns of gene expression which are regulated by signals arising from two key structures in the early embryo: the floor plate of the midbrain and the isthmus organiser. Organisation of the VM region is initiated upon formation of these signalling centres.

The floor plate is present along the length of the neural tube and secretes the sonic hedgehog (Shh) signalling protein from around embryonic day (E) 8.5 onwards in the mouse (Echelard et al., 1993; Ho and Scott, 2002; Hynes et al., 1995a). Interestingly, the spatiotemporal expression pattern of *Shh* in the VM has been shown to contribute to the diverse populations of VM DA neurons, with the 'early medial pool' giving rise primarily to VTA, and very few SNc, DA neurons and the 'later intermediate pool' giving rise to DA neurons of all three subgroups, but largely contributing to the SNc (Joksimovic et al., 2009a). In the floor plate, the bHLH (basic helix–loop–helix) transcription factor Hes1 (also expressed by the isthmus organiser) has been shown to suppress proneural gene expression and induce cell cycle exit (Baek et al., 2006; Ono et al., 2010). Null mutation of *Hes1* results in a transient increase in the number of VM DA neurons between E11.5 and E12.5, followed by a significant reduction in their number from E13.5, compared to the wild type (Kameda et al., 2011). Interestingly, another bHLH transcription factor expressed in the floor plate, Nato3, has been shown to repress *Hes1* expression, and mutation of *Nato3* has been shown to result in a reduction in the number of VM DA neurons generated due to unchecked Hes-1-mediated suppression of proneural genes and the induction of cell cycle arrest (Ono et al., 2010).

The isthmus organiser is a unique signalling centre that separates the midbrain from the hindbrain and is necessary for the development of both of these brain regions (Liu and Joyner, 2001; Rhinn and Brand, 2001). The correct positioning of the isthmus organiser at the midbrain-hindbrain boundary is dependent on the mutual repression of two opposing homeodomain transcription factors: *Otx2* and *Gbx2* (Martinez-Barbera et al., 2001). *Otx2* is expressed in the forebrain and midbrain of the developing anterior neural tube (Acampora et al., 1997; Matsuo et al., 1995; Simeone et al., 1992), while *Gbx2* is expressed more posteriorly in the anterior hindbrain (Wassarman et al., 1997). *Gbx2* expression at the posterior border limits *Otx2* expression which creates the sharp boundary between the midbrain and the hindbrain (Millet et al., 1999).

Fibroblast growth factor 8 (FGF8) is a diffusible factor secreted by the isthmus organiser (Rhinn and Brand, 2001), from around E8 until at least E12.5 in the mouse midbrain-hindbrain boundary (Crossley and Martin, 1995). Surprisingly, although *Otx2* and *Gbx2* are critical for the correct positing of the isthmus organiser, they are not required for the expression of *FGF8*, or for the induction of other isthmus organiser-genes, however they are essential for the correct positioning of the expression domains of these genes (Brodski et al., 2003; Liu and Joyner, 2001). This is highlighted by studies showing that if the position of the isthmus organiser is moved caudally as a result of ectopic *Otx2* expression in hindbrain, there is an increase in the number of VM DA neurons (Brodski et al., 2003). Similarly if its position is moved rostrally by depleting *Otx2* in the midbrain, there is a decrease in the number of VM DA neurons (Brodski et al., 2003), demonstrating the critical importance of isthmus organiser positioning for normal VM DA generation.

As *Otx2*- and *Gbx2*-dependent sharpening of the borders of the isthmus is occuring, a second group of transcription factors begin to be expressed in the isthmus organiser. These include the paired box gene *Pax2* (Urbanek et al., 1997), the lim-homeodomain factor *Lmx1b* (Adams et al., 2000; Smidt et al., 2000), the secreted glycoprotein *Wnt1* (Adams et al., 2000; Crossley and Martin, 1995, Davis and Joyner, 1988; Wilkinson et al., 1987), and *Engrailed-1* (*En1*) (Davis and Joyner, 1988). Of these, *Pax2* is required for the induction of *FGF8* expression by the isthmus, whereas *Wnt1* and *En1* function cooperatively with *Otx2* and *Gbx2* to further refine the position of the expression domain of *FGF8* at the isthmus (Ye et al., 2001).

Shortly after the induction and positioning of *FGF8* expression, *Engrailed-2* (*En2*) and *Pax5* start to be expressed in the midbrain-hindbrain boundary. These genes play critical roles in the regional specification of the VM, and homozygous mutant mice null for *Otx2* (Acampora et al., 1995; Ang et al., 1996), *Wnt1* (McMahon and Bradley, 1990; Prakash et al., 2006), *Pax2* and *Pax5* (double mutant) (Schwarz et al., 1997), *En1* and *En2* (double mutant) (Liu and Joyner, 2001; Simon et al., 2001), or *Lmx1b* (Smidt et al., 2000) all display major VM defects, including partial or total loss of VM DA neurons (see Table 1).

Identity of ventral midbrain dopaminergic neural precursors

Once the appropriate patterning of the VM region has occurred, a developmental programme involving a sequential pattern of gene expression establishes the identity of VM DA neural precursors (NPs) that ultimately generate VM DA neurons (Fig. 1). The identity of these VM DA NPs has been the focus of intensive research in recent years, largely due to their potential to be used as a cell source to generate DA neurons for cell replacement therapy in PD (Kim, 2011; Morizane et al., 2008; Toulouse and Sullivan, 2008).

The origin of VM DA NPs has been debated for many years, with regions such as the diencephalon (Marin et al., 2005), isthmus (Marchand and Poirier, 1983) and VM basal plate (Hynes et al., 1995a, 1995b) emerging as potential candidates. Despite this research, the precise identity of VM DA NPs remained elusive until recently, when a study showed that floor plate cells in the murine VM become neurogenic and subsequently give rise to DA neurons (Ono et al., 2007). This discovery was surprising as the floor plate was thought to consist of specialised non-neurogenic glial type cells that were largely involved in ventralizing the neural tube, mainly by secreting Shh (Fuccillo et al., 2006; Jessell, 2000; Placzek and Briscoe, 2005). This role in ventralisation seems to remains the main function for floor plate cells caudal to the midbrain, as the hindbrain floor plate has been shown to be non-neurogenic (Joksimovic et al., 2009b; Ono et al., 2007). However, the VM floor plate is different to its caudal counterparts and attains neurogenic potential. Ono et al. (2007) demonstrated that *Otx2*, which is critical for the positioning of the isthmus organiser, is also essential for the neurogenic potential of VM floor

Table 1
Genetic mutations affecting the development of VM DA neurons.

Mutation(s)	Effect(s) on DA development	Reference(s)
Otx2 –/–	Loss of VM DA neurons (midbrain absent)	Acampora et al. (1995), Ang et al. (1996)
Wnt1 –/–	Severe reduction in VM DA neurons generated	McMahon and Bradley (1990), Prakash et al. (2006)
Pax2 –/–: **Pax5** –/–	Loss of VM DA neurons (midbrain absent)	Schwarz et al. (1997)
En1 –/–: **En2** –/–	VM DA neurons lost by E14 via apoptosis	Alberi et al. (2004), Liu and Joyner (2001), Simon et al. (2001)
Lmx1b –/–	Substantial reduction in VM DA neurons generated, and disappearance of Pitx3^{-} VM DA neurons by birth	Deng et al. (2011), Smidt et al. (2000)
Hes1 –/–	Transient increase (~E12) in VM DA neurons, followed by their significant reduction (from E13.5). Dorsal migration and hindbrain invasion	Kameda et al. (2011)
Nato3 –/–	Reduction in VM DA neurons generated	Ono et al. (2010)
FGFR –/–	Deficit of VM DA neurons	Lahti et al. (2012), Saarimaki-Vire et al. (2007)
FGF2 –/–	Peri-/post-natal increase in VM DA neurons	Ratzka et al. (2012)
Lmx1a –/–	Substantial reduction in VM DA neurons generated	Deng et al. (2011), Ono et al. (2007)
En1$^{Cre/+}$; **Otx2**$^{flox/flox}$	Reduction in VM DA neurons due to failure of VM DA NP induction	Omodei et al. (2008), Puelles et al. (2004)
Ngn2 –/–	Reduction in mature VM DA neurons generated	Kele et al. (2006)
Oc1 –/–: **Oc2** –/–	Reduction in VM DA neurons generated	Chakrabarty et al. (2012)
Gli1 –/–: **Gli2** –/–	Deficient VM DA neurogenesis (more severe than Gli2 –/–)	Park et al. (2000)
Gli2 –/–	Deficient VM DA neurogenesis	Park et al. (2000)
Lmx1a –/–; **Shh**$^{Cre/+}$; **Lmx1b** –/–	Substantial reduction in VM DA neurons generated (more severe than Lmx1a –/–)	Yan et al. (2011)
Lmx1a –/–: **Lmx1b** +/–	Substantial reduction in the generation of VM DA neurons (more severe than Lmx1a –/–)	Deng et al. (2011)
Lrp6 –/–	Delayed differentiation of VM DA neurons	Castelo-Branco et al. (2010), Pinson et al. (2000)
Fzd3 –/–: **Fzd6** –/–	Deficient VM DA neurogenesis (severe midbrain defects)	Stuebner et al. (2010)
Fzd3 –/–	Transient reduction in VM DA neuron generated	Stuebner et al. (2010)
Wnt2 –/–	Reduction in VM DA neurons generated	Sousa et al. (2010)
β-catenin (Th-IRES-Cre; β-Ctn$^{Ex3/+}$ **mutant)**	Reduction in VM DA neurogenesis	Tang et al. (2009)
Dkk1 –/–	Severe loss of VM DA neurons	Ribeiro et al. (2011)
L1 –/–	Positional abnormalities of VM DA neurons	Demyanenko et al. (2001)
Reelin –/–	Failure of VM DA neuron lateral migration	Nishikawa et al. (2003)
DCC –/–	Aberrant VM DA neuron migration, dorsal shifting of ventral striatal DA projections, aberrant crossing of MFB fibres at caudal diencephalic midline, and reduction of prefrontal cortex DA innervation	Xu et al. (2010)
Ebf1 –/–	Impaired tangential migration of VM DA neurons	Yin et al. (2009)
Nurr1 –/–	Lack TH, AADC, VMAT2 and DAT expression in VM DA neurons, and their subsequently loss	Castillo et al. (1998), Filippi et al. (2007), Saucedo-Cardenas et al. (1998), Smits et al. (2003), Wallen et al. (1999)
Nurr1 –/– **(at late stage of DA dev.)**	VM DA neuron degeneration (snc more vulnerable)	Kadkhodaei et al. (2009)
FoxA2 –/–	Increase in numbers of Nurr1^{+} TH^{-} neurons in VM	Ferri et al. (2007)
FoxA2 +/–	Aged mice develop PD-like symptoms and pathologies	Kittappa et al. (2007)
Pitx3 –/–	Deficit of snc DA neurons from E12.5 (VTA largely unaffected)	Hwang et al. (2003), Nunes et al. (2003), Smidt et al. (2004), Van Den Munckhof et al. (2003)
En1 +/–: **En2** +/+	Progressive degeneration of VM DA neurons (8–24 weeks)	Sonnier et al. (2007)
En1 +/–: **En2** –/–	Progressive degeneration of VM DA neurons (more pronounced in snc)	Sgado et al. (2006), Sonnier et al. (2007)
Nkx2.1 –/–	Aberrant crossing of MFB fibres at caudal diencephalic midline	Kawano et al. (2003)
DCC +/–	Increased branching of VM DA fibres in prefrontal cortex	Manitt et al. (2011)
Pbx1a –/–	Partial misrouting of VM DA fibres	Sgado et al. (2012)
Pax6 –/–	Dorsal deflection of MFB fibres in the diencephalon	Vitalis et al. (2000)
Slit1 –/–: **Slit2** –/–	Aberrant crossing of MFB fibres at caudal diencephalic midline	Bagri et al. (2002), Dugan et al. (2011)
Robo1 –/–: **Robo2** –/–	Aberrant crossing of MFB fibres at caudal diencephalic midline, and abnormal dorsal trajectories of VM DA fibres	Dugan et al. (2011), Lopez-Bendito et al. (2007)

plate cells. This finding expands the importance of this gene in determining the overall structure of the VM region.

However, although the region from which VM DA NPs arise has been determined, the specific floor plate cell type which is a DA NP cell remains to be identified. Fate-mapping studies using a marker for radial glia-specific marker GLAST demonstrated that radial glial-like cells in the floor plate of the mouse VM were DA NPs (Bonilla et al., 2008). Shortly thereafter, a similar study using human VM tissue demonstrated that DA NPs in the VM floor plate showed radial glial characteristics, that is they expressed the radial glial markers, vimentin and BLBP, and displayed a radial morphology (Hebsgaard et al., 2009). VM DA neurons therefore arise from floor plate radial glial-like NPs. The radial-glial origin of DA neurons should not be considered peculiar due to its departure from the classical view of radial glia as a supportive glial cell type. Indeed, the separate identities of radial neuroectodermal stem cells and radial glial cells is regularly challenged in the literature, with some authors suggesting that

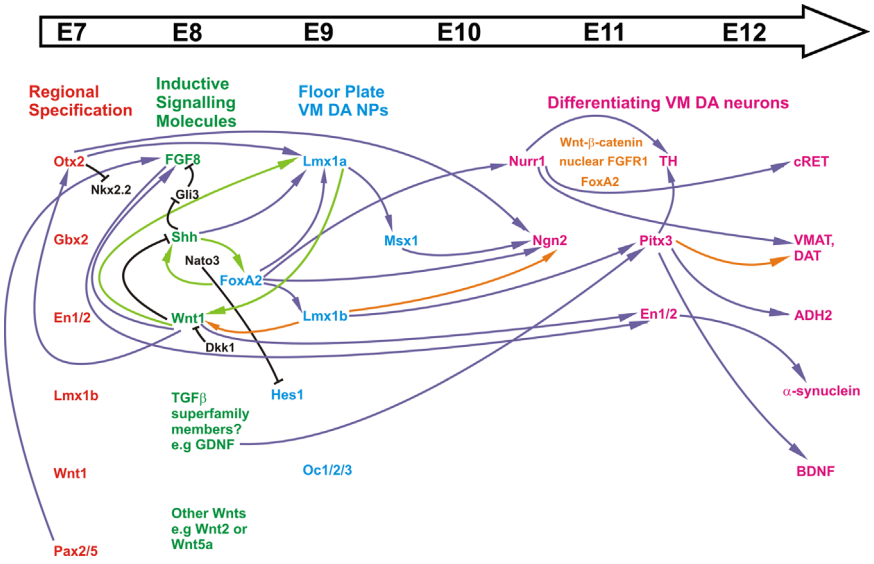

Fig. 1. Molecular factors involved in the development of VM DA neurons. *Molecular interactions in the genesis of VM DA neurons*: The sequence of appearance (see time-course arrow) of each of the factors involved in VM DA neuronal development, and their effects on each other. (Note: the molecules in black text are not shown at the time point at which they appear.) The arrows denote the effect on expression: purple=positive regulation, green=autoregulatory loop, orange=cooperative regulation, and black=negative regulation. The factors are colour-coded as per their role (listed above molecules). Otx2, Gbx2, En1/2, Lmx1b, Wnt1 and Pax2/5 play vital roles in the establishment of the midbrain/hindbrain region, including the isthmus organizer and VM floor plate, and the majority also play direct roles in VM DA neurogenesis. The diffusible signalling factors FGF8, Shh and Wnt1 induce VM DA neurogenesis in radial glial-like floor plate cells, through the induction of FoxA2, Lmx1a/1b, and Msx1 expression. Two autoregulatory loops, Shh–FoxA2 and Wnt1–Lmx1a, contribute to this process, with Hes1 being involved in the Shh–FoxA2 autoregulatory loop, and Lmx1b functioning cooperatively with Lmx1a. The expression of Nurr1 and Pitx3 promotes the differentiation of VM DA NPs into post-mitotic neurons. Nurr1 induces the expression of proteins that are key to the neurotransmitter phenotype of VM DA neurons. A number of factors facilitate the induction of TH by Nurr1 (listed in orange), and Pitx3 has been shown to cooperatively regulate a number of important genes involved in VM DA neurogenesis with Nurr1.

they are in fact the same cell type (Kriegstein and Alvarez-Buylla, 2009).

Induction of a dopaminergic phenotype in ventral midbrain neural precursors

While the floor plate and isthmus organiser are critical determinants of VM patterning, they are also crucial for the induction of a VM DA phenotype. Their role in induction of a DA phenotype is dependent upon the interaction of floor plate-secreted Shh and isthmus-secreted FGF8 (Hynes et al., 1997; Ye et al., 1998).

FGF8

The mechanism by which FGF8 regulates VM DA development is still under investigation, however a recent study suggested that FGF8 is required to induce the correct patterning of VM DA NPs, as the loss of FGFRs (FGF receptors) resulted in altered patterning of the VM and failure of VM DA neuron maturation, with the DA domain adopting diencephalic characteristics (Lahti et al., 2012). In support of this, a similar study that mutated the *FGFRs* reported a reduction in the generation of VM DA NPs, and a disturbance in the maturation of VM DA neurons (Saarimaki-Vire et al., 2007). Another FGF, FGF2, has been shown to function in the regulation of SNc DA NPs proliferation, and also in the developmental cell death of mature SNc DA neurons (Ratzka et al., 2012).

Sonic hedgehog (Shh) signalling and Lmx1 expression

The first sign of a DA phenotype in VM NPs is the initiation of expression of two key determinants of VM DA cell fate, the lim-homeodomain factor *Lmx1a* and the homeodomain transcription factor *Msx1*, at ~E9 in the mouse (Alavian et al., 2008). Shh induces the expression of *Lmx1a*, which subsequently induces the expression of its downstream effector Msx1 (Andersson et al., 2006). The overexpression of *Lmx1a* in the anterior VM results in the ectopic generation of DA neurons, while reduced expression results in a loss of VM DA neurons (Andersson et al., 2006). Additionally, null mutation of *Lmx1a* or the spontaneous mutation of *Lmx1a* in *dreher* mice results in substantial reductions in the numbers of VM DA neurons generated (Deng et al., 2011; Ono et al., 2007). *Lmx1a* expression is maintained in post-mitotic VM DA neurons until postnatal day (P) 180 in mice (Zou et al., 2009); however *Msx1* expression is confined to VM DA NPs (Andersson et al., 2006; Failli et al., 2002). This is surprising considering that *Lmx1a* is upstream of *Msx1*, suggesting that the post-mitotic repression of *Msx1* expression somehow overrides the inductive effect of Lmx1a. *Msx1* contributes to DA neurogenesis by inducing the expression of the proneural gene, *neurogenin* (*Ngn*) *2*, and thus neuronal differentiation. *Ngn2* expression in VM NPs also appears to be under the control of *Otx2* expression, as conditional *Otx2* mutant mice display a loss of *Ngn2* expression in DA NPs (Vernay et al., 2005). This finding is not surprising, considering that *Otx2* induces the expression of *Lmx1a* in VM floor plate cells, suggesting that the loss of *Ngn2* expression in the *Otx2* knockout mouse is due to a failure of *Lmx1a* induction and subsequently *Msx1* expression, rather than a direct effect on *Ngn2* expression, but this remains to be determined (Ono et al., 2007). This suggestion is supported by recent findings showing that in the absence of *Otx2*, VM NPs fail to activate the expression of *Lmx1a*, *Msx1* and *Ngn2*, and therefore largely fail to differentiate into VM DA neurons (Omodei et al., 2008).

Support for the role of Ngn2 in DA induction comes from studies showing that loss of Ngn2 in mice results in a severe reduction in the expression of post-mitotic VM DA markers *Nurr1* and *tyrosine hydroxylase* (*TH*) (discussed later), demonstrating its importance in the generation of mature VM DA neurons (Kele et al., 2006). However, the role of Ngn2 is likely to be generally proneural, rather than specific for VM DA neuronal differentiation.

In support of this, overexpression of *Ngn2* induces neurogenesis but not a DA phenotype in cultured VM NPs (Kim et al., 2007). These data suggest the existence of a developmental programme consisting of an inductive effect of floorplate-secreted Shh on *Lmx1a* expression which subsequently induces the expression of *Msx1*, which in turn induces the expression of *Ngn2* which is required for neuronal differentiation of VM DA NPs. How and where *Otx2* fits into this cascade is unclear, but it is known that *Otx2* is required for the expression of *Lmx1a*. It has yet to be determined whether this is a parallel pathway cooperating with Shh or whether *Otx2* is a master regulator of Shh-induced *Lmx1a* expression. In support of a role of *Otx2* as a master regulator, studies involving the conditional knockout of *Otx2* in the midbrain have suggested that *Otx2* controls the fate of VM progenitors through the repression of *Nkx2.2* and maintenance of *Nkx6.1* expression (Puelles et al., 2004). FoxA2, involved in a feedback loop with Shh (discussed later), induces *Lmx1a* expression and also inhibits *Nkx2.2* (Lin et al., 2009). It is possible that *Otx2* functions via a similar pathway to FoxA2, or indeed that FoxA2 may function downstream of *Otx2* and Shh in the regulation of *Lmx1a* and *Nkx2.2* expression during VM DA neurogenesis.

Interestingly, a recent genome-wide gene expression profiling study has expanded the regulatory role of Lmx1a in this process by identifying novel transcription factors involved in the generation of the VM DA neuronal field. The Oc transcription factors, *Oc1*, *2* and *3*, display similar expression profiles to that of *Lmx1a* in the developing VM, and their loss resulted in diminished generation of VM DA neurons (Chakrabarty et al., 2012). Whether these Oc transcription factors are regulated by Lmx1a, which is plausible given their overlapping expression patterns, or whether they act in parallel to regulate neuronal differentiation in the VM, will be an important question for future research.

Similar to Lmx1a, the related protein Lmx1b has also been shown to promote VM DA neurogenesis (Deng et al., 2011; Lin et al., 2009; Yan et al., 2011). *Lmx1a* and *Lmx1b* are co-expressed in VM DA NPs, and have been shown to mediate the initial steps of NP DA specification (Andersson et al., 2006; Smidt et al., 2000). Furthermore, *Lmx1a* and *Lmx1b* are co-expressed in the P0 VM, suggesting that they may function in the maturation of VM DA neurons also (Zou et al., 2009). Similar to *Lmx1a*, *Lmx1b* can induce the ectopic production of VM DA neurons when ectopically expressed (Nakatani et al., 2010), and its loss results in a substantial reduction in the number of VM DA neurons (Deng et al., 2011; Smidt et al., 2000). A recent study using conditional knockout of *Lmx1a* and *Lmx1b* in mice demonstrated that Lmx1a and Lmx1b function cooperatively to regulate the proliferation of VM DA NPs and Ngn2 expression (Yan et al., 2011). This suggestion is supported by studies on *Lmx1a* null mice carrying one mutant *Lmx1b* allele (as double null mutations are embryonically lethal) which found that *Lmx1a* and *Lmx1b* function cooperatively in the generation of VM DA neurons. This study also showed that *Lmx1b* is involved in the generation of ocular motor neurons and red nucleus neurons in the VM (Deng et al., 2011) and it has been suggested that *Lmx1b* partially compensates for *Lmx1a* function in *dreher* mice, as only 46% of VM DA neurons are lost in these mutants (Ono et al., 2007). It will be important to understand whether Lmx1b exerts its effects in precisely the same way as Lmx1a, for example by modulating *Msx1* expression or that of *Oc1*, *2* and *3* (Chakrabarty et al., 2012).

Sonic hedgehog (Shh) signalling and FoxA expression

Floor plate-derived Shh has been shown to play a key role in induction of a DA phenotype by modulating the expression of the transcriptional regulator *FoxA2*, a well-known floor plate marker. The modulation of *FoxA2* expression is mediated by the downstream effector of Shh signalling, *Gli1* (Hynes et al., 1997). *Gli1* expression is upregulated by a related molecule Gli2 (activator) in response to Shh signalling, which is required for generation of VM DA neurons, while *Gli3* (repressor) is suppressed by Shh to allow the de-repression of *FGF8* expression (Blaess et al., 2006). *Gli2* homozygous null mutants demonstrate the importance of Gli2 in inducing ventral phenotypes, as these mice display clear deficits in VM DA neurogenesis (Park et al., 2000). Loss of both *Gli2* and *Gli1* resulted in a more severe phenotype (Park et al., 2000). *FoxA2*, along with *FoxA1*, is expressed in the VM and in differentiated DA neurons during development. Both have been shown to regulate the expression of *Ngn2* and to maintain the expression of *Lmx1a* and *Lmx1b*, which promotes VM DA neurogenesis (Bayly et al., 2012; Ferri et al., 2007; Lin et al., 2009). As aforementioned, Gli1 has been shown to induce the expression of *FoxA2* (Hynes et al., 1997), with *FoxA2* being reported as a downstream target of Shh signalling (Chung et al., 2009). However, *FoxA2* expression precedes that of *Shh* in the ventral neural tube and is proposed to regulate *Shh* expression (Echelard et al., 1993). These findings likely reflect a regulatory feedback loop between *Shh* and *FoxA2* expression, with Gli1 functioning downstream of Shh in this loop. In addition to Gli1, Nato3, a bHLH transcription factor that contributes to VM DA neurogenesis through the repression of *Hes1* (Ono et al., 2010), has been shown to integrate with the Shh–FoxA2 regulatory feedback loop in the SN4741 dopaminergic cell line (Nissim-Eliraz et al., in press). It has recently been suggested that Shh is necessary and sufficient for lateral floor plate generation, and necessary but not sufficient for medial floor plate generation, while FoxA2 is necessary and sufficient to specify the entire floor plate, acting through both Shh-dependent and independent mechanisms (Bayly et al., 2012). This induction of *FoxA2* expression by Shh has also been proposed to function cooperatively with Lmx1a and Lmx1b in the generation of DA neurons from VM floor plate NPs (Nakatani et al., 2010). This is not surprising, considering that Shh and FoxA2 positively regulate *Lmx1a* and *Lmx1b* expression. *FoxA2* mutant mice have a defective floor plate, as well as notochord, and die at E9.5 (Ang and Rossant, 1994; Sasaki and Hogan, 1994), which precludes examination of their role in DA induction. Given the recent identification of VM radial-glial progenitors, it will be interesting to use targeted strategies to conditionally remove *FoxA2* in the midbrain while preserving its expression in the floor plate (possibly through the use of *GLAST*-cre mice), and thus examine its inductive effect on *Shh* expression and its specific role in DA neurogenesis.

Wnt signalling

The Wnt family of secreted glycoproteins have become increasingly recognised as key regulators of DA neuron induction. *Wnt1* is expressed in the isthmus organiser, in an area rostral to *FGF8* at E9.5 in mice (Wilkinson et al., 1987), and is also expressed in the developing midbrain (Davis and Joyner, 1988; Wilkinson et al., 1987), along with other members of the Wnt family (Andersson et al., 2008; Parr et al., 1993; Rawal et al., 2006). In vitro, Wnt1 has been shown to regulate the proliferation of VM DA NPs and to increase the number of DA neurons generated from these cells. Wnt3a has been shown to enhance VM DA NP proliferation but to inhibit their terminal DA differentiation, whereas Wnt5a regulates the acquisition of a DA phenotype to increase DA neuronal numbers (Castelo-Branco et al., 2003). Wnt5a in particular has recently been demonstrated to play a role in the acquisition of a DA phenotype in VM DA NPs in vivo (Andersson et al., 2008). The effect of Wnt5a on DA differentiation has been suggested to be regulated by the Rac1 guanosine exchange factor, Tiam1 (Cajanek et al., 2013), and Wnt5a has been proposed to be an important mediator of the DA inductive activity of VM glia (Castelo-Branco

et al., 2006). Another Wnt, Wnt2, has been implicated as a novel regulator of VM DA NP proliferation as *Wnt2* null mice displayed reductions in DA neurogenesis (Sousa et al., 2010).

Given that *Wnt1* is expressed in the isthmus and developing midbrain, it is perhaps not surprising that null mice displayed a loss of most of the midbrain and the DA neurons therein (McMahon and Bradley, 1990). Subsequently it was shown that although *Wnt1* null mice develop VM DA NPs, these NPs fail to proliferate and differentiate appropriately, and the few DA neurons that are generated are lost shortly thereafter (Prakash et al., 2006). This is in agreement with data describing Wnt1 as a key regulator of VM DA NPs proliferation and subsequent differentiation (Castelo-Branco et al., 2003). There is now a large body of evidence describing the key role of Wnt signalling in DA generation. Loss of the Wnt receptor *Lrp6* replicates some of the *Wnt1* developmental abnormalities (Castelo-Branco et al., 2010; Pinson et al., 2000). Similarly, loss of the Wnt receptors, *frizzled (Fzd) 3* and *Fzd6*, severely impairs midbrain morphogenesis (Stuebner et al., 2010). Interestingly, the null mutation of *Fzd3* results in a transient reduction in the numbers of VM DA neurons generated, similar to that seen in the *Lrp6* null mutant (Castelo-Branco et al., 2010; Stuebner et al., 2010). Furthermore, the specific inactivation of β-catenin, which mediates canonical Wnt signalling, mimics the midbrain-hindbrain deficits observed in *Wnt1* null mice (Brault et al., 2001; Chilov et al., 2010), suggesting that Wnt1 acts via β-catenin during midbrain-hindbrain development.

As a result of these studies, the molecular bases of Wnt-induced DA differentiation have been the focus of intensive research. Wnt1 has been shown to be essential for the maintenance of *En1* and *En2* expression (Danielian and McMahon, 1996; McGrew et al., 1999; McMahon et al., 1992), with *En1*/*En2* double knockout mice displaying a similar defective VM phenotype as the *Wnt1* null mutants (Simon et al., 2001), suggesting that the effects of loss of *Wnt1* may be due to a loss of *En* expression. This was subsequently confirmed when it was shown that *En1* was sufficient to rescue early midbrain deficits in *Wnt1* mutant mice (Danielian and McMahon, 1996). *En1* and *En2* are expressed in the ventral mesencephalon at the same time as *Wnt1* (~E8.0 in mice), however the overlapping expression domains of these three genes become restricted by E12 (Davis and Joyner, 1988). The expression of *Wnt1* in the *En1* expression domain (*En1-Wnt1* knock-in) causes a ventro-rostral and ventro-caudal expansion of *Wnt1* expression, which is usually restricted to the caudal VM (Danielian and McMahon, 1996), and results in an expansion of the most ventro-rostral DA cell group (Panhuysen et al., 2004). This cell group corresponds to the SNc, thus these studies demonstrate that this cell group is the most robustly influenced by Wnt1 signalling. *En1* expression is detectable in VM DA neurons from the time point at which they initiate their differentiation and persists into adulthood, while *En2* is only expressed in a subset of DA neurons (Simon et al., 2001; Zhong et al., 2010).

As mentioned earlier, *Wnt1* is expressed in the isthmus organiser, in an area rostral to where *FGF8* is expressed (Wilkinson et al., 1987). Interestingly, FGF8 signalling has also been shown to regulate *En1* expression in the developing VM (Lahti et al., 2012). It is tempting to speculate that this may be achieved through the induction of Wnt1. In support of this suggestion, a functional link between FGF8 and Wnt signalling has recently been described, where it was shown that Wnt-β-catenin signalling positively regulated *FGF8* expression in the midbrain-rhombomere1 region (Chilov et al., 2010). It is possible that this may be an autoregulatory loop, similar to that of Shh and FoxA2, with FGF 8 inducing the expression of *Wnt1* and subsequently *En1*/*En2* expression in the midbrain. Interestingly, new data have now shown that Lmx1a and Lmx1b function cooperatively to control the proliferation of VM DA NPs through the regulation of *Wnt1* expression (Yan et al., 2011).

A link between Shh and Wnt signalling has recently emerged with the proposal that canonical Wnt-β-catenin signalling is required to antagonise *Shh*, and that the subsequent reduced *Shh* levels allow the induction of VM DA NPs and the promotion of DA neurogenesis (Joksimovic et al., 2009b). The finding that Shh inhibits DA neurogenesis (Joksimovic et al., 2009b) is surprising, considering its well-established role in the induction of VM DA neurogenesis (Andersson et al., 2006; Blaess et al., 2006; Hynes et al., 1995a; Ye et al., 1998). However, Joksimovic et al., 2009a, 2009bsuggest that Shh is initially required for the early establishment of the VM DA NP pool, but that later it inhibits VM DA NP proliferation and neurogenesis. The current model suggests that once the Shh-induced VM DA NP pool has been established, Wnt-β-catenin signalling suppresses *Shh* levels in the VM to facilitate DA neurogenesis. Additionally Wnt signalling has been shown to induce *Otx2* and *Lmx1a* expression (Joksimovic et al., 2009b; Prakash et al., 2006). Furthermore, a Wnt1–Lmx1a autoregulatory loop has been identified which is proposed to regulate *Otx2* expression via β-catenin during VM DA neurogenesis (Chung et al., 2009). Interestingly, Otx2 has recently been suggested to regulate the proliferation of VM DA NPs via Wnt1 regulation (Omodei et al., 2008), suggesting a possible Otx2-Wnt1 regulatory feedback loop. In contrast to Joksimovic et al. (2009); Chung et al. (2009) suggested a mechanism by which the Wnt1–Lmx1a autoregulatory loop and a Shh–FoxA2 autoregulatory loop control VM DA neurogenesis synergistically. Despite this finding, a more recent paper described an antagonistic relationship between Wnt-β-catenin signalling and Shh signalling that is important in the progression of DA NPs into VM DA neurons (Tang et al., 2010), thus supporting the Joksimovic et al. (2009) theory. Furthermore, the stabilisation of β-catenin in VM NPs, by the inhibition of GSK3β, leads to an increase in DA differentiation (Castelo-Branco et al., 2004; Tang et al., 2009) and targeted deletion of *β-catenin* in VM NPs (Th-IRES-Cre; β-Ctn$^{Ex3/+}$ mutant) results in reduced VM DA neurogenesis (Tang et al., 2009). Surprisingly, mice with mutations in the Wnt/β-catenin inhibitor *Dkk1* actually have a reduction in VM DA neurons (Ribeiro et al., 2011). This is surprising, given that the stabilisation of β-catenin in VM NPs, through the inhibition of GSK3β, leads to an increase in DA differentiation (Castelo-Branco et al., 2004; Tang et al., 2009). Collectively these data largely support the theory that Wnt signalling is required for DA induction, but it is also clear that this is likely to involve a complex interplay with Shh and FGF8 signalling, and potentially other extrinsic signalling factors which have been suggested to induce VM DA neurogenesis, including TGFβs (Farkas et al., 2003; Roussa et al., 2009, 2006).

Development of post-mitotic ventral midbrain dopaminergic neurons

Once NPs of the VM floor plate are specified towards a DA phenotype, these DA NPs gradually become post-mitotic from E10-E14 in mice (E12–E16 in rats) (Lauder and Bloom, 1974; Lumsden and Krumlauf, 1996), with the greatest proportion of VM DA NPs undergoing their final division at E12 in the rat (Gates et al., 2006). The induction of *TH* expression, the rate-limiting enzyme for DA synthesis, is the first sign of the acquisition of the DA neuronal phenotype, and occurs shortly after the final mitosis of VM DA NPs while they are actively migrating to their final positions (Puelles and Verney, 1998, Specht et al., 1981a; Specht et al., 1981b). This process of migration of VM DA neurons from the floor plate ventricular zone to the presumptive VTA and SNc involves two steps: firstly, DA neurons migrate ventrally along tenascin-expressing radial glial processes which project to the pial surface, and secondly, once they have reached the basal part of the VM, they migrate laterally along

tangentially orientated fibres to form the VTA and SNc (Kawano et al., 1995; Shults et al., 1990). These tangentially-orientated fibres express the neural cell adhesion molecule L1, while VM DA neurons express the chondroitin sulphate proteoglycan 6B4. A heterophilic interaction between L1 and 6B4 has been proposed to facilitate this process of lateral migration of VM DA neurons (Ohyama et al., 1998). There have been a variety of studies that show that this process of migration is crucial for the normal positioning of VM DA neurons. Specifically, VM DA neurons are abnormally located in *L1* knockout mice (Demyanenko et al., 2001). A role for Wnt signalling in this process has been implicated by a study showing that the targeted deletion of β-*catenin* in the VM disrupts the integrity of these radial glia, resulting in perturbed migration of VM DA neurons (Tang et al., 2009). It is unclear whether these migratory defects are secondary to a disrupted radial glial scaffold or whether Wnt signalling can also directly affect this process of migration. A number of other molecules involved in neuronal migration in the developing CNS also appear to be involved in the migration of VM DA neurons. These include the well-known migrational regulator *Reelin*, as VM DA neurons fail to migrate laterally to the SNc, in *reeler* (*reelin null*) mice (Nishikawa et al., 2003), and the netrin receptor, *DCC*, which is expressed by migrating VM DA neurons in mice, and its loss results in aberrant migration of these neurons (Xu et al., 2010). In terms of the molecular regulatory networks that control this migration, there have been a number of studies describing roles for Ebf1 (early B-cell factor 1) (Yin et al., 2009) and Hes1 (Kameda et al., 2011) in this process. A key goal for future research will be to understand the molecular networks that control this process of VM DA migration and how newly-identified molecules such as Ebf1 and Hes1 "fit" within this network. While much work has focused on identifying the molecular signals that are required for neuronal migration, it will also be important to understand what positional cues inhibit these processes so that VM DA neurons "know" when to stop.

Several transcription factors have been identified which are essential for the differentiation and subsequent long-term survival of VM DA neurons. These include Lmx1b, Nurr1, Pitx3, En1 and En2. Each of these factors are not individually capable of inducing a complete DA phenotype, suggesting that they function as part of a network (Fig. 1).

Lmx1b

The lim-homeodomain factor *Lmx1b* is broadly expressed in the presumptive midbrain before neural tube closure, and its expression becomes restricted to VM DA NPs at E10.5 in mice, where it is co-expressed with *Lmx1a* and *Msx1* (Andersson et al., 2006; Smidt et al., 2000). Surprisingly, *Lmx1b* expression disappears in the VM at around E11.5, but reappears at E16 in post-mitotic VM DA neurons. *Lmx1b* is subsequently co-expressed with *Pitx3* and *TH* into adulthood in the VM (Dai et al., 2008). Although loss of *Lmx1b* leads to a loss of VM DA neurons (Smidt et al., 2000), *Lmx1b* mutant mice express *Nurr1* and *TH* normally during early development, but fail to express *Pitx3*. These TH-positive VM neurons, which lack *Pitx3* expression, are lost by birth, suggesting a role for Lmx1b in the regulation of *Pitx3* expression and VM DA neuronal survival. Similarly in *Wnt1* null mice, the few TH-positive VM neurons generated lack *Pitx3* expression, and are subsequently lost before E12.5 (Prakash et al., 2006). A similar regulatory loop may exist between Wnt1 and Lmx1b, as Lmx1b induces and/or maintains the expression of *Wnt1*, an important extrinsic factor in VM DA neurogenesis (see above), around the midbrain-hindbrain boundary (Adams et al., 2000; Matsunaga et al., 2002). Wnt1 has also been proposed to act downstream of Lmx1b in the potential regulation of *Pitx3* expression (Prakash et al., 2006). This maintenance of *Wnt1* expression by Lmx1b may be important in the generation of post-mitotic DA neurons, as Wnt1 is required for the terminal differentiation of VM DA neurons at later stages of embryogenesis (Prakash et al., 2006).

Nurr1

Nurr1 is a member of the nuclear receptor superfamily of steroid-thyroid hormone-activated transcription factors (Law et al., 1992), which atypically lacks both a ligand cavity and a canonical coactivator-binding site (Wang et al., 2003). *Nurr1* is expressed in the VM from E10.5 in the mouse, as VM DA NPs begin to become post-mitotic, one day before the appearance of *TH* (Zetterstrom et al., 1996), and *Nurr1* expression is maintained into adulthood (Backman et al., 1999). *Nurr1* expression levels show a sharp peak between E13 and E15 in the rat, at a stage of development when most VM DA neurons are undergoing terminal differentiation (Volpicelli et al., 2004). VM DA neurons in *Nurr1*-deficient animals do not express *TH*, l-aromatic amino acid decarboxylase (*AADC*), the vesicle monoamine transporter 2 (*VMAT2*) or the dopamine transporter (*DAT*), all markers of a DA neuron that has acquired its DA neurotransmitter identity (Castillo et al., 1998; Filippi et al., 2007; Smits et al., 2003). Nurr1 has been shown to play a direct role in regulating the expression of these genes, and a number of well-established signalling pathways in VM DA development cooperate with Nurr1 to mediate this induction. Specifically, Nurr1 has been shown to induce *TH* expression by binding to a NRBE (NGFI-B response element) sequence in the *TH* promoter (Kim et al., 2003; Sakurada et al., 1999), and is known to induce *DAT* expression via an NRBE-independent mechanism (Sacchetti et al., 2001). These effects of Nurr1 on the expression of these genes are enhanced by Wnt-activated β-catenin, which has been shown to promote Nurr1-induced *TH* promoter activation, by interacting with Nurr1 at NRBEs, causing the dissociation of transcriptional co-repressors and recruitment of transcriptional co-activators (Kitagawa et al., 2007). Similarly, nuclear FGFR1 has been shown to cooperate with Nurr1 to promote activation of the *TH* promoter (Baron et al., 2012a). Collectively these data show that Nurr1 functions as a master regulator in the induction of the neurotransmitter phenotypic identity of VM DA neurons, and controls the expression of the molecules that regulate the synthesis, vesicle packaging, axonal transport and reuptake of DA.

Aside from this role in DA identity, Nurr1 has also been shown to be crucial for long-term VM DA neuron survival. In *Nurr1*-deficient animals, VM DA neurons adopt a correct ventral position and express the DA markers *Lmx1b*, *Pitx3* and *En1* (Saucedo-Cardenas et al., 1998; Wallen et al., 1999), demonstrating that *Nurr1* is not required for all aspects of VM DA specification and differentiation. However, these *Pitx3*-expressing VM DA neurons are lost in *Nurr1*-deficient animals during later development (Saucedo-Cardenas et al., 1998), suggesting a role for *Nurr1* in the survival and maintenance of VM DA neurons. In support of these findings, *Nurr1* is expressed throughout the life of VM DA neurons and its heterozygous mutation increases the vulnerability of VM DA neurons to the parkinsonian toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-HCl (MPTP) (Le et al., 1999). Furthermore, the conditional ablation of *Nurr1* at a late stage of VM DA neuron development or in the adult brain results in loss of striatal DA, loss of VM DA markers and DA neurodegeneration, with SNc DA neurons more vulnerable than those of the VTA (Kadkhodaei et al., 2009).

While Nurr1 has been shown to directly regulate the expression of *TH* and *DAT*, Nurr1 may promote VM DA neuron survival indirectly, by inducing the expression of genes essential for their survival. Nurr1 has been found to regulate the expression of the glial cell line-derived neurotrophic factor (GDNF) receptor, *cRet*, whose expression is lost in *Nurr1*-deficient animals (Castillo et al., 1998). GDNF is a well-known survival-promoting factor for VM DA

neurons (Lin et al., 1993; Toulouse and Sullivan, 2008; Yasuhara et al., 2007). *Nurr1* has also been shown to regulate the expression of *VIP* (vasoactive intestinal peptide), which has been proposed to function in the survival of VM DA neurons (Luo et al., 2007). It remains to be determined whether the VM DA neuronal death in *Nurr1* null mutants is as a direct consequence of the absence of a Nurr1-mediated survival-promoting effect, and/or is induced by a lack of neurotransmission by these cells. Aside from its role in DA survival, Nurr1 has been suggested to play a role in target innervation by VM DA neurons (Wallen et al., 1999); however, this finding has been challenged (Witta et al., 2000). It will be important to assess the role of Nurr1 in striatal innervation in vivo using an approach where DA neurons can survive long term in the absence of Nurr1. Such a strategy has been employed successfully in the PNS, where Bax-deficient mice were used to analyse specifically the effects on a gene of interest on target innervation independent of this genes role in neuronal survival (Barker et al., 2001; Glebova and Ginty, 2004; Middleton and Davies, 2001).

The molecular mechanisms by which *Nurr1* expression is induced and regulated in the VM are largely unknown, but Nurr1 has been shown to function independently of FGF8 and Shh signalling (Sakurada et al., 1999). However, recent data suggests that FoxA1 and FoxA2 may be critical to *Nurr1* induction. Through the analysis of single and double mutants, *FoxA1* and *FoxA2* have been reported to regulate the expression of *Nurr1* in a dose-dependent manner, with a dramatic decrease in *Nurr1* expression observed in double mutants (Ferri et al., 2007). In addition to this, a gain-of-function study has demonstrated that *FoxA2* mediates *Nurr1* expression (Lee et al., 2010). Lee et al. (2010) further demonstrated that FoxA2 acts synergistically cooperates with Nurr1 during VM DA neurogenesis, with both factors binding to the *TH* promoter. In support of this, the loss of *FoxA2* resulted in an increase in the numbers of Nurr1-positive, TH-negative, cells in the VM (Ferri et al., 2007). These more recent data suggest that Nurr1 function may not be independent of Shh signalling, as *FoxA2* is a downstream target of Shh; however FoxA2 can function independently of Shh. Similar to Nurr1, FoxA2 appears to be involved in the survival and maintenance of VM DA neurons, as aged mice with a heterozygous mutation in *FoxA2* develop PD-like symptoms and pathologies (Kittappa et al., 2007), but this maybe due to altered FoxA2-induction of Nurr1 expression.

Pitx3

Pitx3 is a bicoid-related, homeodomain-containing transcription factor that is exclusively expressed in the mouse VM from E11.5, at a time when VM DA neurons are beginning to appear (Smidt et al., 1997). VM DA neurons only begin to express *Pitx3* when they arrive at their final ventral position, suggesting that Pitx3 is not involved in the early development or migration of VM DA neurons (Smidt et al., 2004). GDNF has been suggested to induce the expression of *Pitx3* in the VM (Lei et al., 2011; Peng et al., 2011) which is interesting as Nurr1 is known to regulate the expression of the GDNF receptor *cRet*, and is expressed before *Pitx3* in the VM. Nurr1 may therefore play an indirect, non-essential (*Pitx3* expression is retained in *Nurr1* null mutants) role in the induction of *Pitx3* expression. Pitx3 and Nurr1 have been shown to function cooperatively in the regulation of target genes involved in VM DA neurogenesis (discussed later) (Chakrabarty et al., 2012; Hwang et al., 2009; Jacobs et al., 2009a; Jacobs et al., 2009b). Pitx3 is co-expressed in the TH-positive neurons of the VM (Smidt et al., 1997; Van Den Munckhof et al., 2003; Zhao et al., 2004). In *aphakia* mice, which lack *Pitx3* expression due to deletions in the *Pitx3* gene, there is unaltered VM DA development until E12.5, at which time a deficit is observable in the lateral population of VM DA neurons which constitute the presumptive SNc (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003). VTA DA neurons are largely unaffected in these mice. The specific absence of SNc DA neurons in *Pitx3* null (*aphakia*) mice results in a loss of nigrostriatal projections to the dorsal striatum (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004; Van Den Munckhof et al., 2003), and suggests distinct developmental programmes for SNc and VTA DA neurons. Interestingly, it has been reported that lateral VM DA neurons express *Pitx3* prior to *TH*, while the medial VM DA neurons express *Pitx3* coincidently with *TH* (Maxwell et al., 2005). In addition to this, Pitx3 has been suggested to regulate *TH* expression (Cazorla et al., 2000; Lebel et al., 2001; Maxwell et al., 2005). Pitx3 may therefore be critical for the induction of *TH* expression in SNc DA neurons, but not those of the VTA. In support of this, the absence of *Pitx3* results in a failure of SNc DA neurons to express *TH*, while VTA neurons do so. Interestingly, *Pitx3* expression has been reported to be six times higher in VTA DA neurons then in those of the SNc (Korotkova et al., 2005). Perhaps this lower expression level of Pitx3 functions in the induction of *TH* expression in SNc DA neurons, while it may also contribute to their inherent sensitivity. In support of the proposed role for Pitx3 in *TH* expression, Pitx3 has been shown to contribute to the neurotransmitter phenotype of VM DA neurons by inducing the expression of *DAT* and *VMAT2* (Hwang et al., 2009). However, the loss of TH-positive neurons in the ventro-lateral VM is not due to the loss of TH mRNA expression, but to neuronal loss (Hwang et al., 2003; Nunes et al., 2003; Smidt et al., 2004). Perhaps the selective neurodegeneration of SNc DA neurons in *aphakia* mice is not as a result of a failure of DA neurogenesis in the absence of *Pitx3*, but may reflect the characteristic sensitivity of this VM population in comparison to those of the VTA. Indeed, the VTA DA neurons in *aphakia* mice display a normal DA phenotype (Smidt et al., 2004). A recent study has demonstrated that Pitx3 induces the expression of *brain-derived neurotrophic factor* (*BDNF*) in SNc DA neurons, which may be important in the survival of these neurons (Peng et al., 2011). Peng et al. (2011) showed that loss of *BDNF* expression correlates with the SNc neuronal loss in *Pitx3* null mice, and that BDNF treatment induces the survival of *Pitx3* (−/−) VM DA neurons and protects them against the dopaminergic neurotoxin 6-hydroxydopamine. Pitx3 may therefore be critical in the maintenance and survival of SNc DA neurons, acting via BDNF. Similarly, *BDNF* has been identified as a target gene of Nurr1 (Volpicelli et al., 2007).

Despite the lack of a direct role for Nurr1 in *Pitx3* expression, a recent set of studies has shown that Nurr1 regulates target gene expression cooperatively with Pitx3 during VM DA neurogenesis, with Pitx3 potentiating Nurr1 activity by releasing it from SMRT-mediated repression (Jacobs et al., 2009a, 2009b). Similarly, the same group demonstrated that Nurr1 and Pitx3 cooperatively regulate the expression of two cholinergic receptors, *Chrna3* and *Chrnb6*, which may play non-essential roles in VM DA neurogenesis (Chakrabarty et al., 2012). Furthermore, Pitx3 has been shown to induce the expression of the Nurr1-target genes, *VMAT2* and *DAT*, potentially in coordination with Nurr1 (Hwang et al., 2009), with Pitx3 also inducing the expression of aldehyde dehydrogenase 2 (*ADH2*), an enzyme which is highly expressed in SNc DA neurons (Chung et al., 2005). In support of this combinatorial function, Nurr1 and Pitx3 have been shown to cooperatively promote terminal maturation of VM DA neurons in stem cell cultures (Martinat et al., 2006) Collectively, these data suggest Nurr1 and Pitx3 may cooperate to promote VM DA survival and acquisition of a mature DA neurotransmitter phenotype by cooperatively regulating the expression of DA neurotrophic factors, *BDNF* and *GDNF*, and of genes involved in DA neurotransmission respectively.

En1 and En2

En1 and *En2* are important in the formation of the isthmus organiser and in the generation of VM DA neurons (Liu and Joyner, 2001; Simon et al., 2001). Following their initial expression in the midbrain-hindbrain boundary (Davis and Joyner, 1988), VM DA neurons begin to express *En1* and *En2* between E11.5 and E14 in mice, and this expression is maintained into and throughout adulthood (Alberi et al., 2004). Interestingly, in *En1* and *En2* double mutants, VM DA neurons develop normally initially, but are lost by E14 due to caspase-dependent apoptosis, just after the expression of *En* begins in the wild type (Alberi et al., 2004; Simon et al., 2001). Alberi et al. (2004) demonstrated that *En1* and *En2* are required cell-autonomously in post-mitotic VM DA neurons to prevent apoptosis. However, further studies are required to ascertain that VM DA neuronal loss in the *En* double mutants is not as a result of the large midbrain/hindbrain deletion in these mice. Despite this possibility, these data strongly suggest a role for *En1* and *En2* in the maintenance and survival of VM DA neurons. Indeed, intermediate genotypes between wild type and double *En* mutants show varying degrees of VM DA neuronal deficiencies (Sgado et al., 2006; Simon et al., 2001; Sonnier et al., 2007), as has been well-described in recent reviews (Alavian et al., 2008; Alves Dos Santos and Smidt, 2011). The most notable phenotype was observed in *En1* (+/−)/*En2* (+/+) mutant mice, which display a progressive degeneration (between 8 and 24 weeks) of VM DA neurons that can be antagonised by recombinant En2 protein infusion (Sonnier et al., 2007). The progressive degeneration of VM DA neurons in *En1* heterozygotes (*En2* null background in Sgado et al. (2006) study) is more pronounced in the SNc and results in reduced striatal DA and motor deficits, as is characteristic of PD pathology (Sgado et al., 2006; Sonnier et al., 2007). These findings further support the theory that *En1*/*En2* function as important survival-promoting factors for VM DA neurons.

Diversity in genetic regulation of DA neuron development

As mentioned earlier, the molecular mechanisms controlling phenotypic and functional diversity between the various VM DA neuronal subpopulations remain poorly understood. However, recent work has implicated *Otx2* as a factor that may contribute to these distinct developmental pathways. *FoxA2*, *En1*, *Lmx1b*, *Nurr1* and *Pitx3* are ubiquitously expressed in post-mitotic VM DA neurons throughout life; however *Otx2* expression is restricted to VTA DA neurons in the adult brain (Di Salvio et al., 2010b). *Otx2* was shown to regulate subtype identity in the VTA by antagonising the expression of *Girk2* and *DAT*, and was also shown to antagonise the neurotoxic effect of the MPTP in these VTA neurons (Di Salvio et al., 2010a). Interestingly, ectopic *Otx2* expression also provides SNc neurons with neuroprotection to MPTP (Di Salvio et al., 2010a). This potential role of *Otx2* in VM DA neuronal subtype identity has been comprehensively described in a recent review (Simeone et al., 2011).

It is also necessary to mention that a proportion of DA neurons arise anterior to the VM in the diencephalon (Gonzalez et al., 1999. Lahti et al., 2012; Marin et al., 2005; Puelles and Verney, 1998; Smits et al., 2006; Verney, 1999; Verney et al., 2001. Vitalis et al., 2000), and develop earlier than those from the VM (Lahti et al., 2012; Marin et al., 2005). The diencephalic DA domain differs to that of the midbrain. The DA NPs in the diencephalon are intermingled with non-DA Pou4f1+FoxP1+ cells, they lack *Pitx3* and *DAT* expression, and lose *En1*/*2* expression by E9.5 (Lahti et al., 2012), unlike those in the midbrain (Alberi et al., 2004). FGF8 regulates the diverse identities of the DA neurons from the VM and caudal diencephalon. This anterior–posterior patterning by FGF8 suppresses diencephalic identity and maintains midbrain identity (Lahti et al., 2012; Scholpp et al., 2003). A study using zebrafish showed that Nodal signalling was required for the specification of ventral diencephalic and pretectal catecholaminergic neurons (Holzschuh et al., 2003). (Holzschuh et al., 2003) also demonstrated that FGF8 signalling was not required for the specification of these neurons but was important for their proliferation or survival, and that Shh signalling is required for pretectal DA development. Diencephalic DA neurons therefore seem to be subject to a different programme of neurogenesis than those of the VM. Genetic fate-mapping studies are needed to verify whether, or to what extent, these diencephalic DA neurons contribute to the DA subpopulations of the VM.

Establishment of dopaminergic projections from the ventral midbrain

Following their generation, post-mitotic VM DA neurons undergo functional maturation, which involves axonal pathfinding and synaptogenesis. Axons from VM DA neurons, which arise at E11 in mice (E13 in rat), initially project dorsally but then deflect ventro-rostrally towards the forebrain, in response to extrinsic directional cues in the dorsal midbrain and repulsive cues in the caudal brain stem (Gates et al., 2004; Nakamura et al., 2000). The reorientated VM DA neuronal axons then extend towards the telencephalon, through the diencephalon, via the medial forebrain bundle (MFB) which has been reported to have a chemo-attractive effect on these axons (Gates et al., 2004). A recent paper has suggested that Nurr1 regulates the axonal extension of VM DA neurons through the regulation of the expression of the axon genesis gene *Topoisomerase IIβ* (*TopIIβ*) (Heng et al., 2012). Furthermore, a study using retrograde labelling suggested that Nurr1 plays a role in target innervation by VM DA neurons (Wallen et al., 1999). However, as mentioned before, this finding has been challenged (Witta et al., 2000). Gates et al. (2004) also demonstrated that the thalamus prevents entry of VM DA axons through the action of contact-dependent inhibitors, which likely function to maintain the orientation of these axons in the MFB. Furthermore, *Nkx2.1* mutant mice display aberrant midline crossing of MFB fibres at the caudal diencephalon, suggesting that chemorepulsive factors involved in maintaining the ipsilateral trajectory of the MFB at the medial part of the caudal diencephalon are lost in this mutant (Kawano et al., 2003).

The VM DA neuronal axons run via the MFB into the telencephalon, where they terminate in the striatum and cerebral cortex (Specht et al., 1981a, 1981b; Zhao et al., 2004). In the case of the nigrostriatal pathway, chemoattraction from the striatum and chemorepulsion from the cortex have been suggested to facilitate appropriate striatal innervation by nigral DA neurons (Gates et al., 2004). The molecular signals that guide the axons of the various populations of VM DA neurons remain to be characterised; however a relatively recent review has comprehensively described the current understanding of the development of VM DA circuitry (Van Den Heuvel, Pasterkamp, 2008). Molecules which are known to be involved in the establishment of the VM DA circuit are illustrated in Fig. 2.

Despite the current paucity of studies determining the molecular basis of the formation of VM DA projections, several molecules have been implicated to play a role in this process. *EphrinB2* and its receptor *EphB1* have been shown to be expressed in a complementary pattern to facilitate nigro-striatal innervation, with *EphB1* expressed by VM DA neurons (with highest expression in the SNc) and *ephrinB2* expressed in the striatum (Yue et al., 1999). Cell-surface tethered ephrins, and their Eph receptor tyrosine kinases, are known to play important roles in axonal guidance (Egea and Klein, 2007). Furthermore, Yue et al. (1999)

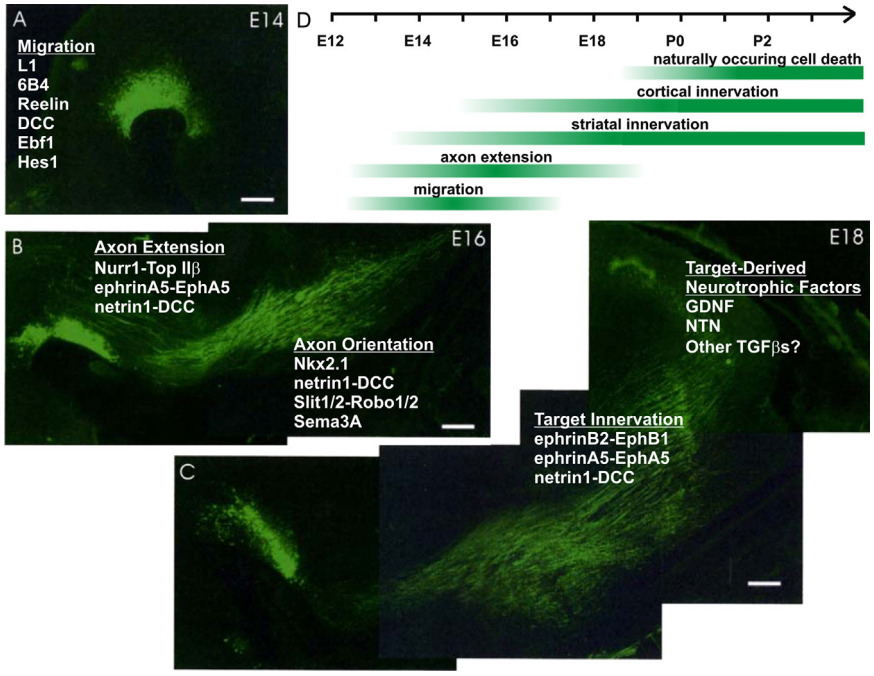

Fig. 2. Molecules involved in the formation of the nigrostriatal DA circuitry. *Development of the rat nigrostriatal pathway*: Representative photomicrographs showing cryosections through the developing rat nigrostriatal pathway at (A) E14, (B) E16 and (C) E18, immunostained for TH. Molecules involved in the migration (A), axon extension (B), axon orientation (B), target innervation (C) and survival (C) of VM DA neurons are labelled on the images where appropriate. Scale bar = 100 μm. (D) Graphical representation of the time-course of DA circuitry formation. VM DA neurons begin to migrate and extend axons at E13 in the rat. These DA fibres begin to reach the striatum by E14, and the cortex at E16. The innervation of these targets continues into the first week after birth. Naturally occurring cell death begins close to birth, reaching a peak at P2 and P14, before subsiding around P20.

showed that co-culture with ephrinB2-expressing NIH-3T3 cells reduced neurite outgrowth and induced death of SNc, but not VTA, DA neurons. These results suggest that the interaction between ephrinB2 and EphB1 in the striatum ensures that SNc DA neurons are confined to the dorsal striatum. Conversely, *EphB1* expression has been shown to disappear in the SNc from E18, and its null mutation resulted in no observable defects in the nigrostriatal pathway (Richards et al., 2007). These results challenge the role for EphB1 in the formation of the nigrostriatal pathway; however other Eph receptors may allow SNc DA axons to detect ephrinB2 in the striatum. In support of a role for ephrinB2 in the correct target innervation of nigral DA neurons, the application of ephrinB2 to VM cultures resulted in an upregulation of *Nurr1* (Calo et al., 2005). This action by ephrinB2 could function to support and maintain correctly-innervated DA neurons. However, this effect was suggested to be mediated by the EphB1 receptor (Calo et al., 2005). Other ephrins and Ephs have also been implicated in DA pathway formation. For example, studies on genetically-altered EphAs and ephrinAs have shown that these molecules are important in the formation of VM DA projections (Halladay et al., 2004; Sieber et al., 2004; Van Den Heuvel, Pasterkamp, 2008), with *ephrinA5* expression being reduced in the forebrain of *Nkx2.1* mutants (described above) (Marin et al., 2002). *EphrinA5* has been shown to be expressed in the developing telencephalon and striatum, in the vicinity of VM DA axons, and to have a repulsive effect on these axons, likely through the action of EphA5 (Deschamps et al., 2009). Conversely, another study has shown that ephrinA5-EphA5 signalling promotes DA axonal growth in vitro (Cooper et al., 2009). Perhaps ephrinA5 initially functions in the establishment of VM DA projections, but later functions to restrict these axons to their targets. Semaphorin signalling has also been proposed to function in VM DA axonal pathfinding (Hernandez-Montiel et al., 2008; Kolk et al., 2009, Tamariz et al., 2010; Torre et al., 2010), with a number of semaphorins and their receptors being expressed in VM DA neurons (Torre et al., 2010). Furthermore, *Sema3A* expression is reduced in *Nkx2.1* mutants (Kawano et al., 2003), and the expression of its co-receptor *Neuropilin1* has been shown to be regulated by Nurr1 in the developing midbrain (Hermanson et al., 2006).

The netrin receptor *DCC* has been demonstrated to play an important role in the formation of VM DA axonal projections. *DCC* is expressed in the VM and in cultured VM DA neurons, as well as in VM DA targets, such as the striatum and prefrontal cortex (Lin et al., 2005; Livesey and Hunt, 1997; Xu et al., 2010), while *netrin1* is expressed in a complimentary fashion in VM, striatal and cortical neurons (Hamasaki et al., 2001; Livesey and Hunt, 1997; Manitt et al., 2011). Studies of heterozygous and homozygous *DCC* mutants have provided insights into how netrin-DCC signalling may regulate the formation of VM DA neuronal projections (Flores et al., 2005; Xu et al., 2010). DA innervation of the dorsal striatum is not affected in heterozygous and homozygous *DCC* mutants, while the ventral striatal DA projections are aberrantly shifted to a more dorsal location in null mutants. The innervation of the prefrontal cortex by VM DA neurons is significantly reduced in null mutants, suggesting that *DCC* is an important mediator of VM DA axonal guidance. Xu et al. (2010) also propose that DCC signalling is an important negative regulator of DA axon arborisation, demonstrating that DA innervation is maintained/increased despite significant VM DA neuronal loss in *DCC* deficient animals. In support of this, analysis of heterozygous *DCC* mutants has shown that DCC can selectively influence the branching of VM DA fibres in the prefrontal cortex at puberty, with a significant increase in the number of TH-positive varicosities present post-puberty in these heterozygotes (Manitt et al., 2011). This proposed role of DCC contradicts previous reports which had suggested that DCC mediates netrin1-promotion of axonal outgrowth in VM DA neuronal cultures (Lin et al., 2005). However, these contrasting results may reflect differences between the responses of VM DA neurons in vitro and in vivo. Furthermore, DCC receptors are known to mediate both attraction and repulsion aspects of the

axon growth-promoting effects of netrins (Round and Stein, 2007). The atypical homeoprotein Pbx1a has been shown to regulate the expression of *DCC* in VM DA neurons, and its deficiency results in partial misrouting of VM DA fibres (Sgado et al., 2012). Interestingly, *DCC* null mice also display aberrant midline crossing of MFB DA fibres at the caudal diencephalon, which is similar to that observed in *Nkx2.1* mutant mice (Xu et al., 2010), likely reflecting a loss of chemorepellant(s) at the ventral midline. In *Pax6* null mice, which display a ventro-dorsal expansion of netrin1 expression, VM DA axons within the MFB are deflected dorsally in the diencephalon, seemingly avoiding areas of ectopic *netrin1* expression (Vitalis et al., 2000). Netrin-DCC signalling may therefore act at the ventral midline of the diencephalon to repel VM DA projections, ensuring that they maintain their ipsilateral course in the MFB. Similarly, mice deficient in both *Slit1* and *Slit2* display abnormal ventral midline crossing of MFB fibres in the diencephalon, suggesting they may also act as ventral midline chemorepellants (Bagri et al., 2002; Dugan et al., 2011). In support of this theory, Slit2 repels VM DA neuronal axons and inhibits their growth in vitro (Dugan et al., 2011; Lin et al., 2005). *Nkx2.1* mutant mice display altered *Slit1* and *Slit2* expression, and a more severe phenotype than that of *Slit1*/*Slit2* double mutants (Marin et al., 2002). This suggests that *Nkx2.1* may regulate the expression of a number of important chemorepellants at the diencephalic ventral midline, such as *Slit1*/*Slit2*, *Sema3A*, *ephrinA5* and perhaps *netrin1*. However, *netrin1* expression is maintained in the subpallium of the *Nkx2.1* mutants (Marin et al., 2002), and has been reported to attract diencephalic (A11) DA neurons towards the ventral midline in the absence of repulsive Slit signals (Kastenhuber et al., 2009). Mice deficient in the Slit receptors, *Robo1* and *Robo2*, also display guidance errors in the MFB tract similar to those in the *Slit* double mutant (Dugan et al., 2011; Lopez-Bendito et al., 2007). However, Dugan et al. (2011) also demonstrated abnormal dorsal trajectories of VM DA fibres in *Robo1*/*2* knockout mice, which does not occur in the *Slit1*/*2* mutant, suggesting that these Robos also function Slit-independently. The expression patterns of Slits also propose a role for these molecules in VM DA axonal guidance, with *Slit1* expressed in the dorsal midbrain (Nakamura et al., 2000) and *Slit3* expressed in the caudal midbrain (Gates et al., 2004), suggesting that these Slits contribute to the ventro-rostral trajectory of VM DA fibres.

Upon innervation of their targets, the axons of VM DA neurons compete to establish functional synapses and survive. There are two peak postnatal periods of naturally-occurring cell death for VM DA neurons. Cell death begins close to birth, reaching an initial peak at P2, before a second peak of apoptosis occurs at P14, with this process largely subsiding around P20 in rodents (Burke, 2003; Jackson-Lewis et al., 2000; Oo and Burke, 1997). This programmed cell death pathway relies on the limited availability of target-derived neurotrophic factors (Burke, 2003), with striatal and prefrontal cortex tissue being shown to promote VM DA neuronal survival when co-cultured in vitro (Hoffmann et al., 1983). The most well-established target-derived neurotrophic factor for VM DA neurons is GDNF (Akerud et al., 1999; Beck et al., 1995; Burke, 2003; Costantini and Isacson, 2000; Gash et al., 1996; Lei et al., 2011; Lin et al., 1993; Redmond et al., 2009; Tomac et al., 1995; Wang et al., 2010). Another member of the GDNF protein family, neurturin (NTN), also acts as a neurotrophic factor for VM DA neurons (Akerud et al., 1999; Horger et al., 1998; Oiwa et al., 2002; Tseng et al., 1998; Zihlmann et al., 2005). Other neurotrophic factors identified for VM DA neurons include transforming growth factor βs (TGFβs) (Farkas et al., 2003), BDNF (Alonso-Vanegas et al., 1999) and growth/differentiation factor 5 (GDF5) (Costello et al., 2012; Hurley et al., 2004; O'Keeffe et al., 2004; O'Sullivan et al., 2010; Sullivan and O'keeffe 2005; Sullivan et al., 1997, 1998). Interestingly, FGF2 has recently been shown to act as a target-derived regulator of VM DA innervation (Baron et al., 2012b).

Concluding remarks and future perspectives

This paper reviews the recent data from molecular studies on VM DA development, and highlights a number of important genetic pathways involved during the neurogenesis of these cells. It is clear that this is a complex developmental programme, complicated further by the fact that VM DA neurons are not a homogenous population of neurons. However, insights are beginning to be made on the molecular mechanisms that may, in part, confer subtype identity within the VM DA circuit, such as those described for Shh, Pitx3 and Otx2. Despite these developments, future studies will be crucial to elucidate the molecular basis of these subtle developmental differences between A8, A9 and A10 DA neuronal identities.

VM DA neurons are now known to arise from floor plate radial glial-like NPs in response to specification by FGF8, Shh and Wnt1. This recent discovery is important if VM DA NPs are to be specifically isolated for use in cell replacement therapies. Additionally, Wnt1 is now accepted as an extrinsic factor for VM DA neurons, along with Shh and FGF8, which has added another dimension to the developmental programme of VM DA neurogenesis. A number of TGFβ superfamily members, and other Wnts, have also been implicated as instructive signalling molecules during VM DA neurogenesis. Similar to the recent studies carried out for Wnt1, these candidates should be investigated thoroughly for their participation in these developmental processes.

The discovery of a number of new candidate transcription factors, for example Oc1/2/3, highlights that there are likely to be other, as yet unidentified, molecular pathways involved in regulating VM DA neurogenesis. Furthermore, new relationships are being uncovered between the transcription factors and molecular pathways that are well-known to play key roles in DA development. For example, Nurr1 and Pitx3 were previously thought to function independently, however, recent data show that these key transcription factors function cooperatively. These findings highlight that there is still a significant challenge remaining to understand the complexities of the dynamic molecular interactions between the known genetic networks involved in VM DA neurogenesis.

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