

From the Department of Cell and Molecular Biology
Karolinska Institutet, Stockholm, Sweden

RESOLVING DEVELOPING NEURONAL LINEAGES IN THE VENTRAL MIDBRAIN

Nigel Kee



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Resolving Developing Neuronal Lineages in the Ventral Midbrain

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Nigel Kee

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Principal Supervisor:

Professor Thomas Perlmann
Karolinska Institutet
Department of Cell and Molecular Biology

Opponent:

Professor Lorenz Studer
Sloan-Kettering Institute for Cancer Research
Center of Stem Cell Biology

Co-supervisor(s):

Professor Johan Ericson
Karolinska Institutet
Department of Cell and Molecular Biology

Examination Board:

Associate Professor Gonçalo Castelo-Branco
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Associate Professor Eva Hedlund
Karolinska Institutet
Department of Neuroscience

Professor Sten Linnarsson
Karolinska Institutet
Department of Medical Biochemistry and Biophysics

Professor Elena Kozlova
Uppsala Universitet
Department of Neuroscience

ABSTRACT

The numerous cells and cell types of our bodies originate from a fertilised egg. Through the study of the developing embryo, we have come to understand much about how different cells in different tissues arise from this single cell, and how they are related to one another within an expansive family tree. Knowledge of these developmental instructions has birthed the field of regenerative stem cell biology, where efforts aim to engineer therapeutic cell types for clinical use. However, time and again we find that the details are more complicated than first thought. The road to the clinic will require extensive understanding of precisely what cell types we want (or do not want), and precisely what cellular instructions will get us there.

Neurodegenerative disorders such as Parkinson's Disease (PD) are becoming a major and increasing health burden for developing societies. In PD patients, loss of dopamine releasing neurons in the ventral midbrain (vMB) leads to a decline of movement control. While prognosis has improved for PD patients in recent years, transplantation of stem cell derived dopaminergic neurons, to replace those that degenerate, remains an attractive prospect. In this light two questions emerge: i) how may stem cells be instructed towards the dopaminergic lineage? and ii) what criteria precisely identify these dopaminergic cells?

Developmental biology research has uncovered morphogen permutations that specify the dopamine lineage. Thus, stem cell differentiation protocols targeting PD incorporate these extrinsic signaling molecules to instruct stem cells to choose a dopaminergic identity. However, this approach is not completely efficient, often yielding neurons from a mix of different lineages. In paper I we show that forced expression of intrinsic transcriptional determinants can also supply these commitment instructions. Following a lineage specific logic, the transcription factor *Lmx1a* selects for dopaminergic neurons, while *Nkx2.2*, *Phox2b*, and *Olig2* select for serotonergic neurons, visceral motor neurons and somatic motor neurons respectively, however only in permissive morphogen contexts. Moreover, in each scenario untargeted neuronal lineages are depleted, and in some cases combinations of intrinsic transcriptional determinants can override even a conflicting extrinsic environment.

Enrichment alone is not enough for clinical grade transplantable material, in particular if contaminating neuron types are detrimental. In addition to enriching for neurons that we want, we must also identify and eliminate neurons that we do not want. In paper II, we employ single-cell RNA-sequencing technologies to characterise the *Lmx1a*-expressing lineage in the mouse vMB. We show that previous stalwarts of dopaminergic identity, such as *Lmx1a/b*, *FoxA1/2*, *Shh*, and *Wnts*, are in fact all shared with rostrally adjacent glutamatergic lineages. Computational analysis uncovered additional markers specific to these cells, that we further validate *in vitro* and *in vivo* in both mouse and human systems. Finally, in paper III we show that appreciation of this new rostro-caudal division can guide fine-tuning of morphogen application, leading to improvements in existing stem cell protocols targeted to PD.

In summary, we have probed neuron lineage commitment in the vMB, finding that *Lmx1a* can instruct stem cells down the dopaminergic lineage, but only when care is also taken to specify the appropriate rostro-caudal level. Interestingly, regenerative medicine efforts outside the PD field are tackling conceptually identical challenges. Using developmental biology as a guide, efforts aim to derive cells of additional neuron types, including muscle, skin, neural crest and other organs, with high purity and efficacy. Given that bone marrow transplants, a routine hematopoietic stem cell therapy, required several decades from inception to clinic, one can hope that with patience and diligence, more stem cell therapies will, in time, become accessible standard medical procedures.

LIST OF SCIENTIFIC PAPERS

- I. Lia Panman, Elisabet Andersson, Zhanna Alekseenko, Eva Hedlund, **Nigel Kee**, Jamie Mong, Christopher W. Uhde, Qiaolin Deng, Rickard Sandberg, Lawrence W. Stanton, Johan Ericson, and Thomas Perlmann.
Transcription factor-induced lineage selection of stem-cell-derived neural progenitor cells.
Cell Stem Cell (2011) vol. 8 (6) pp. 663-751

- II. **Nigel Kee**, Nikolaos Volakakis, Agnete Kirkeby, Lina Dahl, Helena Storrvall, Sara Nolbrant, Laura Lahti, Åsa K. Björklund, Linda Gillberg, Eliza Joodmardi, Rickard Sandberg, Malin Parmar, and Thomas Perlmann.
Single-Cell Analysis Reveals a Close Relationship between Differentiating Dopamine and Subthalamic Nucleus Neuronal Lineages.
Cell Stem Cell 20, 1–12 January 5, 2017

- III. Agnete Kirkeby, Sara Nolbrant, Katarina Tiklova, Andreas Heuer, **Nigel Kee**, Tiago Cardoso, Daniella Rylander Ottosson, Mariah J. Lelos, Pedro Rifes, Stephen B. Dunnett, Shane Grealish, Thomas Perlmann and Malin Parmar.
Predictive Markers Guide Differentiation to Improve Graft Outcome in Clinical Translation of hESC-Based Therapy for Parkinson's Disease.
Cell Stem Cell 20, 1–14 January 5, 2017

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LIST OF ABBREVIATIONS

α -syn	alpha synuclein
AP	anterior-posterior
bHLH	beta helix-loop-helix
BMP	bone morphogenic protein
DA	dopamine
DAT	dopamine transporter
DBS	DNA binding site
ER	endoplasmic reticulum
ESC	embryonic stem cell
GID	graft induced dyskinesias
GP	globus pallidus
GRN	gene regulatory network
ICM	inner cell mass
IsO	isthmus organiser
KO	knockout
L-DOPA	L-dopamine
LIDs	L-DOPA induced dyskinesias
MSN	medium spiny neuron
NGS	next generation sequencing
PD	Parkinson's disease
PSC	pluripotent stem cell
RA	retinoic acid
ROS	reactive oxygen species
RRF	retrochubral field
SC	spinal cord
SHH	sonic hedgehog
SNc	substantia nigra pars compacta
STN	subthalamic nucleus
TF	transcription factor
TH	tyrosine hydroxylase

UTR	untranslated region
vHB	ventral hindbrain
vMB	ventral midbrain
VTA	ventral tegmental area
WT	wild type

1 INTRODUCTION

1.1 Cells, cell type and gene regulation

There exist many cells within the body; muscle, nerve, liver, fat, skin, alveolar, blood. Each has a specific role in the body, and a diverse and impressive array of cell morphologies exist across tissues. However, while cells are clearly different, they also clearly share common features; a cell membrane, a nuclear genome, organelles, mechanisms of cell division, transcriptional and translational machinery, etc. So, how do we satisfy the human desire to classify cells into types? One appealing definition is rooted in function:

Cell type-A is equal to cell-type B if an organism would function identically were all its cell A's magically changed to cell B's, and all its cell B's magically changed to cell A's. Further, neither cell-type A nor B can be reciprocally swapped in this way for any other cell-type in the organism.

In such a definition, even if cell A and cell B have the unlikely situation of having identical transcriptomes, they would not be considered the same cell type if, for example, the arrangement of the proteins between the two cell types conferred different functions for the organism.

The internal milieu of a cell is complicated; cytoskeletal structure, partitioned surface membrane compartments, dynamic nuclear architecture, fluid mitochondrial network, autophagy-lysosome pathways, etc. This milieu is determined for the most part by the culmination of the genes that are active, and have previously been active, in the cell. Nuclear chromosomes are girdled with structural and regulatory proteins and RNA. The pattern of adornment of these regulatory players directs which of the genes in the genome will be active at a given moment, and thereby directs the function of the cell - that cell's type. On the simplest of levels, one way to silence a gene is for that gene and its regulatory regions to be bound in tight higher order structure with histones, compacted into dense chromosomes, and inaccessible to the transcriptional machinery. This renders the gene incapable of influencing the cell. For it to become active it must first be released from this compacted chromatin, making it sterically accessible and then to be targeted by the transcriptional machinery. The gene's product will then be free to influence the cell's phenotype.

Transcription factors are proteins that direct gene regulation in a sequence dependent manner. A given transcription factor (TF) will have a particular affinity for a DNA sequence, to which it binds. Binding may form a platform to which the transcriptional machinery can subsequently bind. Alternatively, a TF-DNA complex may constitute a permissive or attractive environment for any number of additional layers of other TFs to bind, which may in time constitute a platform for the

transcriptional machinery. Non-coding RNA molecules can also contribute to these molecular scaffolds (Wilusz et al., 2009), while post-translational/transcriptional modifications of DNA, chromatin components or TFs add extra permutations to this regulatory logic. Conversely, by the reverse of the same mechanisms TF-DNA complexes may constitute a non-permissive or repressive influence that inhibits association of the transcriptional machinery, or actively recruits enzymatic modifiers that compact local chromatin and eventually silence the gene (García-González et al., 2016; Ho and Crabtree, 2010).

Clearly transcriptional regulation is exceedingly more complicated than described here, however in general terms these two processes act in opposition, such that the sum of the two will dictate the transcriptional activity of a gene. Genes that are never needed by the cell may be positioned in compacted heterochromatin at the periphery of the nucleus. Genes that are always needed may be positioned in unwound euchromatin in and around transcriptional factories. Genes that are sometimes needed may exist in a flux between these two nuclear compartments, where molecular transduction of extrinsic signals leads to addition or removal of activating or repressive elements on DNA and chromatin that, in the end, associates gene loci together with, or apart from, transcriptional machinery (Fedorova and Zink, 2009; Puschel et al., 2016). These dynamic processes also occur at enhancer DNA elements, at times kilobases away, where extra DNA real estate provides additional regulatory information, in particular the ability to recruit or exclude whole sets of genetic instructions through DNA looping, changing an enhancer's proximity to other enhancers or promoters (Matharu and Ahituv, 2015).

Coordination of a gene regulatory network (GRN) to give rise to a specific cell phenotype can be thought of as a computational task. The cell must interpret levels of present TFs and any participating protein complexes, as well as their affinities to on or off-consensus DNA binding sites (DBS) (Oosterveen et al., 2012). If the kinetics of on-off binding are sufficient to catalyse euchromatin promoting modifications, genomic loci may open, leading to the transcription of new genes and/or exposing new regulatory instructions to the current GRN and thereby beginning a new round of molecular computation. Additionally, combinations of TFs may cooperate to give context specific TF binding profiles (Mullen et al., 2011; Oosterveen et al., 2013), at times requiring strict DBS spacing and order (Reményi et al., 2004), while other regulatory operations are independent of such stoichiometric requirements (Smith et al., 2013; Yao et al., 2016). Further, similar mechanisms can work to instead silence genes, with cross regulation by repressive lineage specific TFs often being deployed to pattern progenitor domains in the developing nervous system (Muhr et al., 2001). However, in essence this computational task is a mechanical one. Cohesin is associated with, and can be actively translocated along, open DNA (Davidson et al., 2016; Stigler et al.,

2016). Once localised, cohesin and associated motor complexes operate to traffic DNA elements around the nucleus, facilitating even long-distance interactions (Guo et al., 2015; Merkenschlager and Odom, 2013) of at times functionally related genes (Downen et al., 2014; Hnisz et al., 2016), such that GRN specified downstream genes are expressed, while undesired genes are not. In this way, the TF profile of a cell drives expression of the downstream genes that are responsible for its phenotype.

1.2 Developmental biology - attractor state transitions through time

The progression from pluripotent cell to adult differentiated cell was illustrated in 1957 by Waddington (Waddington, 1957) as a path of irreversible decision points through time, that inevitably restrict the array of future cell types down to a single possibility. In this view, final adult differentiated cell states have decommissioned the DNA elements required to establish any alternative cell types, such that phenotypic transitions away from the current cell type are effectively impossible. This observed genomic configuration is, in some way, governed by simple molecular energy kinetics; it is the lowest energy state considering the current context and condition of all genetic and epigenetic influences; an attractor state (Enver et al., 2009). Additionally, it is argued that attractor states can hold inherent levels of instability, or biological noise, whereby there exists variation in the phenotype of individual cells that, never the less, are of the same type. Such a phenomenon is typified by the ability of single cells from a group to re-establish all prior observed variation (Chang et al., 2008). Individual cells that at a given moment in time stochastically lie at the outer of the attractor state, will require less input to be coerced into a neighboring one (Kalmar et al., 2009). Attractor state transitions require a sufficiently strong perturbation into the system, either in the form of considerable new extrinsic signals, or release from an attractor state checkpoint. The strength of this perturbation would be proportional to the energy distance between the current and the neighboring attractor states. In cellular terms, this refers to the energy required to execute the molecular silencing and activation of new cohorts of enhancers and genes, and subsequent physical genomic rearrangements. The more changes required, the further the distance, and the more stable the current attractor state. Additionally, the event of one cell making such a transition may serve as a catalyst, changing the attractor state landscape, and lowering the energy required for subsequent cells to make the same transition. Developmental ontology would thus suggest that, as a whole, a development trajectory navigates through a low energy path from the pluripotency attractor state to its final phenotype. One imagines that evolution, through mutation of existing GRNs, generates new paths or path variations of attractor state cascades, while adhering to the constraint that paths for other crucial cell types

remain sufficiently intact, or if not, that a new path with a suitable endpoint is generated to replace it.

1.3 Dopaminergic development

What are the attractor states, the hallmark cell types, through which cells of the dopaminergic (DA) lineage pass?

After fertilisation in mice and humans, several rounds of cell division yield a ball of cells termed a morula. At this stage, all cells are equivalent, and can contribute to all embryonic or extraembryonic tissues, a property termed totipotency, perhaps the first attractor state of the embryo. In line with this, removal of a single cell at this stage exerts no influence on the developing embryo (Hardy et al., 1990; Krzyminska et al., 1990). Shortly thereafter totipotency is lost as the blastocyst forms, where cells commit to either the trophectoderm and primitive endoderm that will give rise to the placenta and yolk sac, or the epiblast, giving rise to all the cells of the body (Lanner, 2014; Petropoulos et al., 2016), a property termed pluripotency. The pluripotent properties of the epiblast have allowed the generation of multiple stem cell lines from multiple species (Nichols and Smith, 2011). Epiblast cells then dismantle the pluripotency GRN (Kalkan and Smith, 2014), and undergo gastrulation to derive the three germ layers; ectoderm, mesoderm, and endoderm (Rivera-Pérez and Hadjantonakis, 2014). While the precise mechanisms remain unclear, in general, gastrulation begins as the primitive node forms at the edge of the epiblast disc, and transgresses inward forming the primitive streak with cells invaginating as they go, thus defining the anterior-posterior and left-right axis of the body. Early in gastrulation, predominantly at the anterior portion of the primitive streak, ingressing cells form definitive endoderm through intercalation and displacement of extraembryonic primitive endoderm at the ventral-most cell layer of the embryo. Prechordal mesoderm cells ingress in parallel with these cellular movements, forming a layer between the epidermal and endodermal layers (Viotti et al., 2014; Wilson and Houart, 2004). Crucial for DA neurogenesis at this stage is the process where ectoderm receives secreted bone morphogenic protein (BMP) inhibitors from the underlying newly invaginated mesoderm, such as chordin and noggin, that then drives a neurectodermal commitment (Kelly and Melton, 1995). More lateral ectoderm that does not receive these signals undergoes epidermal commitment, and cells at the interface between neurectoderm and epiderm can commit to the neural crest when also receiving posterior cues (Villanueva et al., 2002). Later in gastrulation at more posterior levels where considerable axis elongation occurs, a common neuromesodermal progenitor gives rise to both spinal cord (SC) neural progenitors, and somitic mesoderm (Gouti et al., 2015). Additionally, cells at the midline of the primitive node invaginate as the node regresses posteriorly, laying down a strip of mesodermal cells that condense into the notochord (Satoh et al., 2012). By the end of

neurulation, from the level of the midbrain down, an internalised neural tube lies atop the notochord and in between paraxial somites. These structures play roles as local organising centers that release sonic hedgehog (SHH) and retinoic acid (RA) respectively, which are themselves both effective neuralisers (Bain et al., 1995; Maye et al., 2004).

Even at this early stage the neurectoderm has acquired significant anterior-posterior (AP) patterning (Pfeffer et al., 2000; Rowitch and McMahon, 1995; Schwarz et al., 1999), and interestingly this regionalisation partly occurs also in the absence of gastrulation (Liguori et al., 2003). The anterior-posterior and dorsal-ventral identity of neural progenitors is subsequently refined. During and following gastrulation, caudal gradients of the SC AP morphogens RA (Durstion et al., 1989), Fgf2 (Kengaku and Okamoto, 1995), and GDF11 (Liu, 2006), do not impact the vMB, where mesencephalic DA neurons arise. Instead, cooperative actions between Fgf8 (Chi et al., 2003; Guo et al., 2010; Meyers et al., 1998) and Wnt-1 (McMahon and Bradley, 1990; McMahon et al., 1992; Thomas and Capecchi, 1990) initiate formation of the Isthmus Organiser (IsO). Over time the IsO is refined to a tight boundary through cross repressive mechanisms between Otx2 and Gbx2 (Millet et al., 1999), defining the border between the midbrain and the hindbrain (Hidalgo-Sánchez et al., 1999; Li and Joyner, 2001). In addition to IsO establishment, Fgf8 secretion is also crucial for the specification of vMB DA neurons and posteriorly adjacent ventral hindbrain (vHB) serotonergic neurons (Guo et al., 2010; Ye et al., 1998). Roof-plate BMP gradients also do not reach to the vMB (Chizhikov and Millen, 2004). Instead, SHH secreted from the notochord imparts a crucial ventral midline floorplate identity, an organiser structure that is non-neurogenic at all AP levels except for the vMB (Joksimovic et al., 2009b). In the vHB and SC, strong early notochordal SHH signaling induces non-neurogenic floorplate (Ribes et al., 2010), while floorplate KO of SHH induces ectopic neurogenesis throughout the AP-axis (Joksimovic et al., 2009b). In particular, SHH induces the transcription factor FoxA2 (Roelink et al., 1995), which works in a feedforward loop through induction of FoxA1, Lmx1a/b and endogenous SHH to stabilise floorplate identity in the vMB (Ferri et al., 2007; Mavromatakis et al., 2011; Metzakopian et al., 2012). Uniquely in the floorplate of the vMB, Wnt-1 expression overlaps SHH expression, where it antagonises SHH activity and de-represses neurogenesis, promoting DA differentiation (Joksimovic et al., 2009b; Tang et al., 2010). In line with this, forced expression of Wnt-1 in the non-neurogenic rostral hindbrain floorplate induces ectopic DA neurogenesis (Joksimovic et al., 2009b; 2012; Prakash et al., 2006). However, SHH activity, while crucial for early ventral midline patterning, is unnecessary for later DA development and is downregulated by E11.5, while a requirement for Wnt-1 signaling persists (Arenas, 2014; Joksimovic et al., 2009b; Mesman et al., 2014).

The coordinated and combinatorial action of SHH and Wnt-1 induce the expression of Lmx1a at the midline of the vMB (Andersson et al., 2006), a crucial transcriptional determinant for DA progenitors, beginning around E9.0. Lmx1a expression is first preceded by its paralogue Lmx1b, whose expression extends more laterally. Lmx1a/b act cooperatively in the vMB to maintain Wnt-1 expression and specify DA neurons (Chung et al., 2009). Here, a feedforward loop ensues, where Wnt-1 mediated induction of Lmx1a in turn induces further expression of Wnt-1, Msx1, Lmx1b, and itself. Lmx1b also induces expression of Wnt-1, Msx1, Lmx1a, but not itself. Further, Msx-1 expression in lateral Lmx1a progenitors is crucial for the repression of neighboring non-DA progenitors, and its loss results in decreased DA neurogenesis and the ventral expansion of lateral Nkx6-2 expression. Thus, significant but incomplete redundancy exists between Lmx1a and Lmx1b; Lmx1a KO results in a modest reduction of DA neurons, floorplate specific KO of Lmx1b has minimal impact of DA neuron numbers, while KO of both causes an almost complete loss of DA neurogenesis (Deng et al., 2011; Yan et al., 2011). Further, Lmx1a expression is required for postmitotic Lmx1b expression (Andersson et al., 2006), while midline DA neurogenesis appears particularly sensitive to the loss of Lmx1a (Deng et al., 2011).

Once specified, proliferating DA progenitors are next required to initiate cell cycle exit, thereby generating post-mitotic neuroblasts that migrate to their final destination. The pro-neural beta-helix-loop-helix (bHLH) gene Mash1 is expressed broadly in vMB progenitors, and precedes expression of another proneural bHLH gene, Ngn2 (Andersson et al., 2006). Ngn2 can substitute for Mash1 during DA neurogenesis, but not vice versa (Kele et al., 2006). Additionally, Nato3 is required at the midline, where it antagonises Hes1 activity, de-repressing Ngn2 and facilitating neurogenesis (Ono et al., 2010). Upon cell cycle exit, first occurring around E10.0 in the mouse and finishing around E14.5, Lmx1 activity in DA neuroblasts upregulates Nr4a2 and Pitx3 (Chung et al., 2009), both crucial TFs required for DA neuron development (Nunes et al., 2003; Zetterström et al., 1997) and expression of mature DA neurotransmitter genes including Tyrosine Hydroxylase (TH), the Dopamine Transporter (DAT), and the monoamine transporter VMAT2 (Kadkhodaei et al., 2009; Prakash and Wurst, 2006). Further neuroblast migration is achieved through the action of the Cxcr4 receptor, responding to Cxcl12 ligand that is secreted from the underlying meninges (Yang et al., 2013), while axonal targeting of DA neurons cells to the striatum is driven in part through netrin-1/DCC (Li et al., 2014) and slit/Robo interactions (Lin et al., 2005).

1.4 Dopaminergic subtypes and basal ganglia circuitry

Three major populations of DA neurons exist in the vMB of adult mice; the retrorubral field (RRF), Substantia Nigra pars Compacta (SNc) and Ventral Tegmental Area (VTA), designated A8, A9 and A10 respectively (Björklund and Dunnett, 2007). During embryonic stages, clear distinctions exist

between SNc and VTA progenitors (Panman et al., 2014), while at the transcriptional level finer subdivisions emerge at later embryonic and early postnatal periods (La Manno et al., 2016; Poulin et al., 2014). However, further resolution of these novel subtype-specific transcriptional profiles and their relationship to existing anatomical and functional data, will be required to understand how these novel DA subtypes influence specific animal behaviors. That said, of note to the use of stem cells in PD, SNc DA neurons are enriched for *Aldh1a1*, *Girk2*, *Sox6*, and *Glyco-DAT* expression, while VTA neurons are enriched for *Calb1* and *Otx2* expression (Di Salvio et al., 2010; La Manno et al., 2016; Panman et al., 2014; Poulin et al., 2014). Interestingly, SNc neurogenesis peaks slightly earlier in the window of DA neurogenesis, while VTA neurons are born later (Bayer et al., 1995; Bye et al., 2012). VTA DA populations preferentially project to cortex, hippocampus, lateral septum, lateral habenula, locus coeruleus, nucleus accumbens, amygdala and ventral striatum. These same neurons receive inputs from the lateral hypothalamus, paraventricular nucleus, nucleus accumbens, ventral pallidum and the amygdala, implicating VTA DA neurons in emotive behaviors, cognition and reward. In contrast, SNc neurons preferentially project to the dorsal striatum and receive inputs from motor and sensory cortex, central amygdala, globus pallidus, subthalamic nucleus and the dorsal striatum, implicating SNc DA neurons in modulation of motor control (Arenas et al., 2015; Watabe-Uchida et al., 2012). SNc projections to the striatum represent vMB DA contribution to the basal ganglia circuitry, a network of centers that controls motor output, including the Subthalamic Nucleus (STN), internal and external segments of the globus pallidus (GP) and the putamen, caudate and accumbens nuclei of the striatum. The connections between these structures are broadly classified into the direct or indirect pathways (Yelnikoff et al., 2014). DA activation of D1 dopamine receptor expressing medium spiny neurons (MSNs) increases intracellular cyclic-AMP, thereby increasing direct pathway excitability, while the reverse occurs after DA activation of D2 dopamine receptor expressing MSNs of the indirect pathway (Kravitz and Kreitzer, 2012). In line with this, optogenetic activation of the direct pathway promotes movement, while activation of the indirect pathway inhibits movement (Kravitz et al., 2010). Further, this action appears to be achieved through rapid phasic DA release by dorsal-striatum projecting SNc neurons, while the same rapid phasic activity in ventral-striatum projecting VTA neurons instead contributes to reward associated behaviors (Howe and Dombeck, 2016). Thus, while VTA DA neurons are involved in a variety of behaviors imparted by their diverse array of both inputs and outputs, dorsal striatum projecting SNc DA neurons function predominantly as a critical component in motor control.

1.5 *Parkinson's Disease*

The first clinical descriptions of PD were detailed by James Parkinson in 1817 where he observed patients who exhibited tremor, rigidity, bradykinesia and impaired gait (Parkinson, 2002). Later, histological descriptions of lesions in the brainstem, in particular in SNc DA neurons, were seen as drivers of motor deficits in PD (GREENFIELD and BOSANQUET, 1953). PD is a degenerative and progressive disease (Hoehn and Yahr, 1967), age is by far the strongest risk factor, while men are one and a half times more likely than women to be affected by the disease (Twelves et al., 2003). At initial diagnosis, patients have likely already undergone significant loss of DA neurotransmission (Kordower et al., 2013). This loss is dramatic, such that 5-10 years after diagnosis only 10-20% of DA neurons remain, a plateau level that can persist for a decade or more. TH and DAT decline slightly ahead of neuromelanin, a pigment identifying midbrain DA neurons, indicating that DA neurotransmission is lost before cell death. Thus, PD patients may live for extended periods of time with reduced levels of dopaminergic neurotransmission, during which symptoms continue to decline.

The major histological hallmark of PD is the presence of SNCA (α -syn) inclusions; Lewy bodies and neurofibrillary tangles present in, but not restricted to, vMB DA neurons (Mezey et al., 1998; Trojanowski and Lee, 1998). α -syn is expressed in many tissues, but is highly expressed in the brain (Jakes et al., 1994). In healthy cells α -syn is soluble, and binds to intracellular lipid membranes at synaptic terminals, the nucleus and mitochondrial membranes (Burré, 2015; Guardia-Laguarta et al., 2015). By contrast, In PD, toxic intracellular insoluble aggregates form from elevated concentrations of WT or aggregation-prone forms of α -syn (Singleton, 2003; Wood et al., 1999). These α -syn aggregates impair their own clearance from the cell through inhibition of endoplasmic reticulum (ER) trafficking and the autophagy-lysosome pathway (Chung et al., 2013; Mazzulli et al., 2016; Tardiff et al., 2013), in addition to disruptions in mitochondrial network homeostasis (Su et al., 2010) or DA specific gene transcription (Decressac et al., 2012). The autophagy-lysosome pathway has been genetically implicated in aging, the strongest risk factor in PD (Lapierre et al., 2015; Salminen and Kaarniranta, 2009). In line with these findings, multiple risk alleles implicated in autophagy-lysosome and mitochondrial homeostasis have been associated with sporadic PD, including LRRP2, GBA1, MAPT, α -syn itself and others (Edwards et al., 2010; Nalls et al., 2014). Further, patient specific induced-PSC derived DA neurons harboring familial PD mutations demonstrate increased α -syn accumulation in PD patients' cells (Chung et al., 2016), mitochondrial dysfunction has been implicated in both familial and sporadic PD (Hsieh et al., 2016) and overexpression of GBA1 or TFEB boost autophagy and can ameliorate motor deficits in PD models (Decressac et al., 2013; Rocha et al., 2015a; 2015b). Interestingly, when compared to

PD resistant VTA neurons, PD susceptible SNc neurons appear to have an intrinsically higher energy demand, higher axonal mitochondrial concentration, and a resulting lower energy reserve capacity, leading to higher production of reactive oxygen species (ROS) (Pacelli et al., 2015). Thus, the selective degeneration of SNc neurons in PD may be an unfortunate situation of SNc specific high ROS load and subsequent susceptibility to age induced impairment of ER-Golgi, autophagy-lysosome and mitochondrial homeostasis functions. Deterioration of these processes may then trigger aggregation of α -syn, leading to a feed-forward accumulation of α -syn in Lewy bodies and neurofibrillary tangles and associated cellular toxicity. Presence of any PD susceptibility alleles may then accelerate this process.

Adult specific KO of the DA transcription factors also leads to DA neuron dysfunction; *Nurr1*, *Lmx1a* and *Lmx1b* KO lead to mitochondrial and autophagy-lysosome dysfunction (Doucet-Beaupré et al., 2016; Kadkhodaei et al., 2013; Laguna et al., 2015), while *En1* +/- animals exhibit adult onset degeneration of SNc neurons and impaired translation of mitochondrial transcripts (Alvarez-Fischer et al., 2011; Sonnier et al., 2007). Thus, these cellular housekeeping functions are, at least in part, controlled by developmental transcription factors that remain expressed at adult stages (Doucet-Beaupré and L vesque, 2013). Thereby these TFs may also constitute targets for ameliorating α -syn accumulation in PD settings.

vMB DA neurons are among several brain centers that degenerate in PD (Hedlund and Perlmann, 2009), and interestingly, many neurodegenerative disorders are believed to be propagative (Brettschneider et al., 2015). α -syn aggregates have been seen in grafted TH+ve cells of PD patients transplanted with young fetal derived tissue, where pathology has presumably spread from the host (Brundin et al., 2008). Clinical observations have noted α -syn aggregates are first seen in the brainstem and olfactory bulb, which then spread to the midbrain and cortex (Braak et al., 2003). One school of thought posits that gut environment induced stress can induce α -syn aggregates in the glossopharyngeal and vagal nerves, that then spreads to their projection targets in the brainstem and onwards (Pan-Montojo et al., 2010; Svensson et al., 2015). In line with this, recent studies in mice argue that the gut microflora of PD patients, as opposed to microflora from healthy patients, induces microglial activation in the vMB, and when combined with a genetic mouse model for PD that overexpresses α -syn, causes cellular stress and exacerbates motor deficits (Sampson et al., 2016). Future research will hopefully enlighten the contribution of intracellular mechanisms of α -syn function and such initiation and propagation pathways of α -syn aggregation to PD pathology.

As mentioned previously, loss of striatal DA in PD decreases direct pathway activity and increases indirect pathway activity, leading to the aforementioned motor deficits. L-dopamine (L-DOPA) is a

major first-line treatment, where orally administration of this precursor to DA restores basal ganglia function. Discouragingly, long term use in PD patients can lead to L-DOPA induced dyskinesias (LIDs), uncontrolled movements arising from a combination of L-DOPA administration to a PD altered basal ganglia circuitry (Jenner, 2008). Alternatively, deep brain stimulation through surgical implants of pace-maker probes into either STN or GP dampen activity in these indirect pathway centers and amelioration motor deficits (Benabid et al., 2009). However patient response to deep brain stimulation can diminish over time, even when combined with L-DOPA treatment. For both treatments PD disease symptoms continue to decline. Thus, additional avenues of therapeutic PD treatments warrant exploration.

1.6 Derivation of dopamine neurons from stem cells

The early success of orally administered L-DOPA in alleviating motor symptoms lead to clinical trials of transplantations of human fetal vMB tissue into the brains of PD patients, with the hope of restoring DA neurotransmission (Barker et al., 2013; Lindvall and Björklund, 2004). Unfortunately, after extensive trials, success was variable. While some patients displayed improvements, others did not, and frustratingly, failure to improve can occur even after successful engraftment of fetal DA cells (Kordower et al., 2016). Other detrimental side effects include graft induced dyskinesias (GIDs) arising from contaminating serotonergic neurons unwittingly included in transplanted cells (Politis et al., 2010). In general, older PD patients, likely already experiencing dramatic loss of DA neurons, display diminished improvement when compared to younger patients. Further, patients whose DA neurotransmission loss was restricted to only the striatum, displayed higher improvement when compared to patients exhibiting DA neurotransmission loss also in other brain regions. Thus, current trials hope that patient pre-selection through stratification along such parameters, choosing young individuals with predominantly nigrostriatal DA loss who do not exhibit LIDs, may decrease variability and improve transplantation outcomes.

The logistical and ethical hurdles of using fetal derived DA cells has motivated the long-standing goal of deriving DA neurons *in vitro* from human pluripotent stem cells (PSCs). DA differentiation protocols have improved significantly in recent years, with more accurate recapitulation of embryonic vMB patterning. Manipulation of key aforementioned morphogens is achieved using proteins or small molecules that activate Shh signaling (Fasano et al., 2010) to impart a floorplate identity, and Wnt (Castelo-Branco et al., 2004; Kirkeby et al., 2012; Kriks et al., 2011) and Fgf8 signaling (Cooper et al., 2010; Kriks et al., 2011; Xi et al., 2012) to impart a midbrain identity. Further, significant pivotal improvements were also made through improved neurectodermal commitment (Chambers et al., 2009) at earlier stages of differentiation. Typically, even under these vMB patterning conditions, differentiations yield a mixture of neuronal types. However, forced

expression of *Lmx1a* can substitute for these imperfect morphogen conditions, increasing DA purity (Andersson et al., 2006). Further, forced expression of additional DA transcription factors *En1* and *Otx2* can induce TH⁺ve neurons in morphogen conditions not previously conducive to DA neurogenesis (**paper I**). Moreover, interrogation of vMB development using single-cell RNA sequencing has revealed that mouse ESCs differentiated under *Shh/Wnt-1/Fgf8* conditions generates two distinct lineages of *Lmx1a* expressing cells, with a *Lmx1a/Lmx1b/FoxA1/FoxA2/Otx2/Shh/Wnt-1/Wnt-5a* marker profile common to both glutamatergic or dopaminergic *Lmx1a* progenitors (**paper II**). Thus, this study revealed that these common markers previously used to assay successful engineering of DA neurons are in fact not uniquely expressed in DA progenitors. Further, modulation of Wnt-1 signaling can selectively enrich for either of these two populations, with low or high signaling levels enriching for glutamatergic or DA progenitors respectively. Moreover, DA *Lmx1a* progenitors arise caudal to glutamatergic *Lmx1a* progenitors, and appreciation for this rostro-caudal distinction when deriving DA cells from PSCs can improve grafting outcome in mouse PD models (**paper III**).

In addition to morphogen permutation and concentration, the timing of morphogen exposure can drastically alter cell fate choices during stem cell differentiations in many settings (Calder et al., 2015; Dias et al., 2014; Fasano et al., 2010; Gouti et al., 2014; Lippmann et al., 2015; Takasato et al., 2015), in principle due to temporal competence windows within which a cell expresses appropriate signal transduction machinery for the desired morphogen interpretation. Elegant molecular and computational tools have been developed to allow read out of the exact temporal and quantitative exposure of single cells to *Shh* signaling (Balaskas et al., 2012; Uhde and Ericson, 2016). Application of such approaches for *Shh*, *Wnt* and *Fgf8* signaling holds future potential to further resolve patterning events in DA development. Of note, the maturation stage of cells upon engraftment influences their transplantation efficacy (Ganat et al., 2012) and thus care must also be taken to reliably stage differentiations before grafting. Finally, co-transplantation of vMB derived DA progenitors with meningeal cells significantly improved TH⁺ve yield, possibly through a *Cxcr4* dependent mechanism (Soma et al., 2015), demonstrating that trophic support of grafted DA cells has high, and perhaps comparatively unexplored, potential to improve transplant outcome.

Much progress has been made in specifying DA progenitors *in vitro*, such that current protocols for human embryonic stem cell (ESC) derived DA cell grafts display impressive amelioration of movement deficits in animal PD models (Doi et al., 2014; Jaeger et al., 2011; Kirkeby et al., 2012; Kriks et al., 2011); **paper III**). However, less is known regarding how these cells behave upon and after grafting. Rat models of PD show that restoration of amphetamine induced rotation can be achieved with as few as 500-1000 graft derived TH⁺ve cells (Grealish et al., 2014) and this

reduction is as effective as for human fetal vMB tissue. In line with this, fetal grafts containing roughly 140,000 surviving TH+ve cells can induce successful motor improvement after transplantation into a PD patient (Kordower et al., 1998). Encouragingly, graft activity may be fine-tunable through administration of small molecules or optogenetic approaches designed to boost or dampen DA neuron firing (Chen et al., 2016; Dell'Anno et al., 2014; Steinbeck et al., 2015), or inhibit serotonergic neuron firing (Politis et al., 2011) in addition to assessing graft behavior. However, complications can arise. Akin to GIDs in fetal transplanted PD patients, rodent PD models can display upregulation of the serotonin receptor 5HT-6 in transplanted DA neurons, leading to serotonergic mediated stimulation of grafted DA neurons from neighboring contaminating serotonergic neurons, which can then lead to uncontrolled dopamine release and uncontrolled movements (Aldrin-Kirk et al., 2016). Moreover, after transplantation there is comparatively little control over the coordinated migration, axonogenesis, or synaptic and dendritic maturation of transplanted cells (Steinbeck and Studer, 2015). Thus, graft heterogeneity upon transplantation, controlled maturation of grafted cells after transplantation and the aforementioned selective enrichment for SNc DA progenitors, remain major hurdles.

1.7 Single-cell transcriptomics

Reduction of graft heterogeneity will require thorough and reliable identification of progenitor lineage identity prior to transplantation in PD. Historically, early detailed histological analysis of tissues utilised chemically based stains such as silver stains, haematoxylin and eosin staining. Impressively complex biological phenomena have been inferred from the morphology of these snapshots of tissues. Subsequent development of *in situ* hybridisation and immunohistochemistry technologies have allowed for more sophisticated gene-product centric cell type classification. However, the clear constraint in these approaches is the requirement for *a priori* selection of which genes to investigate, and thus the process is susceptible to bias. *In silico* gene expression analysis of next generation sequencing (NGS) data, including mRNA transcriptomes (Wang et al., 2009) or translatomes (King and Gerber, 2016), avoids this gene-selection bias. Further advancement of RNA sequencing technologies that can work with low starting material (Hashimshony et al., 2016; 2012; Islam et al., 2014; Picelli et al., 2013; Ramsköld et al., 2012) have now made transcriptome profiling of single cells possible. Given that a cell's genome is, for the most part, the source of its transcriptome, a single-nucleated cell is the smallest possible biological unit of a tissue. Thus, whole-transcriptome sequencing of single cells, followed by computational analysis, allows resolution of dynamic and heterogeneous tissues such as the developing CNS, where rare, transient or subtly unique cell types have likely gone, and perhaps would always be, unnoticed in bulk RNA analysis.

Single-cell RNA-seq appears particularly powerful when considering the aforementioned future challenges of resolving graft heterogeneity, neuronal maturation, and identification of sub-lineages of DA neurons. Firstly, given that DA stem cell differentiations mimic the morphogen conditions of the developing vMB, contaminating cell types are likely to be from lineages proximal to developing DA neurons, and thus thorough genome-wide single-cell resolution of the developing vMB would facilitate identification of these unwanted cells. Secondly, neurogenesis and likely many other attractor state transitions contain transient cell types, and are thereby challenging to study without single-cell methods. These transitions may be of particular interest, and their elucidation most beneficial, to efficacious stem cell differentiation protocols. Finally, it is possible that DA lineage subtypes may appear almost identical for the vast majority of their transcriptome, in which case gene-by-gene centric interrogation would have limited resolution. Application of single-cell RNA-seq to the *Lmx1a* lineage of the developing vMB thus appears well-suited to inform each of these challenges.

2 AIMS

This thesis aimed to assist efforts targeting the translation of stem-cell derived DA cells into the clinic, through the query of three questions:

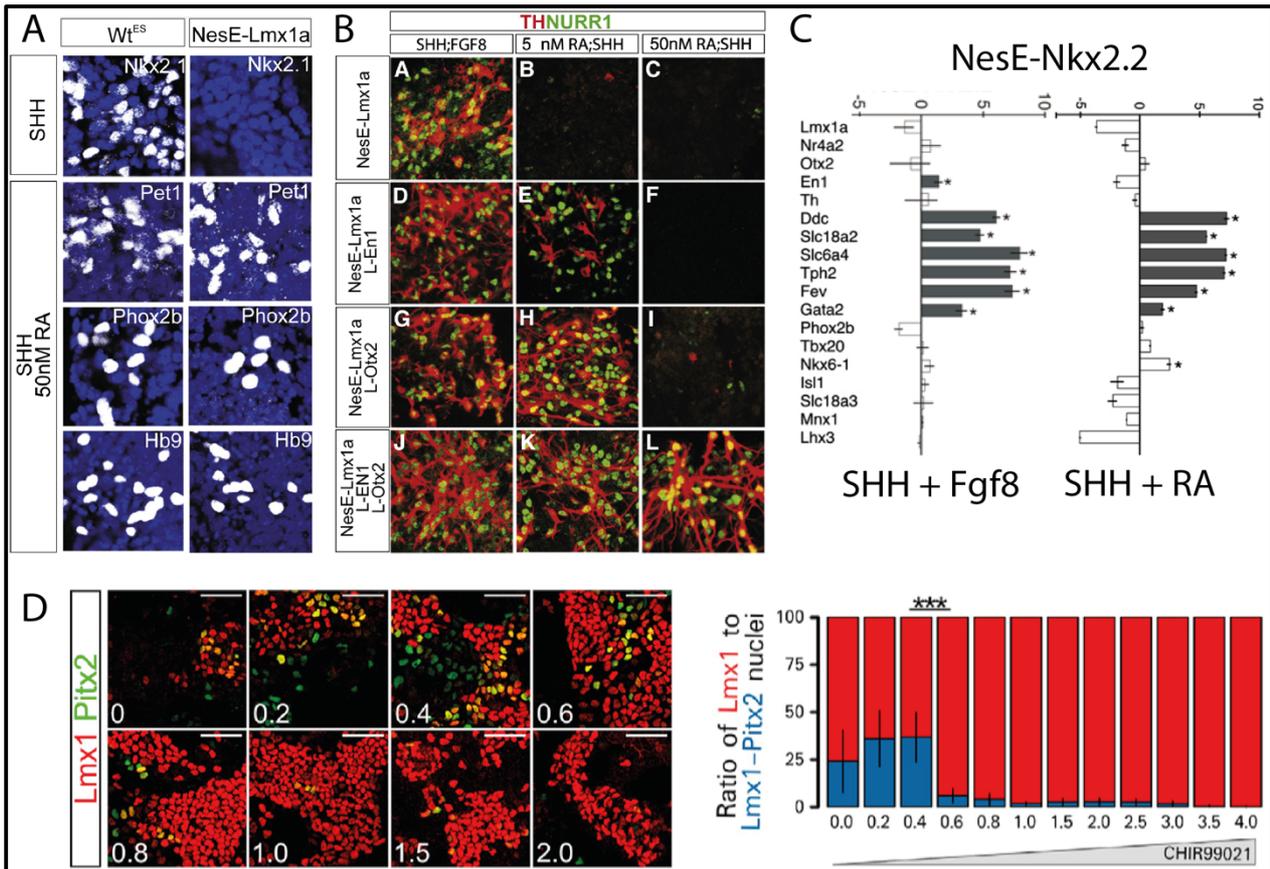
- 1.) To what extent can transcriptional determinants provide instructional cues for neuronal lineage specific commitment during stem cell differentiation?
- 2.) Can single-cell RNA-sequencing resolve Lmx1a-expressing and neighboring neuronal lineages in the developing vMB?
- 3.) Can this resolution inform stem cell differentiations targeting PD?

3 RESULTS AND DISCUSSION

3.1 *Transcription factors and morphogens*

The findings of **paper I** demonstrate that forced expression in neural progenitors of the lineage specific TFs Lmx1a, Phox2b, Olig2 and Nkx2-2 can derive highly selective cultures of DA, vMN, sMN and 5HT neurons respectively, and that this enrichment comes at the expense of non-targeted lineages. Importantly this selection is also dependent on morphogen context. For example, forced expression of Lmx1a decreased the expression of non-Lmx1a lineage markers in Shh conditions, but not after addition of RA (Figure 1A), a morphogen that would not normally impact Lmx1a expressing vMB progenitors. In line with these results, Lmx1a could enrich for Nurr1/TH+ve cells in vMB Shh/Fgf8 conditions, but once again not when Fgf8 was replaced with RA (Figure 1B, top row). However, in these high RA conditions Nurr1/TH+ve cells could be derived after forced expression of Lmx1a together with En1 and Otx2 (Figure 1B, bottom row), two additional vMB TFs that presumably overcome the caudalising effect of RA. Further, forced expression of Nkx2.2 enriched for rostral En1+ve cultures containing 5HT neurons when cultured in Shh/Fgf8 conditions, and caudal En1-ve cultures containing 5HT neurons when cultured in Shh/RA conditions (Figure 1C). Finally, findings from **paper II** describe vMB patterned mouse ESC cultures where Lmx1+ve/Pitx2+ve glutamatergic progenitors are enriched when patterned with low Wnt-1 signaling (low concentrations of the GSK3- β inhibitor, CHIR99021), while Lmx1+ve/Pitx2-ve DA progenitors are enriched when patterned with higher Wnt-1 signaling (higher concentrations of CHIR99021) (Figure 1D). Taken together, these data underscore the importance of appreciating early developmental patterning events that, through deployment of morphogen permutations, confer a context specific framework within which a given transcriptional determinant, that may be expressed in additional neuronal lineages across the CNS, can influence a cell's phenotype.

Figure 1. (A) mouse ESCs harboring a transgene driving expression of Lmx1a under the Nestin enhancer (Nes-Lmx1a) display reduced levels of the non-DA marker Nkx2.1 under SHH conditions, however under SHH/RA conditions non-DA markers Pet1, Phox2b, Hb9 are not reduced. (B - top row) Nes-Lmx1a cells derive TH+ve/Nurr1+ve DA neurons in SHH/Fgf8 conditions, but not when Fgf8 is swapped for RA. (B - bottom row) DA neurogenesis is restored in Nes-Lmx1a cells under RA conditions after lentiviral mediated forced expression of En1 and Otx2. (C) Serotonergic markers are enriched alongside the rhombomere 1 serotonergic marker En1 only in SHH/Fgf8 conditions. (D) Lmx1+ve/Pitx2+ve double positive cells are only observed at low levels of CHIR99021 (left panel), and that are drastically reduced at 0.6uM CHIR99021 and above (right panel).



3.2 Glutamatergic rostral-Lmx1a lineages

The findings of **paper II**, in particular the *Lmx1a*-CreERT2 mediated genetic labelling at E9.5 (Figure 2), strongly point to a ventral diencephalic origin of glutamatergic *Lmx1a*/*Pitx2*, *Lmx1a*/*Barhl1* or *Lmx1a*/*Pitx2*/*Barhl1* expressing hypothalamic neural populations. The thalamus and hypothalamus have historically not been hotspots for developmental biologists, possibly owing to the fact that development of these centers often involves extensive tangential and radial migration and intermingling of progenitors from different lineages, unlike the attractive ordered spatiotemporal patterning of the spinal cord or developing pyramidal neurons of the cortex. STN function continues to be investigated, owing to its role in basal ganglia function. However functional roles for the retromammillary nucleus, premammillary nucleus and posterior hypothalamic area, are less clear. The new marker profiles from **paper II** (e.g. *Lmx1*, *Barhl1/2*, *Pitx2*) will hopefully help guide future efforts exploring the functions of these previously ambiguous populations, including intersectional genetic approaches to derive conditional KO's, or cell-type specific excitatory or inhibitory optogenetic manipulations. Additionally, single-cell RNA-seq of this glutamatergic *Lmx1a* family may uncover further subtype specific markers.

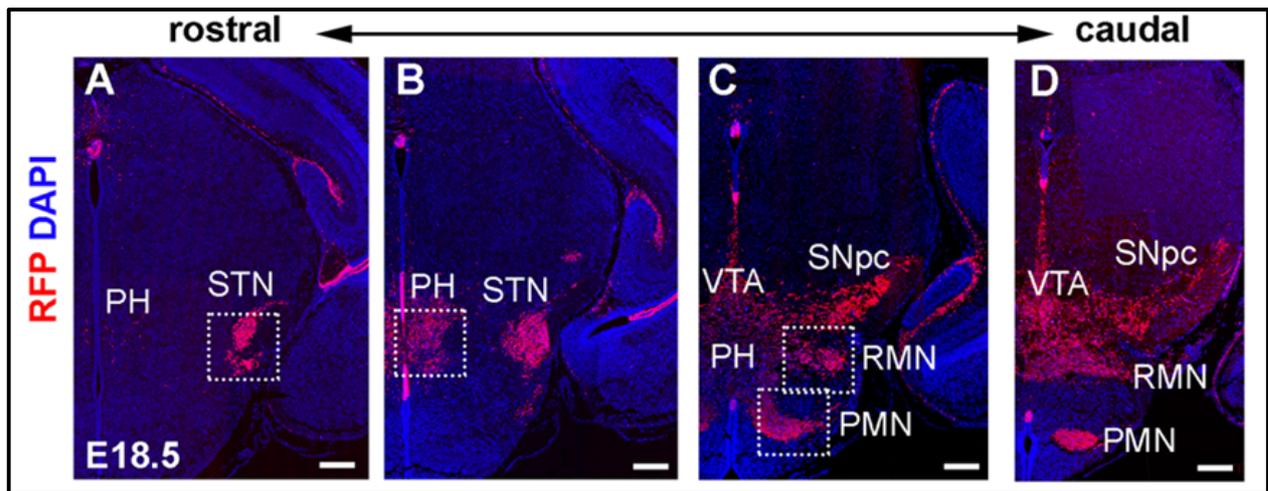


Figure 2. Genetic labelling using $Lmx1a^{CreERT2/+};R26^{TrapC/+}$ mice. Pregnant mothers were tamoxifen treated at E9.5 and coronal sections were analysed from E18.5 pups. Labeled populations include i) rostral glutamatergic $Lmx1a$ populations: the subthalamic nucleus (STN), posterior hypothalamic (PH) area, retromammillary nucleus (RMN), and premammillary nucleus (PMN), and ii) caudal DA $Lmx1a$ populations: the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNpc).

3.3 Developmental biology

Transcriptional determinants that select for DA neurons have been sought-after within the PD field for many years. Upon embarking on the single cell project, we had wondered if new players could be found, but were, perhaps naively, somewhat disappointed to find no new TFs standing out in the DA lineage. It is impressive that the seemingly old-school developmental biology studies based on mutagenic mouse screens, widely used before NGS methodologies, were comprehensive enough to identify so many key protein coding transcriptional determinants, albeit for what nowadays may be considered broad neurotransmitter and/or anatomically defined classifications of neural populations. Interestingly, 3' UTRs (Ramsköld et al., 2009), alternative splicing (Barbosa-Morais et al., 2012), miRNAs (O'Carroll and Schaefer, 2013) and lncRNAs (Cabili et al., 2011) are particularly diverse in the brain, reminiscent of the impressive diversity of CNS neural subtypes, suggesting that precise dynamic regulation of protein coding genes may contribute to this diversity. One can imagine that slightly different levels of expression of a given TF could drive varying downstream arrays of migration, axon-guidance and dendritic arborisation molecules that could have profound effects on a neuronal populations' development, and thereby its functional contribution to behavior. In this light one can appreciate that there exist orders of magnitude fewer TFs than neuronal cell types, and that functional diversity is generated through other mechanisms. Further, in this same way, elucidation of vMB DA sub-lineages may require a high appreciation for levels of TFs and their various isoforms, expression of non-coding RNAs and miRNAs, cellular

context within which neurons are born and their subsequent environmental stimuli, as well as the aforementioned temporal influence of developmental morphogens. The rabbit hole could well be much deeper still, and it is likely that developmental biology will continue to inform stem-cell derivation of DA neurons and other clinically relevant cell types.

3.4 PSC derived DA neurons

There remains potential for further improvement in DA differentiations targeting PD, including overall DA yield and enrichment for dorsal-striatum projecting SNc DA neurons. Of note here, the results of **paper II** and **paper III** describe marker sets that can distinguish undesired rostral glutamatergic Lmx1/Otx2/FoxA2 progenitors of Axis-2 (Barhl1, Pitx2, Epha3, Wnt8b, Dbx1) from desired caudal dopaminergic Lmx1/Otx2/FoxA2 progenitors of Axis-1 (En1/2, Cnpy1) (Figure 3). Importantly, these marker sets also have predictive capability when forecasting grafting outcomes in rodent PD models (**paper III**).

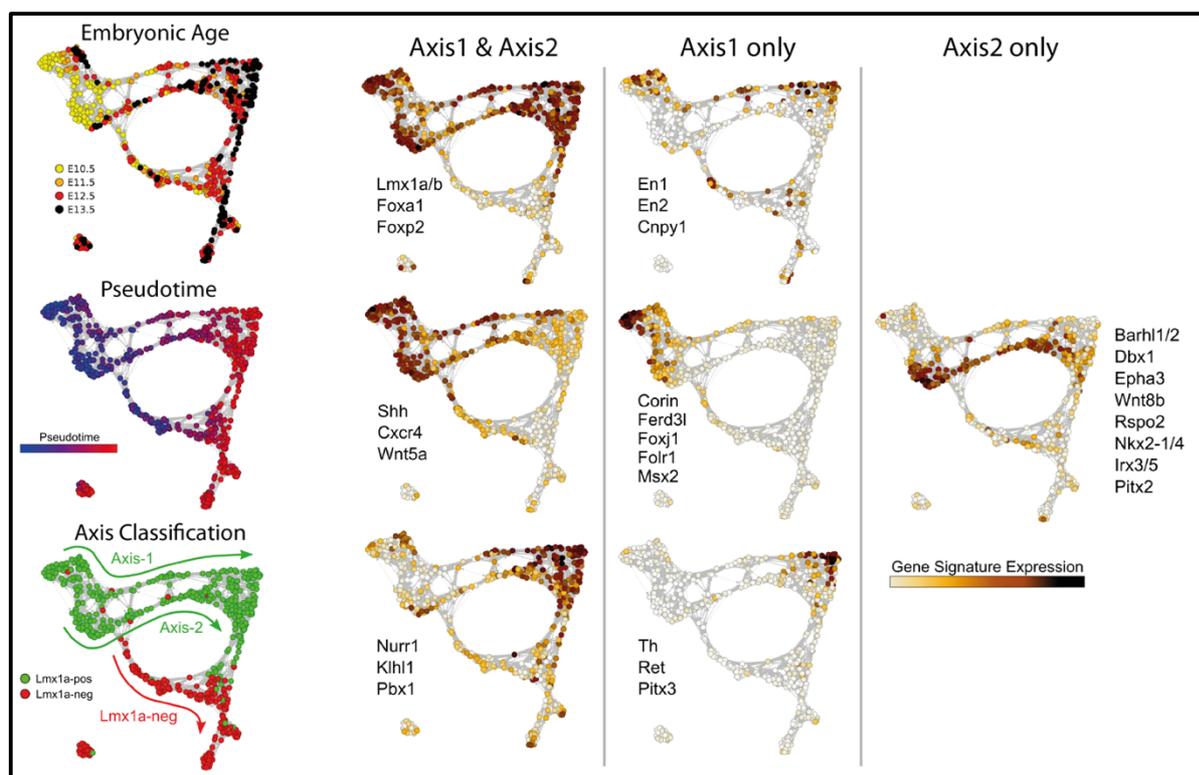


Figure 3. Consideration of cells' embryonic age, pseudotime value and axis classification divided gene sets into three groups, those expressed in (i) both Axis-1 and Axis2, (ii) Axis-1 only and (iii) Axis-2 only.

Interestingly, the single-cell data confirmed earlier observations that, while FoxA2 is expressed both in and lateral to Lmx1a progenitors, FoxA1 appears more specific to the Lmx1a domain (Mavromatakis et al., 2011)(Figure 4). In this light, the timing, duration, and strength of SHH

signaling and its relationship to FoxA1 and FoxA2 levels, may further inform DA progenitor induction and possibly also medio-lateral DA-subtype specification (Joksimovic et al., 2009a). Additionally, while Fgf8 has previously been used during derivation of DA neurons from ESCs (Xi et al., 2012), not all protocols include it (Nielis et al., 2016), possibly due to Fgf8 secretion from caudal IsO cells present in these cultures. Given Fgf8's role in specifying for IsO proximal populations, in particular caudal midbrain DA progenitors, high resolution temporal and dosage assessment of the influence of Fgf8 may bring further improvements in DA yield. Finally, in the single cell data, En1/2 are non-ubiquitously expressed in DA progenitors. Keeping in mind that single-cell RNA-seq protocols can struggle to detect lowly expressed genes, En1/2 expression levels are an inviting starting point to seek out possible DA sub-lineages.

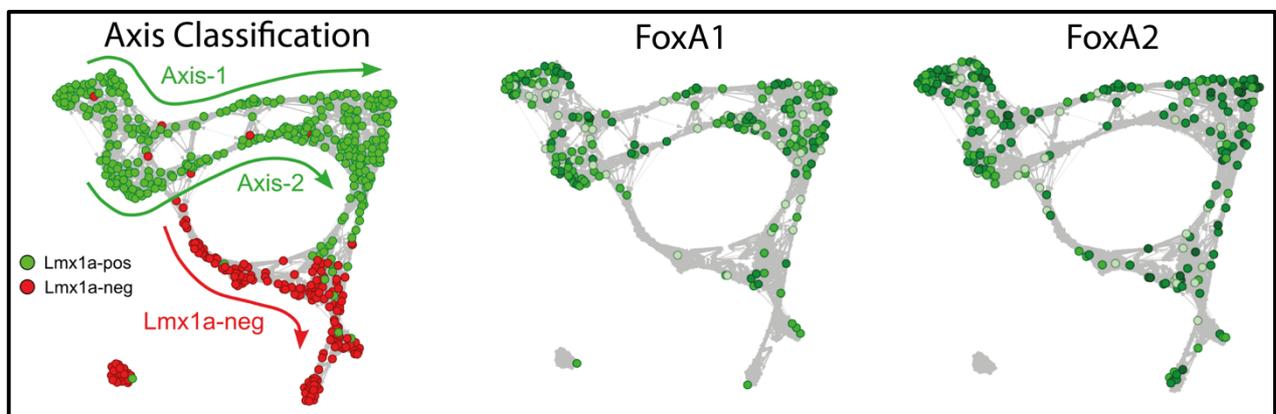


Figure 4. When considering the Lmx1a-neg lineage, FoxA1 is expressed in fewer cells when compared to FoxA2.

3.5 Single-cell RNA-sequencing musings - How many cells? Which genes?

- When a single-cell analysis has been 100% successful, the genes that were considered computationally proved to be informative enough to correctly identify the array of cells that were picked. Take a simplistic example where 500,000 cells are being analysed, within which are a subtype with only three cells present. If only a single gene exists that is exclusively expressed in these three cells, then by considering only this one gene you will correctly identify the group. Unfortunately, however, at the outset of an exploratory project the exact composition of the collected cells is unknown, nor is it known exactly what genes will best inform their identification.

So how does one extract informative genes? Considering the most variable genes is a proven approach, but did not always capture expected lineage genes in our hands. For example, the top 4500 variable genes did not include multiple genes from the Lineage Intersect gene list, (**paper II Figure S2E**), including *Foxa1*, *Corin*, *En1*, *Msx1/2*, *Gata3*, *Isl1*, *Dlx1/2/5*, *Gad1/2*, *Nkx2-2/4*,

Nkx6-1 or *Pax2/5/6*. Neither were over half of the 1354 lineage genes derived from the WGCNA analysis (**paper II Figure S4**). Instead, the top 4500 variable genes comprised mostly of pan-neural differentiation genes. This is not unexpected, given that the neural differentiation trajectory is a large feature of our data set. Also, this difficulty would not be present in data sets containing cell types comprising a single maturation stage. However, it is perhaps an inherent challenge to consider when using single-cell RNA-seq of primary developing tissues to inform stem-cell differentiation protocols aimed at *in vitro* derivation of these same tissues. In these cases, other approaches to derive informative genes that are not involved in differentiation could be considered, as described below. Finally, due to dropout and/or transcriptional bursting (Deng et al., 2014; Picelli et al., 2013), lowly expressed genes may only be sparsely detected, or worse never detected. If a data set's informative genes are, unluckily, not expressed at high levels, then low-coverage sequencing of large numbers of cells may not be as powerful as high-coverage sequencing of fewer cells.

- Clearly it is great if all needed informative genes can be derived from the single-cell data set itself. However, prior or parallel knowledge of gene expression profiles may also prove useful, so long as the possibility of bias is always kept in mind. We found that consideration of the Lineage Intersect list, a rather small gene list of 171 genes, was most informative. Many Axis-1 (*En1*, *Plekhg1*, *Sash1*, *Samd5*, *Foxj1*, *Msx2*, *Cthrc1*, *Ccdc3*, *Plxdc2*, *Bmp7*, *Mlf1*, *Whrn*, *Corin*, *Parm1*, *Folr1*, *Mcc*, *Slc18a2*, *Slc10a4*) and Axis-2 (*Rspo2*, *Epha3*, *Wnt8b*, *Barhl1*, *Nkx2-4*, *Barhl2*, *Dbx1*) confined genes were present on this list. If these genes are removed, the Lmx1a DA and glutamatergic lineages become intermingled as indicated by *En1* and *Barhl1* expression respectively (Figure 5). Thus, analysis of bulk samples containing unknown sub-lineages can uncover sub-lineage specific markers (Usoskin et al., 2015). Moreover, single-cell analysis that utilises prior published gene expression profiles could, in the same way as the Lineage Intersect list, also prove informative.

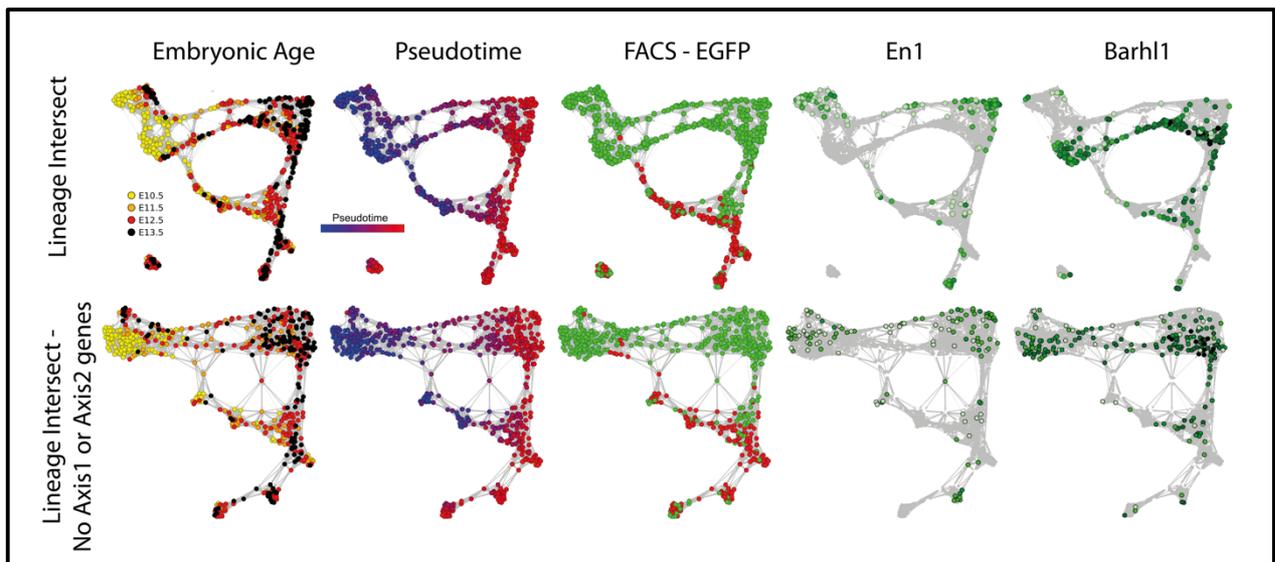


Figure 5. Compared to the complete Lineage Intersect gene list (top), removal of Axis1 and Axis2 genes (bottom) from the analysis leads to mingling of *En1* and *Barhl1* expressing lineages.

- Context is influential, such that big transcriptional differences will drown out subtle differences. To mitigate this, many iterative approaches have been developed that select informative genes from subsets of cells from the data (Grün et al., 2015; Zeisel et al., 2015). This also proved true for our dataset, where upon consideration of cells within a single cluster of, for example, *Gata3*+ve GABAergic neurons, multiple genes implicated in vMB patterning were found to be expressed by only subsets of cells (Figure 6). While too few cells exist in this dataset to warrant further interrogation of these particular observations, in the future it may be interesting to explore statistically comprehensive iterative graph-based clustering approaches that, for example, (i) create a complete graph of all cells, (ii) isolate individual clusters and re-interrogate cells in this reduced context, (iii) integrate the cell-relationship scores from step i and ii, (iv) connect all cells back together as a complete graph, and (v) iterate this approach until the graph structure ceases to change in a meaningful way.

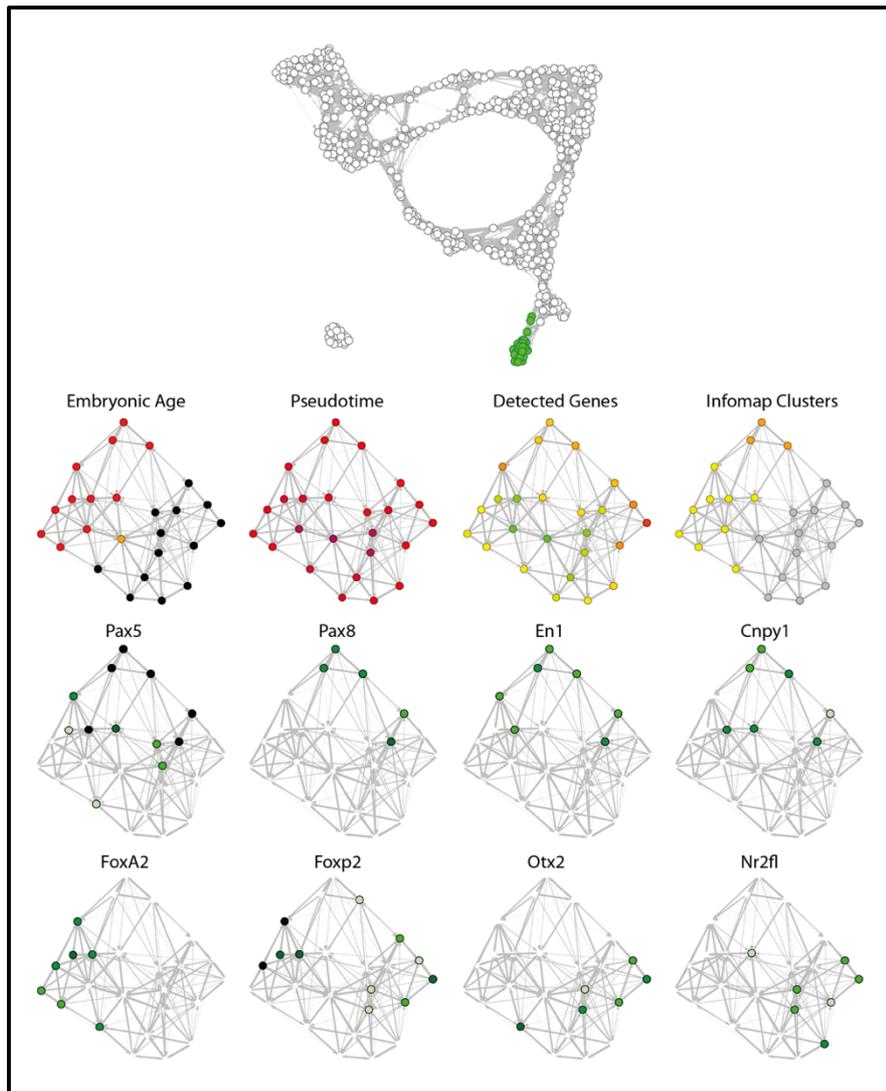


Figure 6. Cells from the green cluster (top graph) were analysed in isolation. MB/HB genes *Pax5*, *Pax8*, *En1* and *Cnpy1* appeared co-expressed in cells towards the top of the graph, while *FoxA2*, *Foxp2*, *Otx2* and *Nr2f1* were expressed in cells at the left or right sides of the graph.

- Many computations consider all genes to be potentially equally informative of cell type, but troublingly, also independent of one another. This is patently incorrect in biology, where many genes are under the control of the same regulatory machinery. If this is not considered, 1000 commonly expressed neural progenitor genes could make two clusters of neural progenitor cells appear much more similar, than 2 differentially expressed lineage specific TF genes would make them look different. From the perspective of a developmental biologist, this is concerning, especially given the biological significance that expression of a single TF can have. A computational consolidation of the 1000 neural progenitor genes down to a single gene co-expression feature has potential to resolve this problem, however, simultaneously introducing the risk of obscuring unrecognised details in an unwittingly inaccurate consolidation paradigm.

3.6 So, how many cells? Which genes?

Empirically, we found it useful to consider a few re-worked questions:

Given a data set that I can afford to generate:

- i) what would I expect to be the biggest differences in the data?*
- ii) in this context, how subtle/strong would be the differences that I am hoping to find?*
- iii) how likely is it that I will be able to (in an unbiased way) extract the genes required to inform my sought-after differences?*

If the cells you hope to find are rare, and their only transcriptional uniqueness is that they express 3 ubiquitously expressed, unsuspected genes at 1.2 times the level present in all other cells, you may need to pick a lot of cells, perhaps tens of thousands. If the cells you hope to find are common and they share high level exclusive expression of 1000 unsuspected genes, then you may need to pick less cells, perhaps 30. In both cases, the better the gene lists you manage to end up considering, the more you will find from the cells that you picked. If you have no preconceived desire to find anything in particular, instead content with an exploratory approach, deeper sequencing may be needed to uncover any differences conferred by lowly expressed genes. Clear as mud.

3.7 Future challenges

More complete transcriptome sequencing chemistries continue to be developed (Abdullayev et al., 2016) that will add a vast array of non-coding genes to single-cell analysis. Genome, methylome, and DNA accessibility data sets from single cells, individually or in parallel, adds further dimensionality to cell identification (Buenrostro et al., 2015; Hou et al., 2016; Hu et al., 2016), as would clonal lineage tracing analysis using cell-barcoding approaches (McKenna et al., 2016). Future protocols may also increase RNA capture efficiency, or decrease sequencing costs, further boosting reliable gene expression detection. To date, single-cell RNA-seq analyses have for the most part focused on cell type classification, with fewer examples aimed at isolating GRNs that drive cell phenotypes (Moignard et al., 2015). Moving forward, one could perhaps imagine a future where reliable cell-type/attractor-state models could be constructed through computational integration of genome-wide transcriptome data, TF/lncRNA consensus and off-consensus DBS profiles, chromatin modification profiles and genome topology. Further modeling that tracks how these cell-types/attractor-states transition through time as lineages develop could provide invaluable tools for future stem-cell based therapeutics.

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