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GENERATION OF CELL DIVERSITY IN THE DEVELOPING BRAINSTEM AND ITS MODELING *IN VITRO*

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Cover illustration: schematic of Nkx2.2^{+} temporal lineage in the ventral hindbrain

Generation of cell diversity in the developing brainstem and its modeling *in vitro*
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family

ABSTRACT

The intersection of spatial and temporal patterning programs underlies the formation of neural cell type diversity in the developing central nervous system (CNS). Deciphering the molecular mechanisms regulating positional and temporal patterning provides a basic framework for how the developing brain can be functionally assembled, and the growing knowledge about these patterning mechanisms offer a powerful tool to effectively control the differentiation of pluripotent stem cells (PSCs) into different neuronal subtypes of clinical importance.

The research described in this thesis is part of a continuing effort to define the molecular mechanisms that modulate the positional and temporal identity of immature neural stem cells (NSCs) and enables these to differentiate into specific subtypes of neurons at defined positions and over specific time-windows in the developing CNS. We have examined the sequential specification of motor neurons (MNs) and serotonergic neurons (5HTNs) by a Nkx2.2^{+} temporal lineage in the ventral hindbrain (HB) and outlined a three-node timer network that conceptually explains how time can be encoded by NSCs. Additionally, and unexpectedly, we show that timed exposure of retinoic acid (RA) can be applied to effectively pattern human PSC (hPSC)-derived neural progenitors into forebrain (FB), midbrain (MB), and HB regional identities. Based on this finding, we developed novel and robust differentiation protocols for production of mesencephalic dopaminergic (mDA) neurons and 5HTNs of the HB. We show that RA-specified human mDA neurons restore motor function after transplantation into a rat model of Parkinson's disease (PD), and that mouse and human 5HTNs can be utilized as cellular platforms to screen small molecules for their capacity to modulate serotonin (5-HT) signaling in 5HTNs.

In **Paper I,** we address how time is encoded by NSCs in temporal patterning processes in the CNS. We focused on a region in the ventral HB where NSCs, defined by expression of the transcription factor (TF) Nkx2.2, sequentially generate MNs, 5HTNs and oligodendrocyte precursors (OLPs). Shh signaling induces the initiation of MN production through induction of the MN-determining TF Phox2b while a delayed activation of transforming growth factor β (Tgfβ) suppresses Phox2b, terminates MN production and trigger the birth of late-born 5HTNs (Dias et al., 2014). In **Paper I** we present a three-node incoherent feed-forward loop (IFFL) circuitry that conceptually explains how time can be measured and set in the Nkx2.2^{+} lineage. By applying a series of *in vivo* and i*n vitro* experiments, in combination with computational modeling, we reveal a progressive decline of *Gli1-3* transcription and bifunctional Gli2-3 TFs over time. Tgfβ is sensitive to transcriptional repressor forms of Gli

proteins (GliR) which prohibit Tgfβ induction by Gli activators (GliA) until GliR has been titrated out. Once activated, the cell non-autonomous activity of Tgfβ counterbalances noise and facilitates a synchronous fate switch of Nkx2.2^{+} NSCs at the population levels.

In **Paper II**, we show that timed delivery of RA can be effectively applied to regionally pattern hPSCs into FB, MB and HB regional territories, in a manner resembling the previously established activity of WNT signaling. However, while WNT signaling is concentration sensitive, it is the duration of RA exposure that is crucial for regional patterning and the response of cells is relatively insensitive to altered RA concentrations. By combining RA- and Shh-signaling we could robustly direct the differentiation of hPSCs into mDA neurons, whose loss underlie motor deficits in PD. When grafted into the striatum of parkinsonian rats, RA-specified cell preparations engraft, differentiate into functional mDA neurons and relieve motor deficits. These data provide proof-of-concept that RA-based protocol for mDA generation could provide a new and alternative route for cell replacement therapy for PD. In addition to mDA neurons, we show that extended exposure of hPSCs to RA results in efficient generation of cranial human MNs and human 5HTNs, suggesting that RA-based regional patterning can be applied to generate several types of clinically relevant neurons from hPSCs.

In **Paper III**, we utilized protocols developed by Dias et al., 2014 and in **Paper II** to produce mouse and human 5HTNs, which dysfunction is strongly linked to various neuropsychiatric disorders and are the target of most prescribed antidepressants. After adaptation of protocols to a screenable format and high-content imaging, we performed an unbiased phenotypic screen to identify small molecules that modulate 5-HT signaling in mouse and human 5HTNs. Out of ~5200 annotated small molecules, we identified and confirmed ~200 hits that modulated 5-HT content in mouse neurons with ~70% of these showing a similar phenotypic response on human 5HTNs. Many hits had previously been associated with the monoaminergic system, but many compounds were not obviously connected to 5HTNs. Among those were the muscarinic acetylcholine receptor (mAChR) antagonist oxybutynin which promoted a notable increase of neuronal 5-HT content and which in subsequent secondary assays acted as an inhibitor of monoamine oxidases in 5HTNs. These data provide proof-of-concept that phenotypic screening on stem cell-derived 5HTNs is a powerful tool to identify new types of compounds with potential antidepressant properties.

LIST OF SCIENTIFIC PAPERS

I. José M. Dias, Zhanna Alekseenko, Ashwini Jeggari, Marcelo Boareto, Jannik Vollmer, **Mariya Kozhevnikova**, Hui Wang, Michael P. Matise, Andrey Alexeyenko, Dagmar Iber, Johan Ericson.
A Shh/Gli-driven three-node timer motif controls temporal identity and fate of neural stem cells.
Science Advances, 2020, 6: eaba8196.

II. Zhanna Alekseenko, José M. Dias*, Andrew F. Adler*, **Mariya Kozhevnikova***, Josina Anna van Lunteren, Sara Nolbrant, Ashwini Jeggari, Svitlana Vasylovska, Takashi Yoshitake, Jan Kehr, Marie Carlén, Andrey Alexeyenko, Malin Parmar, and Johan Ericson.
Robust derivation of transplantable dopamine neurons from human pluripotent stem cells by timed retinoic acid delivery.
Nature Communications, 2022, 13: 3046.

III. **Mariya Kozhevnikova**, Zhanna Alekseenko, Magdalena Otrocka, José M. Dias, Ashwini Jeggari, Natalia Nekhotiaeva, Andrey Alexeyenko, Anna-Lena Gustavsson and Johan Ericson.
A stem cell-based platform for phenotypic identification of compounds modulating serotonin signaling.
Manuscript.

*These authors contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine or serotonin
5HTNs	Serotonergic neurons
6-OHDA	6-hydroxydopamine
AADC	Aromatic amino acid decarboxylase
ALDHs	Retinaldehyde dehydrogenase
AP	Anterior-posterior
AVE	Anterior visceral endoderm
bHLH TFs	Basic-helix-loop-helix transcription factors
BMP	Bone morphogenic protein
CHIR	CHIR99021
CLi	Caudal linear nucleus
CRBPs	Cytoplasmic retinoid binding proteins
CRE	Cis-regulatory elements
DA	Dopaminergic
DAT	Dopamine transporter
DDC	Days in differentiation conditions
DRN	Dorsal raphe nucleus
dSMADi	Dual-SMAD inhibition
DV	Dorso-ventral
FB	Forebrain
FP	Floor plate
GliA	Gli activator
GliFL	Gli full length
GliR	Gli repressor
GPCRs	G protein-coupled receptors
GRNs	Gene regulatory networks
GSK3i	Glycogen synthase kinase 3 inhibitor
HB	Hindbrain
HD TFs	Homeodomain transcription factors
HDAC	Histone deacetylase

(h)PSCs	Human (h) pluripotent stem cells
IFT	Intraflagellar transport
iPSC	Induced pluripotent stem cells
ISH	In situ hybridization
IsO	Isthmic organizer
IZ	Intermediate zone
LP	Lewy pathology
MAO-A	Monoamine oxidase A
MB	Midbrain
mDA	Mesencephalic dopaminergic
MDD	Major depressive disorder
mdFP	Mesodiencephalic floor plate
mdVZ	Mesodiencephalic ventricular zone
mESCs/hESCs	Mouse (m)/human (h) embryonic stem cells
MHB	Midbrain-hindbrain boundary
MNs	Motor neurons
MRN	Median raphe nucleus
MZ	Mantle zone
NC	Notochord
NE	Norepinephrine
NMDA	N-methyl-D-aspartate
NSCs	Neural stem cells
OLPs	Oligodendrocyte precursors
PC	Primary Cilium
PD	Parkinson’s disease
PFC	Prefrontal cortex
Ptch1	Patched 1
RA	Retinoic acid
RAREs	Retinoic acid–response elements
RARs	RA receptors
RBPs	Retinol-binding proteins

RDH/ADH	Retinol/alcohol dehydrogenases
RF	Roff plate
RMg	Raphe magnus nucleus
RNNs	Red nucleus neurons
Rob	Raphe obscurus nucleus
RPa	Raphe pallidus nucleus
RXRs	Retinoid-X receptors
SC	Spinal cord
scRNA-seq	Single-cell RNA sequencing
SERT	5-HT transporter
Shh	Sonic hedgehog
sMNs	Somatic motorneurons
Smo	Smoothened
SNpc	Substantia nigra pars compacta
SNRI	Serotonin norepinephrine reuptake inhibitors
SSRI	Selective serotonin reuptake inhibitor
STN	Subthalamic nucleus
TCA	Tricyclic antidepressants
TFs	Transcription factors
Tgfβ	Transforming growth factor β
TH	Tyrosine hydroxylase
TPH2	Tryptophan hydroxylase 2
VAD	Vitamin A-deficient
vHB	Ventral hindbrain
VLM	Ventrolateral medulla
VLMCs	Vascular leptomeningeal cells
VMAT2	Vesicular monoamine transporter 2
vMB	Ventral midbrain
(v)MNs	Visceral (v) motor neurons
VTA	Ventral tegmental area
ZLI	Zona limitans intrathalamica

1 INTRODUCTION

1.1 THE CONCEPT OF SIGNALING CENTERS IN THE EARLY DEVELOPMENT OF THE CNS

The development of the CNS requires precise coordination between cells in time and space, but the mechanisms underlying cell patterning are complex and not completely understood. The emergence of the CNS takes place during gastrulation (Tam & Behringer, 1997) and is orchestrated by several transient populations of cells, called "partial" organizers, that are spatially and temporally allocated (Martinez Arias & Steventon, 2018; Vieira et al., 2010). These local signaling centers produce biochemical substances called morphogens, that diffuse between cells and evoke specific cellular responses at certain environmental concentrations. The synthesis, release and diffusion of morphogens from these signaling centers provide axial asymmetry and enables to subdivide the embryonic brain into distinct neuroepithelial fields (segments and columns) along anterior-posterior (AP) and dorso-ventral (DV) axis, resulting in the establishment of separate longitudinal and transversal boundaries, respectively (Puelles et al., 2013). A concentration gradient of a morphogen provides cells with positional information where concentration is perceived by transmembrane receptors and translated into the activation of intracellular signaling pathways that, in turn, results in differential expression of target genes encoding activating and repressing TFs (Briscoe & Small, 2015). (Briscoe & Small, 2015).

The synthesis of a morphogen is a dynamic process which creates additional, temporal aspect of pattern formation and as a result, the target gene expression also changes with time (Dessaud et al., 2007). Besides, neuronal patterning is controlled by the molecular mechanisms that are integrated into the transcriptional network motifs that define the timing of target gene expression and the overall precision of patterning (Exelby et al., 2021).

1.2 SHH-MEDIATED SPATIOTEMPORAL PATTERNING OF THE VENTRAL NEURAL TUBE

The DV specification of neural progenitors relies on the establishment of anti-parallel morphogen gradients resulting from the production, secretion, and diffusion of different types of signaling molecules from opposite poles of the neural tube: sonic hedgehog (Shh) which is secreted by the notochord (NC) and the floor plate (FP) cells (ventralizing signal), and bone morphogenic proteins (BMP) and Wnt proteins secreted by the roof plate (RP) cells (dorsalizing signals) (Briscoe & Ericson, 2001; Briscoe & Small, 2015; Ulloa & Martí, 2010) (**Fig. 1**).

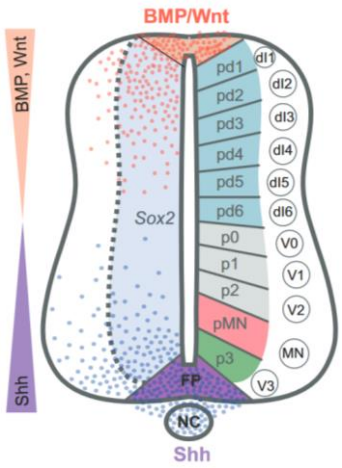

Fig. 1. DV patterning of the neural tube. Opposing gradients of secreted morphogens establish the spatial patterning of the vertebrate neural tube in the form of discrete domains of progenitors (p): ventral p0-p3, pMN and dorsal pd1-pd6 along the DV axis. Shh is released from the NC and FP, while Wnt and BMP from the FP. From Briscoe and Small, Development, 2015, reprinted with permission.

The best-studied role of Shh signaling is to mediate the patterning process in the ventral neural tube that results in the generation of distinct neural progenitor subtypes in stereotypically organized ventral domains (Matise & Wang, 2011; Placzek & Briscoe, 2018). Graded activity of Shh signaling in the neural tube operates in space, through dynamic Shh concentration (Chamberlain et al., 2008; Ericson et al., 1997; Roelink et al., 1995) and in time, through the duration of Shh signaling (Dessaud et al., 2007; Stamataki et al., 2005). How this spatiotemporal gradient of Shh ligand is interpreted at a cellular and molecular level remains unclear. Initially Shh expression occurs in the node and in its derivative, the notochord. Shortly after Shh induces the FP, a transient longitudinal subdivision of the neural tube at the ventral midline that create the ventral polarity within the CNS and extends from the spinal cord (SC) along the HB, MB and terminates in the zona limitans intrathalamica (ZLI) in the caudal diencephalon (Placzek et al., 1993; Vieira et al., 2010).

Shh acts as a long-range morphogen

Shh, secreted from the notochord and the FP, creates a dynamic gradient within the ventral neural tube and mediates its patterning effect as a long-range diffusible peptide acting at a long distance (Briscoe et al., 2001; Chamberlain et al., 2008). Shh gradient triggers in a concentration-dependent fashion the expression of distinct homeodomain (HD) and basic-helix-loop-helix (bHLH) TFs that are involved in inducing and maintaining distinct neuronal subtypes along the DV axis of the neural tube.

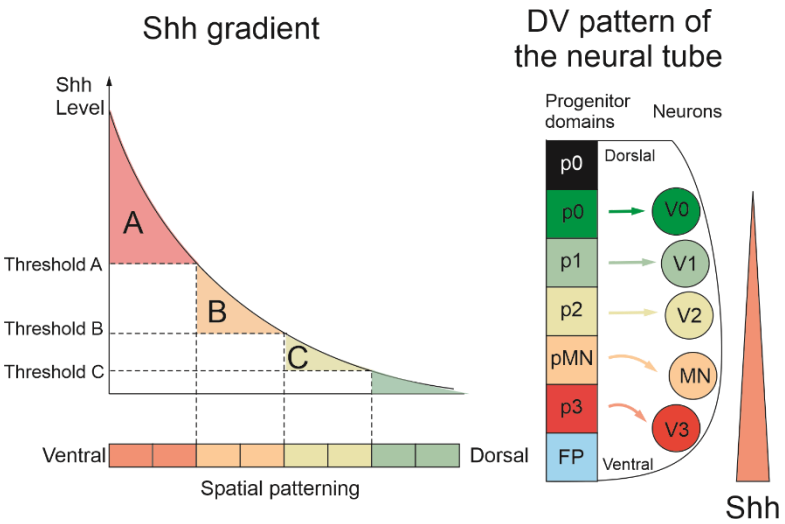

Fig. 2. DV pattern formation in the ventral neural tube by Shh signaling. Distinct thresholds of Shh signaling provide positional information about boundaries between progenitor domains in the ventral tube in a graded manner. Adapted from Dessaud et al., Development, 2008.

The induction of molecularly distinct neural progenitor domains along the neural tube DV axis depends on the threshold requirements for Shh morphogen: more ventral cell types require higher concentrations of Shh than more dorsal (Ericson et al., 1997; Marti et al., 1995; Roelink et al., 1995) (**Fig. 2**).

Production, secretion and diffusion of Shh protein

Glycoprotein Shh is synthesized as a longer precursor protein that undergoes an intramolecular cleavage resulting in a shorter, amino-terminal peptide (ShhNp) that is modified by cholesterol at its carboxy terminus (Porter et al., 1996) and palmitic acid at its amino terminus (Pepinsky et al., 1998) This lipid-modified fragment of Shh precursor protein is responsible for all known signaling activities of the Shh protein (Briscoe & Thérond, 2013). The mode by which modified Shh spreads in the ventral neuroepithelium is not completely resolved. Possibly the spatial dispersion of Shh is facilitated by microtubule-based transport towards the apical surface of the neural progenitors where Shh is accumulated intracellularly and then released (Chamberlain et al., 2008). The diffusion of Shh is facilitated by several extracellular and transmembrane proteins such as enzyme arylsulfatase Sulf1 and heparin sulphate proteoglycans (HSPGs) (Dessaud et al., 2008).

Transduction of Shh signal via Ptch and Smo transmembrane proteins

Cellular response to the Shh signal is governed by Patched 1 (Ptch1) and Smoothened (Smo), where Ptch1 negatively regulates Shh pathway and Smo is a downstream activator, essential for intracellular transduction of the Shh signal. In the absence of Shh, Ptch1 antagonizes Shh signaling by inhibiting Smo activity. When Shh binds to Ptch1, Smo is activated which triggers the activation and translocation of Gli proteins to the nucleus resulting in the transcription of Gli target genes (Carballo et al., 2018; M. H. Chen et al., 2007). Importantly, *Ptch1* is transcriptionally upregulated in response to Shh. Thus, activation of the Shh pathway results in the induction of a negative feedback loop that attenuates Shh signaling by limiting the diffusion of Shh, and therefore the range of direct Shh action within the Shh target field (Dessaud et al., 2007; Jeong & McMahon, 2005; P. Li et al., 2018) (**Fig. 3**).

The central role of primary cilium in Shh signal transduction

Primary cilium (PC) is a highly specialized apical structure emerging from the cell surface of most vertebrate cells and functioning based on bidirectional intraflagellar transport (IFT) machinery (Rosenbaum & Witman, 2002). This structure is essential for sensing and transducing Shh signal (Goetz & Anderson, 2010; Hsiao et al., 2012). In the absence of

ongoing Shh signaling, Ptch1 localizes to the PC. In response to Shh ligand, Ptch1 is internalized and degraded and the disappearance of Ptch1 results in Shh dependent multi-site phosphorylation of carboxy-terminal tail of Smo which, in turn, induces a conformational change of Smo to its active open form and promotes its ciliary localization and accumulation (Chen et al., 2011; Zhao et al., 2007). Ptch1 internalization and Smo activation and translocation to the cilia are a requisite for Gli activation and are associated with ongoing Shh signaling (Goetz & Anderson, 2010; Park et al., 2019).

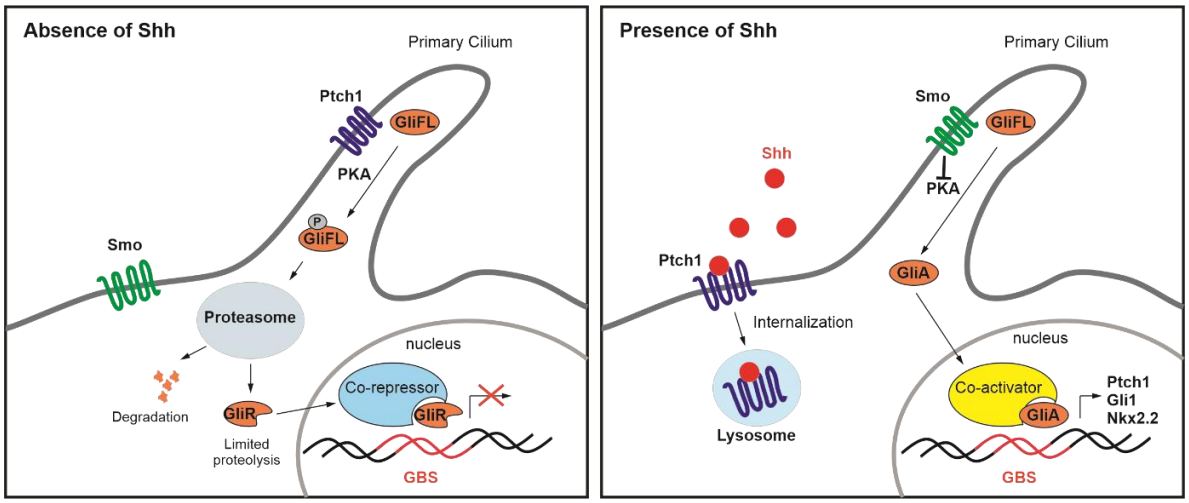

Fig. 3. Schematics of SHH signal transduction.

Post-translational modifications of Gli proteins at the PC

A crucial role in Shh-mediated patterning of the ventral neural tube belongs to Gli proteins that act as the transcriptional effectors of Shh signaling and regulate the expression of Shh-responsive genes. The family of Gli proteins in mammals consists of three proteins (Gli1, Gli2 and Gli3) where Gli1 functions exclusively as a transcriptional activator (GliA), whereas Gli2 and Gli3 act as either activators or repressors (GliR). In the absence of Shh, Smo is inactive and the full-length (FL) forms of Gli2 and Gli3 (GliFL) are constitutively phosphorylated by protein kinase A (PKA), a master negative regulator of Shh signaling (Tuson et al., 2011), and processed into truncated repressive forms (GliR) that keep Shh target genes switched off. Proteosome-dependent processing of Gli2 and Gli3 occurs with different efficiencies: most of Gli2 proteins are fully degraded, whereas Gli3 is mainly cleaved into a shorter Gli3 form (Gli3R). Downstream activation of Smo in the presence of Shh changes the access of Gli2/3 proteins to PKA and allows these TFs to escape from degradation/cleavage pathway and function as GliA. Gli1 is not expressed in the absence of Shh signal and is only transcriptionally induced after Shh signaling has commenced. Thus,

Gli1/2 act predominantly as transcriptional activators (Briscoe & Thérond, 2013; Matise & Wang, 2011; Pan et al., 2006; Tuson et al., 2011).

Gradient of Shh morphogen is interpreted by graded Gli activity

The extracellular ventral-to-dorsal gradient of Shh morphogen regulates the balance between GliA and GliR protein levels in target cells, which in turn evokes a gradient of Gli activity along the DV axis of the neural tube. Thus, the GliA/GliR ratio is proportional to the level of Shh and the balance between GliA and GliR defines the net Gli activity that shapes the response of NSCs to Shh gradient by initiation of the expression of different Gli target genes (Briscoe & Small, 2015; Stamataki et al., 2005). However, the relationship between the concentration of extracellular Shh and the transcriptional activation/repression of the target genes by Gli are more complex and Shh/Gli gradient alone is not sufficient to provide the neural progenitors with precise positional information and establish target gene boundaries (Kerszberg & Wolpert, 2007).

The duration of Shh signaling is integrated in the ventral neural tube patterning

In addition to a concentration gradient, the duration of Shh signaling plays a crucial role in the patterning process. During development, the production and accumulation of Shh protein in the ventral neural tube gradually increases with time, resulting in growing maximum level of Shh morphogen at the ventral pole of the neural tube (Chamberlain et al., 2008; Cohen et al., 2015). Consequently, there is no constant relationship between spatial location of neural progenitor and the specific level of Shh morphogen since the last changes over time. To achieve the higher level of Gli activity needed for transcriptional activation of more ventral target genes, higher concentration of Shh morphogen is required, thus longer periods of Shh production. In the ventral domains that are exposed to higher concentrations of Shh at the final stage, the neural progenitors transiently acquire a gene expression profile associated with the cell identities induced by lower concentrations of Shh, in other words undergo a dorsal-to-ventral progression over time. For instance, the neural progenitors of the p3 domain in the SC that are fated to express Nkx2.2 (requires high GliA level) transiently express Shh responsive TFs Olig2 and Pax6 (requires low GliA level) prior to Nkx2.2 (Balaskas et al., 2012; Dessaud et al., 2007, 2010). Therefore, the mechanisms underlying the spatial and temporal patterning are interconnected.

Cross-repressive interactions between Class I and Class II TFs

The identity specification of neural progenitors within restricted DV domains is linked to the expression of several HD and bHLH TFs that are classified into two classes according to their mode of regulation by Shh signaling: class I TFs (such as Pax3, Pax7, Pax6, Msx1, Irx3) are constitutively expressed by neural progenitors in the absence of Shh and are repressed by Shh signaling, whereas class II TFs (such as Foxa2, Nkx2.2, Nkx6.1, Olig2, Dbx1, Dbx2) require Shh exposure for expression (Briscoe & Novitch, 2008). Many of these TFs predominantly function as transcriptional repressors and they direct cell fate by repressing alternative cell fates (Kutejova et al., 2016; Muhr et al., 2001). Selective cross-repressive interactions between ClassI and ClassII TFs in neighboring domains create gene regulatory networks (GRNs) in the form of bistable switches that restricts activation of target genes to specific designated regions, following all-or-nothing principle and preventing neural progenitors with mixed identities (Balaskas et al., 2012; Briscoe & Small, 2015; Cohen et al., 2013b). As a result, newly generated neurons are located in spatially designated areas (**Fig. 4**).

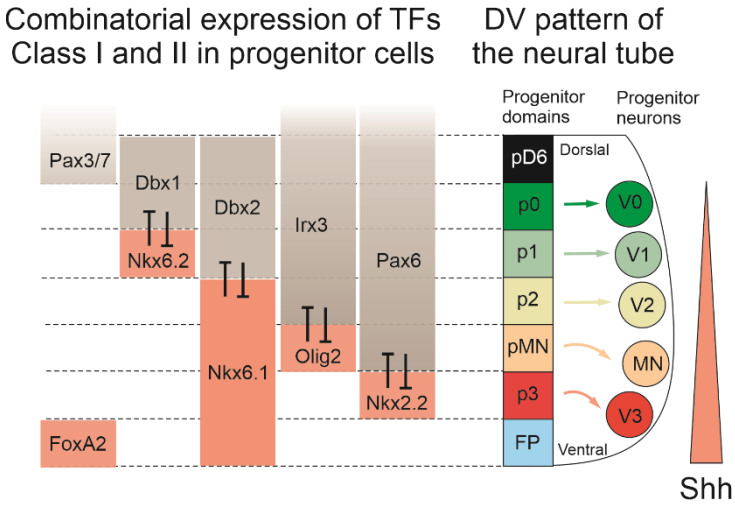

Fig. 4. The spatial expression of Class I and Class II TF. Schematic of the ventral half of the neural tube where the ventral-to-dorsal decreasing gradient of Shh specifies the identity of neural progenitors by inducing a set of genes encoding TF classified in Class I (inhibited by Shh) and Class II (activated by Shh) with clear boundaries of expression. The combinatorial expression of Class I and Class II TFs defines domains of progenitors. Adapted from Dessaud et al., Development, 2008.

Target gene CREs integrate inputs from multiple transcriptional regulators

The precise spatiotemporal control of gene expression is achieved through cis-regulatory elements (CREs) serving as “information integration hubs” that integrate combined inputs from multiple upstream regulators (TFs and other DNA-binding proteins such as effectors and coactivators, histones of certain modifications and others) with either inhibitor or activator function (Buecker & Wysocka, 2012; Levine, 2010). CREs are modular elements that contain binding sites for multiple TFs and are essential components of GRNs that play the important role in refining the appropriate pattern of gene expression in the neural tube development in response to secreted developmental cues (Davidson, 2010).

According to the current model, the individual CREs of the target genes induced by Shh signaling integrate the transcriptional input from three players: the morphogen effectors (Gli proteins), the activity of which depends on the availability of the morphogen; the uniformly expressed TFs that provide general neural specificity to Shh target genes and change their sensitivity to the morphogen (members of SoxB1 family); transcriptional repressors (class I and II TFs) that are under transcriptional control of Gli activity (Cohen et al., 2013, 2014; Oosterveen et al., 2012; Peterson et al., 2012).

Precision of ventral neural tube patterning is governed by the dynamics of the GRNs

Importantly, the gene expression profile acquired by the neural progenitors within appropriate progenitor domain appears to be stable against transient fluctuations in Shh-Gli signaling level. For example, embryos lacking *Gli3* display transient increase in the level of Gli activity (Balaskas et al., 2012), however the ventral regions are patterned normally and *Nkx2.2* expression boundaries in the ventral neural tube stay unchanged (Persson et al., 2002). At the same time, the absence of both Gli3 and Pax6 results in a marked shift in the border of *Nkx2.2* expression and deteriorated precision of the p3-pMN boundary with increased level of intermingling cells (Balaskas et al., 2012). It highlights that Gli input alone is not sufficient to set sharp boundaries between domains, revealing an important role of cross-repressive interactions between pairs of class II TFs in averaging the transient fluctuations in Shh-Gli signaling over time (Peterson et al., 2012).

Temporal aspects of neuronal patterning

Spatial and temporal aspects of neuronal patterning couldn't be separated from each other and are essential for generation of a wide variety of the neurons and glial cells that build the CNS (Okano & Temple, 2009). This variety of cells are derived from a pool of multipotent NSCs (also known as neural progenitor cells or NPCs) that have a developmental potential to produce more than one cell type over a time course. NSCs undergo the specification process during which the progenitors become committed or" biased" to the specific cell fate at certain time point in such a way that they are no longer affected by environmental cues. The timing of this process is intrinsically encoded within individual early progenitor cells allowing to sequentially proceed through several temporal identity states where the transition from one identity to another is irreversible process meaning that the development potential of the progenitors becomes progressively restricted (Holguera & Desplan, 2018; Kohwi & Doe, 2013). The sequential production of different cell types from the same progenitor pool can be found in the Drosophila nervous system (Doe, 2017), vertebrate cortex (Telley et al., 2019),

HB (Pattyn, Vallstedt, Dias, Samad, et al., 2003), MB (Deng et al., 2011). Usually neurons are generated first, followed by glial cells (Miller & Gauthier, 2007; Rowitch & Kriegstein, 2010). However, the mechanisms that regulate the timing of temporal switches that are responsible for shifts in temporal identity remain elusive.

The sequential switches of temporal fates in Nkx2.2$^+$ temporal lineage in the HB

The vertebrate HB is an attractive system to address how temporal progression of neurogenesis is controlled. High level of Shh emanating from FP cells in the ventral HB induce Nkx2.2$^+$ progenitors (Matise & Wang, 2011; Pattyn, Vallstedt, Dias, Sander, et al., 2003) that sequentially produce MNs, 5HTNs and oligodendrocyte precursors (OLPs) (Pattyn, Vallstedt, Dias, Samad, et al., 2003). In Nkx2.2$^+$ differentiation lineage Phox2b plays a role of temporal fate determinant that is required for the specification of early-born MNs and defines the temporal window of MN production (J. M. M. Dias et al., 2014; Pattyn et al., 2000; Pattyn, Vallstedt, Dias, Samad, et al., 2003). Despite a broader developmental potential of early Nkx2.2$^+$ progenitors, they do not generate late-born 5HTNs due to the presence of Phox2b and downregulation of Phox2b is a prerequisite for the transition from MN to 5HTN temporal fate.

Tgfβ signaling regulates the pace of temporal patterning in the developing CNS

Tgfβ signaling pathway is implicated in the timing of temporal switches in several parts of the developing CNS, where it induces the generation of late-born neuronal subtypes. For example, in the vMB activation of Tgfβ signaling promotes transition from early-born ocular MNs to late-born red nucleus neurons (RNNs) or at the SC level progressive upregulation of *Tgfβ2* expression in Olig2$^+$ pMN progenitors correlates with suppression of somatic MN (sMN) fate and induction of OLP fate (J. M. M. Dias et al., 2014). The recent data reveals that temporal patterning programs are universal and employ the conserved and limited set of global patterning TFs that are induced in neuronal progenitors in a sequential order and allocate them to early-, intermediate-, and late-born cell types respectively in many regions of the developing CNS. The pace of this temporal patterning program is approximately the same throughout the developing CNS and the series of switches from production of early-born neurons to later appearing cell types are implemented through activation of Tgfβ signaling pathway (Sagner et al., 2021). Importantly, the progression of temporal patterning is maintained even in the absence of active Tgfβ signaling pathway, for instance the knock-out of *Tgfbr1* delays but does not cancel the MN-to-5HTN fate switch in the ventral HB (Dias et

al., 2014; Paper I). This observation suggests the presence of other extrinsic or cell-intrinsic factors that are required to advance temporal patterning.

Mathematical modeling integrates the spatiotemporal patterning and the dynamics of GRNs

The temporal adaptation model postulates a progressive decrease in the sensitivity of neural progenitors to ongoing Shh exposure (Dessaud et al., 2007). Initially cells are very sensitive to Shh and the level of transcriptional activation of the target genes by Gli proteins is high even in the presence of low concentration of Shh. But with time cells adapt and finally become desensitized to ongoing Shh exposure, coinciding with decline in the level of Gli transcriptional activity. It remains unclear what contributes to the desensitization of cells to Shh signal in time, but the following mechanisms might be involved in adapting dynamics of downstream Gli transcriptional activity: the induction of negative feedback mechanisms, for example through *Ptch1* transcriptional upregulation; downregulation of *Gli* expression by transcriptional repressors induced by ongoing Shh signaling; dynamics of GliA and GliR levels (Cohen et al., 2015; Junker et al., 2014). But how exactly does the system respond to different levels or/and durations of Shh signal by means of these mechanisms? Mathematical models based on the experimental data help to integrate the mechanisms that operate in the patterning networks with graded spatial information and explain the logic behind the dynamics of the transcriptional networks. The power of mathematical modeling could be proven by accurate prediction and recapitulation of the sequence of gene expression observed in the neural progenitors with time (Balaskas et al., 2012; Cohen et al., 2014; Panovska-Griffiths et al., 2013).

The concept of hysteresis in developmental biology

Dynamical systems theory provides tools and concepts that help to describe the behavior of complex patterning systems and visualize it (Jaeger et al., 2008; Jaeger & Monk, 2014; Strogatz, 2014). One of the concepts taken from the dynamical systems theory is known as hysteresis phenomenon (Noori 2014; Strogatz, 2014). It allows to describe the dynamical biological systems that are governed by highly non-linear and multistable processes triggering switches between discreet states. Modeling based on hysteresis phenomenon defines the dynamics based on the history of inputs the system has received, including the current one. Utilization of mathematical models of hysteresis in developmental biology helps to describe biological switches and memory-dependent patterning systems with spatiotemporal resolution (Noori 2014). One of the examples when the memory of the past

inputs shapes the final output is the induction of Nkx2.2 TF in the p3 progenitor domain of the ventral SC. The initiation of *Nkx2.2* expression requires the repression of Pax6 and Olig2 at early states. Once *Nkx2.2* is induced by high level of GliA achieved with time due to ongoing Shh signaling, Nkx2.2 TF inhibits the expression of *Pax6* and *Olig2* and since then Nkx2.2 expression is sustained at lower levels of Shh-Gli signaling (Balaskas et al., 2012). The intracellular GRNs govern the positional specification in time where the cellular state with its specific gene expression dynamics feeds back on positional information encoded by Shh gradient.

1.3 THE ROLE OF RETINOIC ACID SIGNALING IN THE ANTERIOR-POSTERIOR PATTERNING OF THE CNS

RA is an endogenous morphogen that is widely known as an inductive signal involved in the caudalization of neural progenitors during CNS development (Maden, 2002) and as a molecule commonly used in differentiation protocols to induce more caudal neural identities from PSCs, e.g., MNs of the SC (Wichterle et al., 2002). Besides, ongoing RA signaling promotes neuronal differentiation and patterning in the SC primordium and is required for initiation of neurogenesis and specification of V1/V2 interneuron and MN progenitors in the forming SC (Diez del Corral et al., 2003). Additionally, *in vitro* studies have shown that RA is involved in dissolution of pluripotency (Gonzales et al., 2015) and promotion of neural fate by inducing the expression of neural genes and repressing the expression of mesodermal genes (Bain et al., 1996; Okada et al., 2004).

RA acts in a region-specific manner, acquiring local organizer functions during secondary gastrulation stage

The newly formed paraxial mesoderm starts to play a role of RA signaling center during secondary gastrulation stage where RA is most strongly expressed in that part of the presomitic mesoderm that is adjacent to the anterior SC with the rostral margins at the presumptive first somite. RA synthesized in the presomitic mesoderm is released in paracrine manner and diffuses into the adjacent posterior neuroectoderm where it plays a crucial role in the patterning of posterior HB, SC and trunk tissues along AP axis (Duester, 2008).

RA metabolism

RA is a metabolite of vitamin A (retinol) and represents a small lipophilic molecule that diffuses rapidly across membranes and acts as a morphogen. Intracellularly, vitamin A is modified subsequently in two steps: first the retinol/alcohol dehydrogenases (RDH/ADH) oxidize retinol to retinaldehyde, and then, the retinaldehyde dehydrogenases (ALDHs,

previously known as RALDHs) oxidize retinal to all-*trans* RA and 9-*cis* RA, two biologically active forms. Oxidation of RA is carried out by the action of three cytochrome P450 enzymes, CYP26A1, CYP26B1 and CYP26C1 (collectively known as CYP26s) and results in its degradation (Kumar & Duester, 2011) (**Fig. 5**).

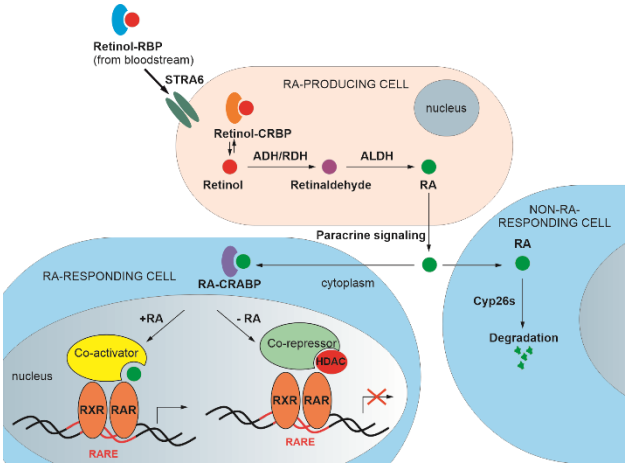

Fig. 5. RA synthesis and signaling. Retinol is carried by Retinol-Binding Proteins (RBPs) in the bloodstream, enters cells via STRA6 receptor and once inside the cell binds to CRBPs, buffering free retinol for storage. RA is released and taken up by surrounding cells with help of cellular-RA-binding proteins (CRABP) that facilitates RA uptake and transfer RA to the nucleus. Cells with high levels of degrading Cyp26s enzymes rapidly degrade RA and do not respond to RA.

Main components of the canonical RA signaling pathway

To convey RA signaling, first RA binds to cytoplasmic retinoid binding proteins (CRBPs) in the cytosol that prevents degradation of RA by Cyp26s and then is transported to the nucleus where RA activates or represses target genes by binding to two types of nuclear receptors, RA receptors (RARs) and retinoid-X receptors (RXRs). RARs form heterodimers with RXRs, with each heterodimer acting as a TF. In the presence of RA, RAR/RXR heterodimers act as transcriptional activators and bind to retinoic acid–response elements (RAREs) in the regulatory regions of RA-responsive genes. In the absence of RA, unliganded RAR/RXR heterodimers recruit a co-repressor complex with histone deacetylase (HDAC) activity, thus acting as transcriptional repressors of RA signaling target genes (Kumar & Duester, 2011).

Autoregulation of RA concentration by ALDH- and Cyp26-mediated negative feedback loops

During embryonic development, the intracellular level of active RA is tightly controlled by RA synthesizing and degrading enzymes (Dubey et al., 2018). Changes in RA availability (absence, insufficiency, or excess) in various species cause severe abnormalities where the developing CNS is particularly vulnerable (Ross et al., 2000). To operate at precise concentrations and ensure the robustness of the RA gradient, RA signaling employs feedback inhibition to self-regulate its level either by inhibition of RA synthesis (Niederreither et al., 1997; Strate et al., 2009) or induction of RA degradation (Ray et al., 1997; Reijntjes et al.,

2005). *Cyp26A1* promoter region contains functional RAREs, which makes it very sensitive to an increase of RA concentrations (Rhinn & Dollé, 2012).

The clearance of RA by Cyp26A1 is a particularly important regulatory mechanism for AP patterning of the developing CNS. Transient *Cyp26A1* expression in the anterior neuroectoderm between E7.0 and E7.5 (de Roos et al., 1999; MacLean et al., 2001) results in the absence of RA in this part of the CNS which is a prerequisite for the proper FB development (Ribes, Fraulob, et al., 2007).

RA is necessary for correct positioning of the HB and SC regions in the CNS

It is well known that RA provides important positional information to cells for proper HB and SC development, where the patterning is guided by the constant supply of RA from the paraxial mesoderm. The later formed more posterior rhombomeres are exposed to RA for longer time and to higher concentrations of RA, which results in activation of more posterior *Hox* genes (Nolte et al., 2019).

The gain- and loss-of-function experiments corroborate a strong caudalizing potency of RA. Loss of both Cyp26A1 and Cyp26C1 enzymes in mice double mutants results in severe truncation of the anterior part of the brain, where posterior identity (*Hoxb1* expression domain in the HB) is expanded rostrally at the expense of anterior identity (*Otx2* and *Meis2* expression domain in the MB) (Uehara et al., 2007). The observed phenotype in *Cyp26A1/Cyp26C1*$^{-/-}$ mice mutants is similar to the phenotype revealed in the pharmacological studies (Bally-Cuif, Gulisano, et al., 1995; Simeone et al., 1995). In contrast, depletion of RA in vitamin A-deficient (VAD) embryos of quail and rodents results in posterior expansion of prospective telencephalic tissue, enlarged anterior HB and disrupted segmentation in the myelencephalon (Gale et al., 1999; Halilagic et al., 2003; Maden et al., 1996; White et al., 2000).

The role of RA in the MB positioning

Mentioned above studies are mainly focused on FB and HB structures and do not specify how alterations in RA signaling affect MB determination and development. Simeone and colleagues, by using a combination of molecular markers of pros-, mes-, met- and myelencephalic regions, demonstrate that RA administration to the mouse embryo at the late streak stage (E7.4) results in a coordinate anteriorization of *Wnt-1* and *En-1* expression domains at the expense of anterior-most neuroectoderm, which was severely reduced (Avantaggiato et al., 1996). Another study showed that mice embryos exposed to RA at early

streak stage (E6.5) demonstrated the anterior shift of the posterior boundary of *Otx2* expression at the MHB (S. Ang et al., 1994). Interestingly, by the early somite stage, *Otx2* expression was no longer responsive to RA, suggesting a transient window of sensitivity to this morphogen, restricted to the primary gastrulation.

Therefore, RA-induced expansion of MB region at the expense of anteriorly adjacent FB and the anterior shift of Otx2 expression domain in the presumptive MB region could suggest a special role that RA may play in correct positioning of presumptive MB respectively to future FB and rostral HB along AP axis during primary gastrulation.

This model, however, is not supported by the study of *Aldh1a2-/-* mice embryos, where RA synthesizing Aldh1a2 enzyme deficiency led to complete lack of embryonic RA and resulted in severe malformations including shortening of the entire posterior region, externally truncated craniofacial and frontonasal regions, and eventual death between E9.5 and 10.5, but did not alter *Otx2* expression pattern, and the MHB was positioned correctly in these mutants (Niederreither et al., 1999).

Repression of RA signaling target genes by unliganded RAR/RXR-co-repressor complex is required for correct positioning of presumptive FB territory

The role of RA signaling in patterning of the anterior part of neuroectoderm before and at the onset of gastrulation remains a controversial issue. Although RA and corresponding enzymes responsible for its synthesis are first detected only within secondary gastrulation stage in the mouse embryo (approximately from E7.5), RARs and RXRs mRNA, encoding two nuclear receptors involved in the transcriptional regulation of RA signaling target genes, are already expressed at pregastrulation stages, both in extraembryonic and embryonic tissues (H. L. Ang & Duester, 1997). Moreover, the expression of *Cyp26A1* in the extraembryonic endoderm of the pre-gastrula mouse embryos (at E6.0) is another very early event in the developmental program linked to RA signaling (de Roos et al., 1999; Fujii et al., 1997; MacLean et al., 2001). The functional significance of so early expression of *Cyp26A1* and *RAR*s/*RXR*s remains unclear, but there are observations supporting the hypothesis that RA signaling might be involved in specification and segregation of presumptive FB and MB territories before and at the onset of gastrulation.

Experiments with RARE-*lacZ* transgenic mice embryos, where RARE-*lacZ* is a sensitive reporter for endogenous RA signaling, show that active RA-mediated signaling is absent in the anterior neuroectoderm during gastrulation (Ribes et al., 2009; Rossant et al., 1991), although RARs and RXRs mRNA are present (H. L. Ang & Duester, 1997). These studies

together with studies of early and transient expression of *Cyp26A1* in the presumptive FB region at the gastrula stage of the mouse embryo suggest that the anterior development must be devoid of active ligand-driven RA signaling during this time window(Abu-Abed et al., 2001). According to Koide and colleagues (Koide et al., 2001), the active derepression of RAR signaling in *Xenopus* embryos results in drastic reduction of the presumptive FB and downregulation of the anterior markers, a similar phenotype observed in the embryos treated with exogenous RA (Avantaggiato et al., 1996). On the contrary, increasing RAR/co-repressor complex-mediated repression by using inverse agonist (block RA binding to RARs and stabilize the RAR-co-repressor complex) leads to anteriorization of the neural plate: expansion of FB structures and up-regulation of anterior neural markers, the phenotype observed also in the VAD embryos (Koide et al., 2001).

These findings support another model where the active repression of RA signaling in RA-free condition is required for specification of presumptive FB region and its correct positioning to adjacent MB along AP axis.

RA signaling is involved in the neural patterning at pre-gastrula stages of avian embryonic development

In the mouse embryo anterior visceral endoderm (AVE), a mammalian analogue of hypoblast, is an important signaling center that initiates the anterior neural patterning and plays a crucial role in distancing the presumptive FB/MB region from caudalizing signals of the anterior primitive streak and the node (Foley et al., 2000; Molè et al., 2020; Stower & Srinivas, 2018). It has been shown that in the avian embryo RA synthesizing enzyme Aldh1a2 starts to be expressed in the anterior part of the hypoblast already at the pre-gastrula stage (Halilagic et al., 2003). The authors also reported that in the early VAD quail embryos in which biologically active RA is absent, prospective telencephalic tissue and the prechordal mesoderm are expanded more posteriorly, suggesting that RA at this early stage is needed for proper boundary formation between the future telencephalon and diencephalon. Involvement of RA in the proper specification and regionalization of the avian prosencephalon is additionally confirmed at later stages, where 100% of VAD embryos lack cephalic flexure and zona limitans intrathalamica (ZLI) (Halilagic et al., 2003).

Taken together, RA could act as a posteriorizing factor responsible for correct boundaries between different regions in the developing brain, but more studies are needed to understand the role of RA in early embryonic development.

1.4 VENTRAL MIDBRAIN AND HINDBRAIN DEVELOPMENT IS GOVERNED BY COMMON SIGNALS

Considerable load of observations supports the hypothesis that the pre-patterning of the anterior neural territory (presumptive FB) starts very early, before gastrulation, partly by the signals releasing from the AVE, while more caudal territories are regionalized as the neuroaxis is extending (Arkell & Tam, 2012; Beddington & Robertson, 1999; Molè et al., 2020; Rivera-Pérez & Hadjantonakis, 2015; Stower & Srinivas, 2018; Tam & Behringer, 1997). Broadly regionalized neuroectoderm with presumptive FB, MB, HB and SC territories is then "stabilized" and further refined into local identities with distinct molecular borders by arising secondary signaling centers (Martinez Arias & Steventon, 2018; Martinez-Ferre & Martinez, 2012; Vieira et al., 2010).

The isthmic organizer (IsO) and the FP are essential secondary signaling centers for MB and rostral HB development

The specification of presumptive MB (mesencephalon) and rostral part of HB (metencephalon) along AP and DV axis tightly relies on local instructive signals produced by two key regional organizing centers: the IsO and the notochord-derived FP (discussed above) (Hynes, Poulsen, et al., 1995; Wurst & Bally-cuif, 2001). The IsO appears within the neural plate stage (approximately at E8.0) at the MB-HB boundary (MHB) that includes the isthmus (the first segment of the HB) and the MB part immediately anterior to the isthmic territory (Martínez, 2001; Nakamura et al., 2008; Watson et al., 2019). The MHB coincides with the caudal expression limit of the gene *Otx2* in the MB region and the rostral expression limit of the gene *Gbx2* in the isthmus. Reciprocal negative interactions between Otx2 and Gbx2 set a correct positioning of the IsO (Broccoli et al., 1999; Joyner et al., 2000; J. Y. H. Li & Joyner, 2001; Simeone et al., 1992).

FGF, Wnt and Shh signaling pathways play central roles for specification of mesencephalic dopaminergic (mDA) neuron and rostral 5HTN progenitors

The establishment of mDA and rostral 5HTN progenitor domains in early neural development is accomplished through the concerted action of three diffusible signaling molecules: Wnt1 and FGF8 produced by the IsO (AP patterning) and Shh produced by the notochord and its derivative, the FP (DV patterning) (Castelo-Branco et al., 2003; Gibbs et al., 2017; Hynes, Porter, et al., 1995; Hynes & Rosenthal, 1999; Irving & Mason, 2000; Matise & Wang, 2011; Wurst & Prakash, 2014; Ye et al., 1998). Wnt1 expression is restricted to a narrow transverse ring encircling the MB part of the MHB and is required to

promote cell proliferation (Dickinson et al., 1994; Panhuysen et al., 2004; Wurst & Prakash, 2014), and to maintain En1 expression (Danielian and McMahon, 1996) and a stable MHB (Bally-Cuif et al., 1995). Besides Wnt1, the isthmus produces a bidirectional gradient of FGF8 signal that directs competent MB and HB territories to select appropriate fates, depending on the local FGF8 concentration (Gibbs et al., 2017; Martínez, 2001; Watson et al., 2017). Higher concentrations of FGF8 in the anterior HB induce a HB identity whereas lower concentrations in the adjacent MB region drive cells to adopt a MB cell fate (Basson et al., 2009; Y. Chen et al., 2009).

1.5 DOPAMINERGIC NEURON DEVELOPMENT

mDA neurons are derived from FP cells

All mDA neurons are initially originate from a common pool of neural progenitors located within the mesodiencephalic FP (mdFP) along the ventral midline of the MB and caudal FB (diencephalon), also called the mesodiencephalic ventricular zone (mdVZ) (Smidt, 2017; Smidt & Burbach, 2007; Smits et al., 2013). The mdFP is neurogenic structure (Bonilla et al., 2008; Ono et al., 2007) which comprise cells that secrete morphogen Shh and give rise to molecularly distinct progenitor pools that sequentially generate different subpopulations of mDA neurons in response to spatiotemporal gradients of local morphogens (Blaess et al., 2011; Joksimovic et al., 2009; Panman et al., 2014). Neurogenic mdFP cells are specified into mDA progenitors by FP-derived Shh morphogen (DV patterning) and the isthmus-derived FGF8 and Wnt signals (AP patterning) during embryonic development (Arenas et al., 2015; Smidt & Burbach, 2007).

mdFP gives rise to different subpopulations of mDA neurons

The embryonic vMB is transiently organized into a reiterated set of discrete territories, MB arcs, arrayed bilateral to the ventral midline. Each of these repetitive territories has a unique molecular identity and precedes the appearance of MB nuclei (Agarwala & Ragsdale, 2002). According to classification based on anatomical distribution of DA neurons in the brain (Björklund & Dunnett, 2007) DA neurons of the MB are located in two biggest and closely positioned nuclei, the substantia nigra pars compacta (SNpc) or A9 group and the ventral tegmental area (VTA) or A10 group, and in one smaller A8 group in the retrorubral field (Dahlstrom and Fuxe, 1964; Björklund & Dunnett, 2007). The caudal parts of both the SNc and the VTA are located in the ventral MB, whereas their rostral parts lie in the diencephalon, across its prosomeres 1, 2, and 3 (Watson et al., 2019).

The mDA neurons innervate distinct parts of the brain and differ molecularly

Despite being close to each other, the SNpc and VTA subtypes of mDA neurons project their afferent nerve fibers to distinct areas of the brain, thus controlling different functions. The SNpc mDA neurons predominantly project to the caudate putamen in the dorsolateral striatum along the nigrostriatal pathway and are implicated in the control of voluntary movements and body posture. The degeneration of these neurons leads to impaired motor function observed in patients with Parkinson's disease (PD). The VTA neurons project to the ventral striatum (nucleus accumbens, amygdala, olfactory tubercle) as a part of mesolimbic system and additionally connect to the prefrontal cortex along mesocortical pathway; the VTA neurons are involved in the modulation of behavioral functions such as motivation, reward associations, pleasure and addiction. Impaired functioning of these two pathways (collectively called the mesocorticolimbic) are associated with several neuropsychiatric disorders such as drug addiction, depression and schizophrenia (Bissonette & Roesch, 2016; Björklund & Dunnett, 2007).

Besides different innervation targets, the SNpc and VTA mDA neurons differ at the molecular level (Afonso-Oramas et al., 2009; Chung et al., 2005; Greene et al., 2005). The complexity and diversity of DA neurons was further confirmed with single-cell transcriptome sequencing methods (Ásgrímsdóttir & Arenas, 2020; J. F. Poulin et al., 2014, 2020). Interestingly, the diversification of DA neurons into subtypes starts early in the development, at neural progenitor stage: SNpc DA neurons arise from medially located $Lmx1a^{+}$ progenitors that express Sox6, whereas VTA DA neurons originates from lateral $Lmx1a^{+}$ progenitor domain and express Otx2 and Nolz1 (di Salvio et al., 2010; Panman et al., 2014). According to La Manno et al., the final refinement of DA neurons into A9 and A10 subtypes takes place only postnatally, during the maturation stage, as a result of interaction of DA precursors with local microenvironment. During this stage cells acquire differential expression of late-onset markers that broadly segregate DA neurons into subtypes (La Manno et al., 2016).

Subtype diversification, terminal differentiation and maintenance of mDA neurons

Proliferating mDA progenitors, born in the mdVZ at E9.5, eventually exit the cell cycle and become post-mitotic mDA precursors around E10.5. Simultaneously with exiting cell cycle mDA precursors leave the mdVZ and start to express the nuclear receptor Nr4a2 (Nurr1) which is essential for establishing the DA neurotransmitter identity (Saucedo-Cardenas et al., 1998; Smits et al., 2003; Zetterström et al., 1996). Following neurogenesis, mDA precursors migrate first along the processes of the radial glia and subsequently along tangentially

arranged nerve fibers, through the intermediate zone (IZ) toward their final destinations in the mantle zone (MZ), establishing afferent connections with striatal and cortical targets around E11.5 (Bissonette & Roesch, 2016; Kawano et al., 1995).

During the migration mDA precursors continue to differentiate and acquire the expression of late TF Pitx3 that is necessary in the final step of the mDA differentiation (Veenvliet et al., 2013). Importantly, *Pitx3* gene is exclusively expressed in all terminally differentiated mDA neurons approximately at E14.5 and the absence of Pitx3 in *Pitx3* mutant animals results in decreased nigrostriatal projections and selective vulnerability towards this mutation between different subpopulations of mDA neurons: it becomes lethal to only those neurons that later normally develop into the SNpc, but not VTA (Nunes et al., 2003; Smidt et al., 2004). It suggests the existence of molecular and functional diversity among mDA neurons (Roeper, 2013; Smidt, 2017). Reaching the MZ, the mDA late precursors undergo the terminal differentiation and becomes mDA neurons characterized by the expression of two sets of markers: (i) the pan-neural markers and (ii) proteins necessary for the synthesis of DA and neurotransmission, such as rate-limiting enzyme tyrosine hydroxylase (TH) or the dopamine transporter (DAT) for the synthesis and uptake of DA into the cell, respectively.

The cellular diversity of embryonic and adult mDA neurons explained by single-cell RNA-sequencing (scRNA-seq)

Single-cell transcriptomic analysis allows to profile single cells from heterogeneous embryonic mDA neuronal population in the murine and human vMB and model the developmental processes in a systematic manner (Ásgrímsdóttir & Arenas, 2020; J. F. Poulin et al., 2020; J.-F. Poulin et al., 2014). This approach provides insight into molecular diversification of the mDA lineage during neurogenesis and reveals the presence of at least three distinct subpopulations of both murine and human embryonic mDA neurons: a very immature DA0 which express *Th*, DA1 which additionally express *Slc5a3*, encoding DAT and DA2 that is distinguished by expression of *Aldh1a1* (classification according to La Manno et al., 2016; see also review Poulin et al., 2020). mDA neurons continue their specification and maturation during the whole embryogenesis and postnatally, migrate along radial glia where axon pathfinding to the target cells and local environment plays the most prominent roles (La Manno et al., 2016). According to the most recent data, there are at least 9 murine (Saunders et al., 2018) and 10 human (Kamath et al., 2022) transcriptionally distinct subpopulations of postnatal DA neurons revealed by single-cell transcriptome profiling. The emergence of specific mDA neuron subtypes postnatally raises the important question of later

acquired heterogeneity of adult DA cell population and apply another level of complexity to stem-cell-based replacement therapy for Parkinson's disease (PD).

mDA neurons and Parkinson's disease (PD)

PD is a progressive neurodegenerative disorder that mainly affects mDA neurons of the SNpc. Depletion of DA in the caudate putamen (the nigrostriatal pathway) in the dorsal striatum due to progressive loss of mDA neurons in the SNpc causes three core locomotor symptoms of PD, such as resting tremor, bradykinesia (slowness of movements with progressive hesitations and halts), muscular rigidity and leads to complete disability at the late stages of the disease (Poewe et al., 2017). Besides the degeneration of mDA neurons, there is another pathological hallmark of PD: the formation of intraneuronal α-synuclein-rich protein aggregates, termed Lewy pathology (LP) (Goedert et al., 2013). Although individuals with PD usually have LP, it is not always the case; besides, LP is found in the brains of asymptomatic individuals (Surmeier et al., 2017). These data reveal that the emergence of LP alone is not suitable for PD diagnosis. There are several lines of experimental evidence that extracellular α-synuclein fibrils are retrogradely spreading from the infected neuron to the healthy one through synaptic connections, thus causing LP (Goedert et al., 2013; Surmeier et al., 2017).

Selective vulnerability of mDA neurons from SNpc: cell-autonomous determinants of PD

mDA neurons from SNpc share several features that probably make them especially vulnerable to aging, genetic mutations or environmental toxins. First, SNpc mDA neurons have long and highly branched unmyelinated axons with compelling amount of transmitter release sites. It makes SNpc mDA neurons extra sensitive to mitochondrial bioenergetic crisis that could lead to eventual death (Bolam & Pissadaki, 2012; Pacelli et al., 2015). Another distinctive trait of SNpc mDA neurons is their physiology: they belong to neuromodulatory networks with characteristic diffuse axonal projections regulating other neurons via activation of slow G protein-coupled receptors (GPCRs). SNpc mDA neurons have slow and broad action potentials and spike autonomously in the absence of any synaptic input. Their slow and rhythmic pacemaker activity is accompanied by oscillations in intracellular Ca^{2+} concentration triggered by the opening of voltage-dependent L-type Ca^{2+} channels (Cav1 channels) and release of Ca^{2+} from endoplasmic reticulum stores into cytosol where level of Ca^{2+} buffering is low due to low level of Ca^{2+}-buffering proteins, such as calbindin. The slow Ca^{2+} oscillations in SNpc DA neurons promote free Ca^{2+} entry into the mitochondria which

stimulates the production of adenosine triphosphate (ATP). The dysfunction of the mitochondria leads to increased production of toxic reactive oxygen species (ROS) and mitochondrial oxidant stress and elevated level of Ca^{2+} in the cytosol promotes α-synuclein aggregation (V. Dias et al., 2013; Poewe et al., 2017; Surmeier et al., 2017).

Cell-replacement therapy in PD

Symptomatic treatment of PD with drugs modulating DA neurotransmission or increase dopamine levels in the striatum (for example supplement via L-DOPA) temporarily relieves some motor symptoms but doesn't prevent the progressive degeneration of mDA neurons (Brichta et al., 2013). Therefore, there is an enormous need for alternative treatment options that allow us to substitute the lost or damaged mDA neurons in PD patients. The clinical studies from late 1980s until late 1990s and early 2000s showed that human fetal mesencephalic tissue rich with DA neuroblasts could be successfully transplanted by microsurgery into caudate putamen (the putamen), the main target area of mDA neurons within the dorsal striatum of patients with PD with the following alleviation of motor deficits (Henchcliffe & Parmar, 2018; Parmar et al., 2020). It serves as proof-of-concept that cell-replacement therapy is a viable strategy (Henchcliffe & Parmar, 2018; Parmar et al., 2020). In some individuals with PD fetal tissue-derived transplants of mDA neuron precursors gave a long-lasting motor improvements with L-dopa treatment withdrawal for several years post-transplantation (Lindvall, 2016). However, the lack of efficacy in double-blind trials, occurrence of dyskinesia (uncontrolled, involuntary movements) in some patients, the limited quantity of human fetal mesencephalic tissue and the difficulty to standardize the preparations of fetal tissue-derived cells led to the halt of clinical testing of mDA cell therapy in PD for more than a decade (R. a. Barker et al., 2015). Development of protocols based on derivation of mDA neurons from hESCs and human (h) induced pluripotent stem cells (iPSCs) (known collectively as hPSCs) and deeper knowledge of mDA lineage specification rekindled the cell-replacement therapy and became a promising strategy that could provide a scalable, standardized and physiological source of mDA neurons (Kim et al., 2020; Parmar et al., 2020).

Pluripotent stem cells as a tool for regenerative medicine

PSCs are the cells that have a remarkable ability to self-renew nearly indefinitely *in vitro* and differentiate into derivatives of all three embryonic germ layers under certain conditions. There are two types of PSCs – ESCs and iPSCs, where mouse ESCs were first reported in 1981 (Evans and Kaufman, 1981; Martin 1981), human ESCs in 1998 (Thomson et al., 1998)

and human iPSC in 2007 (Takahashi et al., 2007). PSCs can exist at least in two distinct and well-characterized states of pluripotency, naïve pluripotency with properties of pre-implantation epiblast, and primed pluripotent state, corresponding to post-implantation epiblast, when cells become primed or responsive to developmental inductive signal from surrounding extraembryonic tissue (Nichols and Smith, 2009). Naïve PSCs have unique transcriptional and epigenetic features and besides their potential to give rise to ectoderm, mesoderm and endoderm, can generate trophectoderm cells, which primed PSCs can't (Dong et al., 2019; Dong et al., 2020). While mESCs have a naïve pluripotency (Nichols and Smith, 2009), hESCs obtained under conventional conditions are developmentally more advanced and have primed pluripotent state, even though they are derived from preimplantation embryos (Nichols and Smith, 2011; Kumari, 2016), with predisposition to neural differentiation (Nakamura et al., 2016; Zhang et al., 2001). The ability of PSCs to differentiate into specific lineages *in vitro* makes them a powerful tool for fundamental developmental biology studies, transplantation medicine, drug discovery and disease modeling. Moreover, PSC-derived cells have advantages over other cell sources such as fetal-derived cells because they can be manufactured in a standardized way to achieve the highest quality and purity and cryopreserved for storage and transportation.

Modeling mDA specification and differentiation *in vitro*

hPSCs is an important regenerative therapeutic tool to model the development of any appropriate cell type *in vitro* (R. a. Barker et al., 2015; Lindvall, 2016). The production of mDA neurons from hPSCs attracts considerable attention due to their great potential for replacement therapy of PD (R. A. Barker et al., 2019; Björklund & Lindvall, 2017; Grealish et al., 2014). The discovery of the FP origin of mDA neurons (Bonilla et al., 2008; Ono et al., 2007) and the following efficient derivation of functional FoxA2-expressing FP intermediate cells from hESCs (Fasano et al., 2010) allowed to develop the first protocol for generation of $FOXA2^{+}/LMX1B^{+}$ mDA progenitors and functional mDA neurons *in vitro* by combining a dual-SMAD inhibition (dSMADi) strategy with activation of Shh and Wnt signaling in a dose-dependent manner (Kriks et al., 2011). dSMADi is based on inhibition of BMP/TGFβ signaling and effectively upregulate neural lineage-specific genes and downregulate genes associated with endodermal and mesodermal lineages (Chambers et al., 2009). Application of BMP/TGFβ signaling inhibitors to the monolayer culture produce rosettes that consist of multipotent NPCs. Activation of canonical WNT signaling by the glycogen synthase kinase 3 inhibitor (GSK3i) CHIR99021 (CHIR), triggers dose-dependent patterning of neural progenitors to the caudal FB, MB, HB and anterior SC cell fates: low concentrations of CHIR

induce progenitors with more anterior identity whereas higher doses of CHIR fate the cells into more caudal identities (Kirkeby et al., 2012). There are two major limitations of CHIR-based patterning strategy: first, it is highly concentration-sensitive and therefore, accurate titrations of every new batch of CHIR are required to direct cells into the desired identity (Nolbrant et al., 2017; Maimaitili, M. et al. 2021); second, the PSC-derived mDA cultures often contain undesired cell populations such as diencephalic progenitors that molecularly can be assigned to subthalamic nucleus (STN) lineage (Kee et al., 2017). To avoid these shortcomings, two strategies were utilized to fine-tune CHIR-based protocols: sequential exposure to high levels of CHIR, called CHIR boost (Kim et al., 2021) and the timed delivery of FGF8 (Kirkeby et al., 2017a; Nolbrant et al., 2017; Xi et al., 2012). Both strategies help to mimic the activity of the IsO, a regional source of caudalizing Wnt1 and FGF8 molecules in the development, and progressively pattern progenitor cells toward a caudal MB identity, thus reducing the number of cells with diencephalic identity. Of note, FGF8 has pleiotropic effect and induces cerebellum fate in rhombomere 1 (r1) (A. Liu et al., 2003), as well as $Gbx2^{+}$ rhombencephalic cells in the caudal FB explants (A. Liu et al., 1999) and HB markers such as *HOXA2* (Kirkeby et al., 2017a). Also, it is important to keep in mind that active WNT signaling pathway is involved in *Otx2* expression maintenance in the vMB domain (Prakash et al., 2006; Puelles et al., 2004) and Otx2, in turn, is known to control VTA mDA differentiation (di Salvio et al., 2010; Grealish et al., 2014; Prakash et al., 2006) and, therefore, in the condition of prolonged active Wnt signaling the presence of VTA-like mDA neurons in the cultures becomes highly possible (Oosterveen et al., 2021).

Despite the advances of currently available stem cell-based protocols, hPSC-derived cultures of mDA neurons contain a mixture of SNpc- and VTA-like subtypes of mDA neurons (Oosterveen et al., 2021; J. F. Poulin et al., 2020; Tiklová et al., 2019). Also, the overall yield of mDA neurons after transplantation is less than 10% (Piao et al., 2021; Song et al., 2020) and the grafts contain non-neural cells, such as the perivascular fibroblast-like cell population called “vascular leptomeningeal cells” or shortly VLMCs (Tiklová et al., 2020)

Taken together, there is a great need for the new alternative protocols that produce disease-relevant SNpc-like subtypes of mDA neurons *in vitro* and significantly improve cell purity of grafts and can also serve for disease modeling and drug discovery.

1.6 SEROTONERGIC NEURON DEVELOPMENT

Rhombomeres and pseudo-rhombomeres

5HTNs are born in the ventral part of the HB, also known as rhombencephalon, between E9.5 and E12.5 in the mouse embryo (E. Deneris & Gaspar, 2018; E. S. Deneris & Wyler, 2012). The HB is an embryonic structure divided into discrete territories called rhombomeres. Overt rhombomeres (r2-r6) are morphologically distinguished as well-defined bulges with clear intersegmental boundaries along the neural tube. The isthmus (r0) and r1, located more rostrally, do not have intersegmental boundaries between each other and form a common morphological field with the caudal MB, however, could be distinguished molecularly (Puelles et al., 2013). Though the development of r0, r1 and the MB is guided by the gradients of the common inductive signals secreted by the IsO, 5HTNs do not arise from the MB (Cordes, 2005; Zervas et al., 2004). Caudally to r6, the HB represents non-segmented region that could be subdivided into five so called pseudo- or crypto-rhombomeres (r7-r11) based on cytoarchitectonic landmarks in mouse medulla oblongata and Hox gene expression (Puelles et al., 2013; Tomás-Roca et al., 2016).

Shh is a strong ventralizing signal needed for patterning of both rostral and caudal subtypes of 5HTNs

Simultaneously with rhombomeric segmentation of the HB along the AP axis, distinct progenitor subpopulations appear at stereotyped positions along the DV axis, known as progenitor domains. The 5HTN progenitors are located in the ventral HB and specified by high doses of Shh emanating from the FP cells (Briscoe & Ericson, 1999; Hynes & Rosenthal, 1999) and the presence of Shh-blocking antibodies results in failure to develop 5HTNs (Ye et al., 1998).

5HTNs are generated from the same pool of progenitors as visceral motor neurons (vMNs)

5HTNs share a common origin with vMNs where both subtypes originate from a neural progenitor population located in the pMNv domain (topologically equivalent to the p3 domain of the SC) of the ventral HB. In the mammalian embryo vMNs and 5HTNs are produced in a sequential order: vMNs are born first between E9.0 and E10.5, followed by generation of 5HTNs between E10.5 and E12.5. This sequential pattern is altered at two levels of AP axis: in r1, which produces approximately half of all 5HTNs, there is no phase of vMN production, and in r4, where exclusively vMNs are generated. Therefore, r4 creates a gap that separates the rostral cluster (r1-r3-derived) from the caudal cluster (r5-r11-derived)

of 5HTNs (J. Briscoe et al., 1999; Pattyn, Vallstedt, Dias, Samad, et al., 2003). However, this widely accepted assumption that r4 lacks any 5HTNs is contradicted by meticulous 5-HT immunostaining correlated with *in situ* hybridization (ISH) at different developmental stages in the mouse embryo. The analysis showed that r4 contributes to 5HTNs of the HB, though it is a minor population that expresses low level of 5-HT and Pet1 (Alonso et al., 2013).

The first 5HTN progenitors appear in r1, where they give rise to the first 5HTNs between E9.5 and E10.5, whereas 5HTNs in r2 and r3 are born about a day later due to initial production of vMNs in these domains (Pattyn, Vallstedt, Dias, Samad, et al., 2003). Simultaneously with rostral subset of 5HTNs, the caudal 5HTNs are born, however there is a 1-2-day delay in the synthesis of 5-HT between these two groups (E. S. Deneris & Wyler, 2012).

5HTNs are anatomically and developmentally heterogeneous cell population

Leaving cell cycle, post-mitotic 5HTNs migrate from the VZ of the embryonic HB in a medial-to-lateral direction to the MZ (Leber & Sanes, 1995) to form clusters called raphe nuclei. The transverse limits of raphe nuclei correlate with molecular boundaries of rhombomeres (Alonso et al., 2013; Puelles et al., 2013; Tomás-Roca et al., 2016; Watson et al., 2019).

Based on the cell soma distribution of mature 5HTNs, nine clusters of 5HTNs (B1-B9) are classified, distributed along AP axis from the caudal medulla oblongata in the caudal HB through the pons to the MB, from caudal B1 to rostral B9, respectively (Dahlstrom and Fuxe, 1964; Wallace & Lauder, Lumsden 1983). Based on cytoarchitectonic structures, 5HTNs are broadly grouped into the rostral (B9-B4) and the caudal (B3-B1) clusters with opposite polarity of their axonal projections (Jacobs & Azmitia, 1992).

The rostral “superior” brainstem group is subdivided into two major nuclei, the median raphe nucleus (MRN, or B8 and B5) and the dorsal raphe nucleus (DRN, or B7 and B6; contain about half of all 5HTNs of the CNS), and into two smaller nuclei, the caudal linear nucleus (CLi) and the B9 nucleus. 5HTNs from the caudal “inferior” cluster comprise the raphe pallidus nucleus (RPa, B1), the raphe obscurus nucleus (Rob, or B2), the raphe magnus nucleus (RMg, or B3) and the ventrolateral medulla (VLM) (Okaty et al., 2019).

Importantly to underpin that each raphe nuclei is heterogeneous and contains a variable mixture of 5HTNs and other subtypes such as DA neurons and GABAergic neurons. For

instance, the percentage of 5HTNs varies between 50% to 21% in the DRN and MRN, respectively (Descarries et al., 1982; Sos et al., 2017).

Development of the HB along AP axis is controlled by the *Hox* gene family

Segmentation process along the AP axis in the HB is tightly controlled by the *Hox* genes which early activation segregate cell population with common origin and similar potential into distinct subpopulations that respond differently to environmental signals. The regionally restricted and overlapping patterns of *Hox* expression, present in all rhombomeres except r1, represent a combinatorial code that specifies the unique identity of each HB segment. The expression of *Hox* genes is induced by dynamic changes in concentration of endogenous RA produced by the paraxial mesoderm (so called RA gradient along the AP axis) that also depends on the time of exposure to RA (more caudal segments of the HB are exposed to RA for longer time) (Nolte et al., 2019; Parker & Krumlauf, 2020; Tümpel et al., 2009).

Patterning and segmentation of the HB are based on reciprocal interactions of Wnt, FGF and RA signaling pathways

Although both rostral and caudal subsets of 5HTNs are born near the FP and their development requires the presence of ventralizing Shh signal (Hynes & Rosenthal, 1999; Ye et al., 1998), there are another three key signaling pathways involved in generation of rostral and caudal 5HTNs – Wnt, FGF and RA. These inductive signals have posteriorizing effect and their expression is dynamic in space and time (Parker & Krumlauf, 2020).

The rostral group of 5HTN progenitors reside near the MHB and, therefore, are highly dependent on the isthmus-derived FGF signaling. *Cyp26* genes are upregulated in response to FGF signaling in the anterior part of the HB, adjacent to the isthmus. The boundaries of *Cyp26* gene expression are dynamic and shifting posteriorly as the segmental patterning proceeds. Also, FGF inhibits *Aldh1a2*, thus acting as a repressive signal that limits the ability of RA to induce *Hox* genes and establish the anterior boundary of *Hoxa2* expression, the most rostrally expressed *Hox* gene, at r1/r2 border, thus making r1 a "Hox-free" territory (Diez et al., 2003; Nolte et al., 2019; Parker & Krumlauf, 2020). More caudal 5HTN progenitors are specified by RA, transiently produced in the cervical somitic mesoderm that bilaterally flank the caudal HB (Rhinn & Dollé, 2012). RA diffuses into the adjacent neuroepithelium and is degraded by anteriorly expressed Cyp26s. RA induces *Hox* patterning genes in a dose-dependent manner where RA gradient is deciphered by RAREs located in the enhancers of key target genes, such as *Hoxa1*, *Hoxb1*, *Hoxb4* and others. As patterning of the HB proceeds and the embryonic axis is elongating, expression of *Aldh1a2* is downregulated in anterior

somites and upregulated in newly organizing posterior somites, which gives a temporally dynamic gradient of RA concentration, with low RA anteriorly and high RA posteriorly (Nolte et al., 2019; Parker & Krumlauf, 2020).

Differentiation of postmitotic 5HTN precursors and their maturation

Postmitotic 5HTN precursors acquire serotonergic identity through expression of 5HTN-specific gene battery of TFs that includes GATA3, LMX1b and PET1. LMX1b and PET1 are expressed in 5HTNs through all their life and are required for the proper neurochemical identity of 5HTNs (E. Deneris & Gaspar, 2018; E. S. Deneris & Wyler, 2012; C. Liu et al., 2010; Wyler et al., 2015). 5HTN phenotype is defined by a set of core proteins that is stably expressed at terminal stages of differentiation and is required to build and maintain the 5-HT neurotransmission, synaptic responsivity to external stimuli and axonal connectivity (**Fig. 6**).

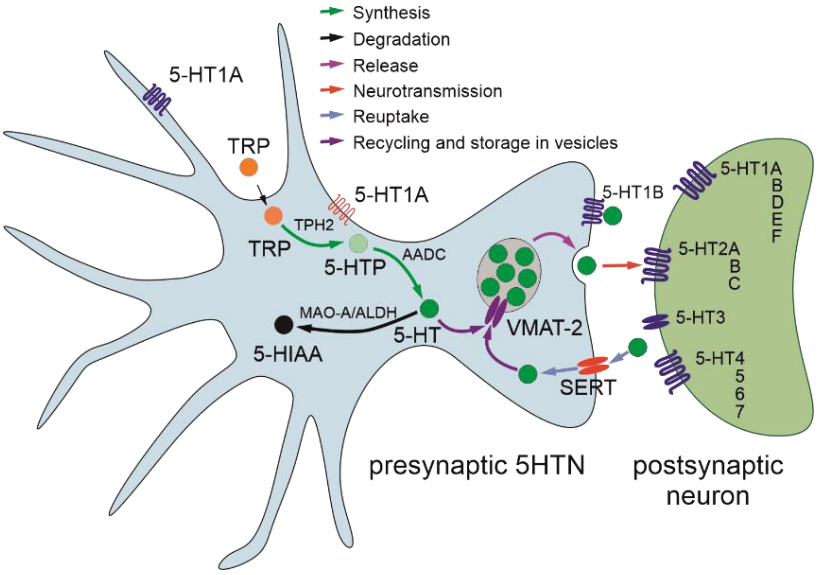

Fig. 6. 5HTN identity. 5-HT is synthesized in two steps: first dietary L-tryptophan (TRP) is converted into 5-hydroxytryptophan (5-HTP) with rate-limiting enzyme TPH2 and then undergo rapid decarboxylation to 5-HT by AADC. 5-HT can be released into the extracellular space and either stimulates neurotransmission via postsynaptic 5-HT receptors or taken up by SERT back into cell. 5-HT further is degraded intracellularly to 5-hydroxyindole acetic acid (5-HIAA) by a combination of the mitochondrial enzyme MAO-A and an aldehyde dehydrogenase (ALDH). Synaptic firing is controlled via somatodendritic 5-HT1A autoreceptors and 5-HT release is modulated via 5-HT1B autoreceptors.

This set includes 5HTN-specific markers involved in the 5-HT synthesis (tryptophan hydroxylase 2 (TPH2); aromatic amino acid decarboxylase (AADC), degradation (monoamine oxidase A (MAO-A)), intracellular loading of 5-HT into vesicles by the vesicular monoamine transporter 2 (VMAT2, gene *Slc18a2*), 5-HT reuptake by 5-HT transporter (SERT, gene *Slc6a4*), autoinhibitory feedback to regulate 5-HT neurotransmission (5-HT1A autoreceptor) (Okaty et al., 2019). None of these proteins is restricted to 5HTNs and only their co-expression with pan-neuronal markers defines 5HTN molecular phenotype (Deneris & Gaspar, 2018). Indeed, even Tph2 is present in non-neuronal cells (Lv et al., 2017), however, at least in the mouse CNS, *Tph2* is uniquely expressed in 5HTNs (Okaty et al., 2019). 5HTN population is molecularly heterogeneous with restricted or variable

expression of a variety of terminal markers such as 5-HT1A autoreceptor, neuropeptides, the vesicular glutamate transporter 3 (VGLUT3) and others (E. Deneris & Gaspar, 2018; Fernandez et al., 2016; Kiyasova et al., 2011, 2013; Okaty et al., 2015). Phenotypic differences between 5HTNs most likely reflect the functional specialization that is tightly connected to physiological and behavioral functions.

5HTNs innervate various regions of the brain and SC

Overall 5HTNs innervate most regions in the brain and SC and, appropriately, participate in a wide array of functions in the CNS. The rostral 5HTNs reside in the ventrolateral tegmentum of the MB and rostral HB and have ascending axonal projections, innervating virtually all areas of the FB and MB such as cerebral cortex, limbic system, basal ganglia and additionally HB including cerebellum and the raphe nuclei themself (Muzerelle et al., 2016). These 5HTNs regulate the emotional responses, circadian rhythms, and energy balance. The caudal 5HTNs give rise to descending projections to major brainstem nuclei and the SC, and control the cardiorespiratory homeostasis, thermoregulation and nociception (Wylie et al., 2010).

5HTNs and psychiatric disorders

Dysfunctional serotonergic system in the brain has been implicated in the development of many psychiatric disorders such as major depressive disorder (MDD) (Blier & El-Mansari, 2013; Morrissette & Stahl, 2014; Vadodaria et al., 2019), anxiety disorders (Griebel & Holmes, 2013), autism spectrum disorder (Müller & Homberg, 2015), schizophrenia (Selvaraj et al., 2014), drug addiction (Müller & Homberg, 2015) and others. Even though the central serotonergic system has become one of the best investigated neurotransmitter systems, the role of 5HTNs in the development of neuropsychiatric disorders is far from being elucidated (Albert et al., 2012; Albert & Benkelfat, 2013).

The monoamine hypothesis of depression

The classic monoamine hypothesis of depression claims that depression arises from a deficiency in 5-HT, norepinephrine (NE) and DA in the brain (Hillhouse & Porter, 2015). This hypothesis is mainly based on two lines of evidence: first, reserpine, an alkaloid that inhibits VMAT2 responsible for uptake of monoamines (5-HT, DA and NE) into synaptic vesicles and thus decreasing the level of 5-HT in the brain, was found to evoke depression in some patients; second, the mechanism of action of classical antidepressant drugs such as MAO inhibitors (MAO-Is) or tricyclic antidepressants (TCAs) that target the monoamine neurotransmission (Hillhouse & Porter, 2015). Selective inhibition of 5-HT/NE uptake by

later developed selective serotonin reuptake inhibitors (SSRIs) and serotonin norepinephrine reuptake inhibitors (SNRIs) has become a centerpiece in the antidepressant treatment for several decades until now. However, the classic monoamine hypothesis doesn't explain why it takes 4-12 weeks for the first-line SSRIs/SNRIs to start the action and why these drugs are ineffective in approximately one-third of patients with depression (Cowen, 2017; Harmer et al., 2017; O'Leary et al., 2015; Uher et al., 2011). Modern monoamine hypothesis partly explains the slow onset of antidepressant action by desensitization of autoreceptors 5-HT1A/1B in 5HTNs and adrenergic heteroreceptors α2 in non-monoaminergic neurons, which takes 2 to 4 weeks and results in sustained release of 5-HT/NE in the synaptic cleft (Harmer et al., 2017; Hen & Nautiyal, 2017; Y. F. Li, 2020). To accelerate the therapeutic effect of SSRIs, new multi-target antidepressant drugs with fast onset that preferentially desensitize autoreceptors were developed and later approved by FDA, such as vilazodone (SSRI and 5-HT1A partial agonist) and vortioxetine (SSRI and 5-HT1A agonist, 5-HT1B partial agonist and 5-HT1D/5-HT3/5-HT7 antagonist) (Artigas et al., 2018; Köhler et al., 2016).

Non-monoamine strategy to treat depression

Functional deficits of 5-HT system in brain circuits and aberrant 5HTN morphology are known to be associated with the pathophysiology of depression which underpins the importance of 5-HT system in the human psychology (Jacobs & Azmitia, 1992; Sharp & Cowen, 2011; Vadodaria et al., 2019). However, the association of various mental disorders to certain neural populations releasing specific neurotransmitters was challenged in recent years. There was a growing understanding that the modeling of a neuropsychiatric disorder solely on the dysfunction of 5-HT system is a simplification and there is rather a complex signaling exchange or interplay between different neurotransmitter systems that precisely modulate behavioral output (Albert et al., 2012). Besides, a delayed onset of SSRI action, limited efficacy (treatment-resistant depression) (Cowen, 2017; Uher et al., 2011) and side-effects such as sexual dysfunction (Clayton et al., 2014) or suicidal thoughts (Larsson, 2017) prompted to seek new alternative agents. Therefore, the monoamine hypothesis was revised, and new non-monoamine strategies were developed such as FDA-approved fast-acting antidepressant S-ketamine, non-competitive N-methyl-D-aspartate (NMDA) receptor channel blocker (Berman et al., 2000; Machado-Vieira et al., 2017; Murrough et al., 2013). Ketamine at subanesthetic doses produces rapid antidepressant effect within 2-72h, reduces core symptoms of depression such as suicidal tendencies, anhedonia, and has clinical therapeutic effects in patients that are resistant to traditional antidepressants and electroconvulsive

therapy (Duman et al., 2016; O'Leary et al., 2015). There are several lines of evidence that antagonism of NMDA receptors increases glutamate neurotransmission via inhibition of NMDA receptors on inhibitory GABAergic interneurons that results in disinhibition of pyramidal neurons in the prefrontal cortex (PFC) and subsequent increase in glutamate release, which in turn activates neuroplasticity pathways (Machado-Vieira et al., 2017). S-ketamine's antidepressant action is based not only on NMDA antagonism, but also is indirectly mediated through mTOR pathway activation and the following mTOR-dependent increase of synaptic protein synthesis in the PFC of rats (Li et al. 2010). Thus, the rapid increase in synaptogenesis in the PFC might reverse the loss of synaptic connectivity in depressed patients and explain the mechanism of the fast-onset antidepressant action of S-ketamine. However, clinical application of S-ketamine is currently limited due to abuse liability, psychotomimetic effect and transient therapeutic effect (Hillhouse & Porter, 2015; Y. F. Li, 2020; O'Leary et al., 2015). Besides, it's important to mention that other NMDA receptor antagonists did not show the same antidepressant efficacy, if any, in comparison to S-ketamine (Ionesc & Papakostas, 2017; Machado-Vieira et al., 2017). Therefore, the discovery of innovative antidepressants that avoid the mentioned undesired effects is greatly needed in modern drug research and development.

Generation of disease-relevant 5HTNs *in vitro* for disease modeling and drug discovery

Patient-derived 5HTNs differentiated from hiPSCs guided by developmental signaling molecules or generated by transdifferentiation directly from fibroblasts represent a new and promising approach for studying cellular and molecular mechanisms that underlie fundamental programs of 5HTN generation in the development of neuropsychiatric disorders (Soliman et al., 2017; Vadodaria et al., 2018). Several research groups, including our lab (**Paper II**), independently developed methods for generating human 5HTNs *in vitro,* which gave almost unlimited access to otherwise very small fraction of neurons in the brain that could be used as a tool for screening therapeutic compounds for drug discovery purpose and for disease modeling (Lu et al., 2016; Vadodaria et al., 2016; Valiulahi et al., 2021; Z. Xu et al., 2016). hiPSC technology made it possible to successfully model neuropsychiatric diseases *in vitro,* such as schizophrenia (Brennand et al., 2011; Siegert et al., 2015), bipolar disorder (Mertens et al., 2015; Madison et al., 2015) and autism spectrum disorders (Marchetto et al., 2017) and shed the light on the cellular and molecular biology of depression. Vadodaira and colleagues were first who demonstrated the utility of this approach in the serotonergic field: 5HTNs derived from patients with treatment-resistant depression

showed altered neurite growth and morphology as compared to 5HTNs derived from healthy controls or SSRI-sensitive patients (Vadodaria et al., 2019).

Accordingly, the recent progress in hiPSC technology combined with development of derivation protocols for 5HTNs make a path towards potential treatment of most prevalent neuropsychiatric disorders.

2 RESEARCH AIMS

The overarching aim of this study is to investigate the molecular mechanisms that govern the temporal aspects of neural patterning within NKX2.2^{+} lineage in the ventral HB and demonstrate how our better understanding of organizing influence of regional signaling centers on the patterning of the neural progenitors *in vivo* and mimicking these centers *in vitro* in a timed manner can lead to the development of new alternative approaches to produce the desired types of clinically relevant neurons in a highly competitive fields of DA and 5HTN research.

Specific aims:

Paper I: To understand how the downregulation of *Gli1-3* transcription is linked to intrinsic activation of extrinsic switch factor TGFβ that controls a timing of MN-to-5HTN fate switch within NKX2.2^{+} temporal lineage in the ventral HB.

Paper II: To show that timed exposure of signaling molecule RA can be applied to regionally pattern hPSC-derived NSCs into FB, MB and HB regional identities and based on that knowledge to establish novel alternative protocol for derivation of clinically important human mDA neurons and 5HTNs of the HB.

Paper III: To utilize the established in **Paper II** protocol based on timed exposure of RA for production of 5HTNs and develop a stem cell-based scalable phenotypic screening platform for searching small-molecule compounds that modulate 5-HT neurotransmission and hold a therapeutic potential for the treatment of neuropsychiatric disorders.

3 RESULTS AND DISCUSSION

3.1 PAPER I: A SHH/GLI-DRIVEN THREE-NODE TIMER MOTIF CONTROLS TEMPORAL IDENTITY AND FATE OF NEURAL STEM CELLS

Temporal patterning is an important phenomenon that contributes to the emergence of neural cell diversity along orthogonal axis in the developing CNS. However, little is known about time-encoding circuitries that establish precise time frames for production of designated neural progenitors within certain temporal lineage and determine point of transitions from one temporal fate to the next one. To approach the aspects of temporal patterning in NSCs we used Nkx2.2^{+} temporal lineage in the developing ventral HB that sequentially produce cranial MNs, 5HTNs, and OLPs (J. M. M. Dias et al., 2014; Pattyn, Vallstedt, Dias, Samad, et al., 2003; Vallstedt et al., 2005). This lineage is induced by ventralizing FP-derived Shh and acquire HB identity by caudalizing presomitic mesoderm-derived RA morphogen.

The generation of MNs, 5HTNs and OLPs from Nkx2.2^{+} NSCs could be recapitulated in the culture of mESCs in response to timed exposure to Shh agonist SAG and all-trans RA. At 3.5 days in differentiation conditions (DDC) >90% of Sox3^{+} NSCs expressed Nkx2.2^{+}. The MN time window in the Nkx2.2^{+} temporal lineage is specified by expression of Phox2b TF. As soon as Phox2b expression is terminated in Nkx2.2^{+} progenitors around 5.5 DDC, 5HTNs are generated by a default program. Is there any intrinsic timer mechanism that governs this MN-to-5HTN fate switch? To answer this question, we performed genome-wide transcriptome analysis and focused on those genes that were significantly down-regulated between 3.5 DDC, when Phox2b expression has been induced, and 5.5 DDC when Phox2b expression is terminated in Nkx2.2^{+} NSCs. We found 27 genes that were profoundly downregulated, including *Gli1*, *Gli2* and *Gli3*. In the absence of Shh Gli2 and Gli3 are either degraded or proteolytically processed into truncated transcriptional repressor form Gli3R and Gli2R (collectively GliR); in response to extrinsic Shh Gli2 and Gli3 are stabilized and act as full-length activators Gli2A and Gli3A (collectively GliA) because of internalization of negative regulator of Shh signaling Ptch1 receptor at the cilia and simultaneous translocation of Smo receptor into cilia. Thus, Gli2 and Gli3 are bimodal TFs that can act as repressors or activators. Gli1 is induced by ongoing Shh signaling and acts solely as an activator. *Ptch1* and *Gli1* are direct target genes of the Shh pathway and has biphasic temporal expression profile: first they are upregulated in the Nkx2.2^{+} temporal lineage between ≈1 DDC – 3 DDC, and then progressively downregulated over MN window (3 DDC to 5.5 DDC). We identified 61 genes with similar biphasic expression and whose expression was inhibited by Smo

antagonist cyclopamine. Among these 61 genes 35 genes were bound by Gli1 or Gli3, including *Phox2b*, meaning that Gli1-3 act as putative activators of Phox2b expression.

Western blot analysis showed a progressive decay of full-length activator forms of Gli2 (Gli2A) and Gli3 (Gli3A) between 2.5 DDC and 5.5 DDC. Immunocytochemical analysis showed clear accumulation of Gli1-3 proteins at the cilia and in the nuclei in NSCs at 3-3.5 DDC, which are signature events of ongoing Shh signaling, however by 5.5 DDC Gli proteins disappear from the nuclei and cilia, which corresponds to progressive downregulation of *Gli* genes and decay of Gli proteins. Inhibition of Shh signaling by treatment of cultures with cyclopamine between 2.5 DDC to 4.5-5 DDC resulted in premature downregulation of Phox2b and increased number of $Gata3^{+}$ 5HTN progenitors. Altogether, the data support the notion that ongoing Shh/Gli signaling is required for Phox2b expression within MN window and inhibition of Shh signaling is sufficient to induce a switch from MN to 5HTN temporal fate.

The presence of repressor forms of Gli2 (Gli2R) and Gli3 (Gli3R) is declining in $Nkx2.2^{+}$ progenitor cells in a similar way as their corresponding activator forms between 2.5 DDC and 5.5 DDC. This temporal expression profile of *Gli* genes could be theoretically explained by the presence of Ptch1-dependent negative feedback regulation of *Gli* expression. To test the hypothesis, we analyzed differentiating $Ptch1^{-/-}$ NSCs in culture and observed active Shh/Gli signaling in condition of no SAG treatment ($Ptch1^{-/-}$ − SAG) with corresponding patterning into $Nkx2.2^{+}$ lineage but didn't detect any changes in the temporal biphasic expression profiles of *Gli* genes or GliA proteins in comparison to control (WT + SAG). Besides, we were able to detect GliR proteins in $Ptch1^{-/-}$ cultures either treated or not treated with SAG. These data implies that though Ptch1 negatively controls the initiation of Shh signaling, it does not regulate the following temporal gene-expression patterns, and second, in the condition of fully activated Smo and complete absence of Ptch1 certain portion of full-length Gli proteins at the cilia are anyway processed into GliR. Thus, decline in expression of GliA and GliR proteins over MN window is Ptch1-independent and rather is a result of the downregulation of *Gli* genes.

To assess the effect of constant supply of GliA on temporal patterning of $Nkx2.2^{+}$ NSCs, we generated mice with constitutively expressed Gli1 (hereafter termed $Gli1^{ON}$ mice). Forced expression of Gli1 in $Nkx2.2^{+}$ NSCs sustained the expression of Ptch1 and evoked mild spatial patterning phenotype with slight extension of $Isl1^{+}$MN production and decreased number of $Pet1^{+}$ 5HTNs at E11.5, a time when MN generation is terminated. This mild phenotype was also accompanied by upregulation of *Tgfβ2* at E11.5, revealing a positive

feed-forward regulation of *Tgfβ2* expression by GliA. This finding proposes the transcriptional regulatory network which belongs to the three-node incoherent feed-forward loop (IFFL) class, where GliA activates not only Phox2b but also Tgfβ node that negatively regulates Phox2b. To confirm our assumption that the temporal gene expression pattern observed in Nkx2.2^{+} NSCs is governed by suggested IFFL, we crossed Gli1^{ON} mice with a Tgfbr1$^{-/-}$mice (Gli1^{ON}:Tgfbr1$^{-/-}$) and observed prominent temporal phenotype with massive accumulation of premigratory and migratory Isl1^{+} MNs, sustained expression of *Phox2b* in NSCs and complete absence of Pet1^{+} 5HTNs at E11.5. Besides, *Tgfβ2* expression was markedly reduced in Gli1^{ON}:Tgfbr1$^{-/-}$ mice, implying that positive feed-back regulation by Tgfβ plays an important role in induction of *Tgfβ2* downstream of GliA.

If GliA promotes both *Phox2b* and *Tgfβ2* expression, why *Tgfβ2* expression is delayed in time? A possible answer to this question is the presence of regulatory mechanism based on molecular titration that makes the system ultrasensitive to even very low level of inhibitor (Buchler & Louis, 2008). Indeed, in situ hybridization showed that there was a mutually exclusive relationship between the presence of *Tgfβ2* and *Gli2*, *Gli3* transcripts at r2/3 vHB of the mouse embryo and as soon as the most ventral cells stopped to express *Gli2* and *Gli3*, *Tgfβ2* expression initiated. We assumed that Gli2^{+}/ Gli3^{+} environment provides GliR to suppress *Tgfβ2* expression. The epistasis experiments with forced expression of GliR clearly supported our assumption. Collectively, the presented data support a hypothesis that *Tgfβ2* expression is ultrasensitive to any GliR and suppressed until GliR is completely titrated out, which represents the "all-or-none" type of response. We also suggested that *Phox2b* and other Shh-regulated target genes have certain sensitivity to GliR, but this sensitivity is lower in comparison to *Tgfβ2* which allows to upregulate *Phox2b* earlier.

The suggested Gli inhibitor-titration regulation of *Tgfβ2* expression implies that in condition of complete absence of *Gli2*/*Gli3* expression at time of a MN-to-5HTN temporal switch, Gli1 becomes the main form of GliA that robustly promotes the expression of *Tgfβ2*. Indeed, feed-forward activation of *Gli1* by Gli2, Gli3 proteins is designed to prolong GliA activity in Nkx2.2^{+} temporal lineage and is required to induce Tgfβ2 expression around a time of MN-to-5HTN switch. In support of this assumption, we revealed that Tgfβ2 wasn't induced on time in Gli1$^{-/-}$ mice but appeared with 24-hour delay and finally reached as high level of expression as in controls most likely due to self-propagation of Tgfβ2 expression.

Taken together, the described intrinsic timer mechanism is based on three-node IFFL and involves the intrinsically programmed delayed upregulation of extrinsic switch factor Tgfβ. We know that there are variable fluctuations in the expression level of Gli1 and Gli2 between

individual Nkx2.2^{+} nuclei. How does the system counterbalance the gene expression noise and provide a robust temporal tissue patterning? How does the transition from one cell fate to another happen within such a short period of time and in a relatively synchronized way? We proposed that the nature of Tgfβ as a self-promoting diffusible switch factor is crucial for precisely timed and quick MN-to-5HTN transition in Nkx2.2^{+} temporal lineage. To support this notion, we employed mathematical modelling which showed that the self-activation property of Tgfβ node steepens the response, especially when the positive feedback represents a hysteretic switch. But it doesn't shorten the transition time. Considering that Tgfβ is a diffusible protein and that paracrine Tgfβ signaling facilitates synchronization of the fate switch in the neighboring progenitors, we introduced the spatial averaging in our model. We revealed that spatial averaging together with the positive feedback regulation of Tgfβ expression improves the robustness to noise and that hysteretic switch in this model allows a substantially shorter transition time. GliA is another component of the described timer network; this node lacks spatial averaging due to intracellular localization and functioning. According to our model the highest robustness is provided with low GliA threshold needed for activation of Phox2b, meaning that the moment when Phox2b expression starts to decline is set by activation of paracrine Tgfβ signaling. The modeling of different Gli decay rate shows the proportional inverse changes of the time point at which the fate switch occurs.

Taken together, our mathematical modelling clearly supports the hypothesis that diffusible and self-activating Tgfβ inhibits Phox2b at the population level via paracrine signaling thus reducing the effect of stochastic fluctuations in gene expression and providing a needed precision and reproducibility of temporal tissue patterning.

3.2 PAPER II: ROBUST DERIVATION OF TRANSPLANTABLE DOPAMINE NEURONS FROM HUMAN PLURIPOTENT STEM CELLS BY TIMED RETINOIC ACID DELIVERY

The current strategies to produce hPSC-derived transplantabe mDA progenitors and functional mDA neurons are mainly focused on activation of Wnt and/or FGF signaling pathways that mimic the activity of the IsO. However, the IsO is a regional signaling center that appears during secondary gastrulation stage, when the presumptive FB, MB, HB and SC territories are already defined. Besides, the IsO plays a crucial role not only in the MB but also in the cerebellum and HB development (A. Liu & Joyner, 2001), and as a result, the ongoing activation of Wnt signaling *in vitro* might lead to appearance of undesired cell types (Kee et al., 2017; Kirkeby et al., 2017). We know that RA signaling is somehow involved in the correct positioning of presumptive FB and MB territories very early in the development,

upstream of local developmental cues emanating from the regional signaling center IsO, such as Wnt1 and FGF8 (Ang et al., 1994; Avantaggiato et al., 1996; Ribes et al., 2007).

In **Paper II** we presented the alternative strategy for generation of transplantable mDA neurons from hPSCs by using RA signaling activation within certain time frames which principally differ from current CHIR-based protocols aimed at dose-dependent activation of canonical WNT signaling (Tao & Zhang, 2016).

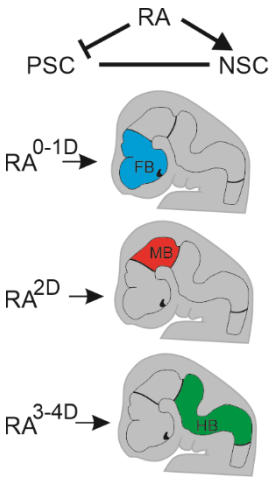

Fig. 7. Summary of the effects of RA-pulse duration on NSC's regional identity. Alekseenko et al., Nature Communications, 2022.

Depending on the duration of RA exposure we could direct NSCs to different regional identities along AP axis: cells grown in dSMADi-only condition and not treated with RA (RA^{0D}) acquired $FOXG1^{+}/OTX2^{+}/HOXA2^{-}$ FB-like identity; cells exposed to RA for the first 24 hours (RA^{1D}) also acquired FB-like identity but with lower level of *FOXG1* expression; 48-hour exposure to RA (RA^{2D}) induced $FOXG1^{-}/OTX2^{+}/HOXA2^{-}$ MB-like cell fate; cells exposed to RA for 3 and 4 days (RA^{3-4D}) acquired $FOXG1^{-}/OTX2^{-}/HOXA2^{+}/$ $HOXB4^{-}$ rostral HB and $FOXG1^{-}/OTX2^{-}/HOXA2^{+}/HOXB4^{+}$ caudal HB identities, respectively (**Fig. 7**). Importantly, RA pulse within the first 48 hours or longer induced rapid conversion of $OCT4^{+}/SOX1^{-}$ hPSCs into $OCT4^{-}/SOX1^{+}$ NSCs in combination with dSMADi within the first 2-3 days.

We next showed that combined RA and Shh signaling (RA^{2D} + SAG), where SAG is a small-molecule agonist of Shh signaling, imposed a vMB identity to hPSC-derived NSCs starting to express classical mdFP markers $LMX1A^{+}/LMX1B^{+}/FOXA2^{+}/OTX2^{+}$ specific to mDA neuron progenitors. To exclude the presence of ventral progenitors of the caudal diencephalon that give rise to mdFP-derived STN precursors (Kee et al., 2017; Kirkeby et al., 2017), we analyzed RA^{2D} + SAG cultures for expression of specific diencephalic STN-progenitors markers BARHL1, BARHL2, PITX2, and NKX2.1 and detected only rare $LMX1A/1B^{+}$ cells that co-expressed PITX2 and NKX2.1 at 14 days in the culture.

Of note, $LMX1A^{+}/FOXA2^{+}/OTX2^{+}$ mDA neuron progenitors induced by timed exposure of RA and SAG, acquire $EN1^{-/LOW}$ rostral-like vMB identity and that specification of vMB cell fate occur independently of WNT1/FGF8 signaling.

Next, we addressed the issue of reproducibility and consistency of RA-induced vMB patterning of NSCs across four different hPSC lines (two hESC lines HS980, HS401 and two

hiPSC lines SM55 and SM56) and showed consistent and high-yield production of $LMX1A^{+}/FOXA2^{+}/OTX2^{+}/NKX2.1^{-}$ vMB progenitors without need to adjust concentration or time exposure of RA, which is an advantage over current CHIR-based protocols that often require titration of ventralizing agent for different hPSC lines (Nolbrant et al., 2017). Then we asked, did altered concentration of RA change the patterning output? To answer this question, we exposed cells cultured in RA^{2D} + SAG-conditions to RA in the range of 100–800 nM and concluded that within the range of 200-400 nM RA the vast majority of NSCs acquired $LMX1A^{+}/NKX2.1^{-}$ vMB-like identity at DDC 9. $LMX1A^{+}/NKX2.1^{-}$ vMB-like progenitors were also generated outside this concentration range, but at a lower yield. This remarkable tolerance of RA-driven patterning of NSCs to different concentrations of this morphogen might be explained by the presence of well-known in the early development of the CNS adaptation mechanism based on negative feedback regulation of the level of RA via induction of RA-degrading CYP26 enzymes involved in the shaping of the RA distribution in the developing brain (Schilling et al., 2012). Indeed, we observed adaptive temporal expression profile of *CYP26A1* in hPSC cultures, when increasing concentration of RA in dSMADi + RA^{2D} condition evoked higher level of expression of *CYP26A1*. To further explore this phenomenon, we questioned how changed activity of CYP26A1 via selective inhibition and the following altered bioavailability of RA in NSCs could change the patterning output in response to timed RA exposure. In RA^{1D} + SAG or RA^{2D} + SAG cultures the inhibition of CYP26 activity with 500nM of the selective antagonist R115866 triggered a $PHOX2B^{+}$ $HOXA2^{+}$ $HOXB4^{+}$ caudal HB-identity, corresponding to regional identity in RA^{4D} + SAG condition; when concentration of R115866 was reduced to 100nM, cell culture was composed of mixture of $LMX1A^{+}/NKX2.1^{-}$ vMB and $PHOX2B^{+}$ HB cells, thus suggesting the intermediate caudalizing effect of intracellular level of non-degraded RA obtained at this concentration of CYP26 inhibitor. These data show the importance of CYP26-based adaptation mechanism that regulates RA bioavailability in cells. As soon as RA avoids degradation, it makes RA signaling activated for a longer period and has caudalizing effect on patterning output.

With our developed approach based on early activation of RA signaling and timed exposure of PSCs to RA morphogen we were able to generate FP cells (peaked at 12-14 DDC) followed by their neuralization that resulted in production of vMB progenitors (peaked at 21 DDC) and then post-mitotic mDA neurons that had undergone the sequential stages of maturation *in vitro* (up to 45 DDC). Thus, ~80% of all HuC/D^{+} neurons expressed TH^{+}, ~10% of neurons expressed GABA (some $GABA^{+}$ cells co-expressed TH) and only rare cells expressed 5-HT or the MN marker PRPH at 35 DDC.

Despite undetectable or very low expression of *EN1* in RA-driven mDA neuron progenitors *in vitro*, temporal mRNA expression analysis revealed a progressive upregulation of *EN1* in the long-term cultures, which was confirmed by immunocytochemistry. This finding shows that EN1 is upregulated within maturation process of mDA precursors, which is in line with previous observations showing the importance of EN1 for survival and maintenance of mDA neurons in mice but not for their specification (Simon et al., 2001). Acquisition of functional maturity by post-mitotic mDA neurons *in vitro* was confirmed by expression of DAT, GIRK2, synaptophysin, CALB1, SOX6, VMAT2 and ALDH1A1 and generation of spontaneous action potentials, evoked action potentials and voltage-dependent Na^+ and K^+ currents at 40-45 DDC.

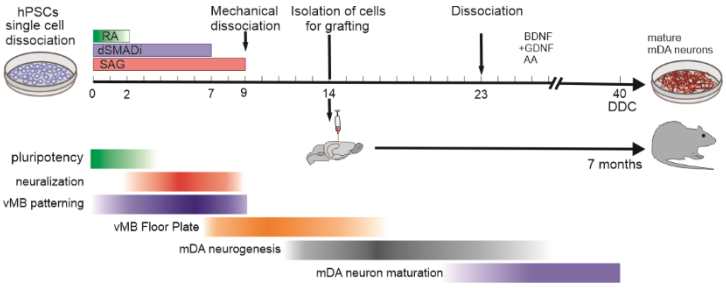

Fig. 8. Schematic summary of differentiation timeline. Alekseenko et al., Nature Communications, 2022.

To reveal the performance of mDA progenitors *in vivo* and, therefore, their utility for cell-replacement therapies of PD, cells were isolated at 14 DDC and grafted to the striatum of athymic (nude) rats with prior unilateral lesion to the nigrostriatal DA system using neurotoxin 6-OHDA which mimicked the loss of DA neurons in patients with Parkinson's disease (a rat model of PD). Seven months post-transplantation analysis showed that all nine rats survived and had TH^+/$HuNu^+$ rich grafts with extensive innervation of dorsolateral striatum and prefrontal cortex, where ~70% and more TH^+ neurons expressed EN1, PITX3, GIRK2 and SOX6, known as markers, enriched in therapeutic SNpc-subtype of mDA neurons (Brignani & Pasterkamp, 2017) (**Fig. 8**). Transplant-induced functional recovery was assessed with standardized amphetamine-induced rotation test: the damage of DA neurons triggered an asymmetry in motor functioning in control rats, but transplantation of RA-specified vMN progenitors resulted in restoring motor deficits.

Timed RA exposure can be used as a viable strategy to specify hPSC-derived neural progenitors into clinically relevant neurons, such as 5HTNs. RA exposure for 3 and 4 days in combination with SHH signaling activation induced $NKX2.2^+$ HB-like progenitors which sequentially generated cranial $PHOX2B^+$ MNs and 5HTNs *in vitro* (**Fig. 9**). Using this approach, we could achieve ~60–65% of total HuC/D+ neurons expressing 5-HT and molecular markers of 5HTNs such as GATA3, LMX1B, tryptophan hydroxylase 2 (TPH2), serotonin transporter (SERT), and the 5-HT1A autoreceptor (5-HT1AR).

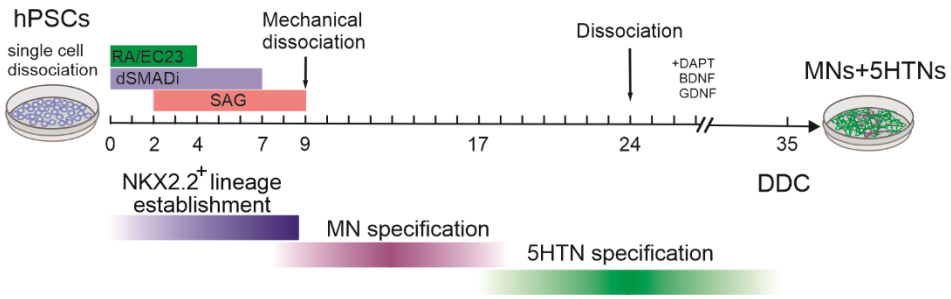

Fig. 9. Schematic summary of differentiation conditions and processes timeline during cranial MN and 5HTN generation. Alekseenko et al., Nature Communications, 2022.

Collectively, identification of RA as a concentration-independent in certain range but tied to time MB patterning signal triggering relatively synchronized differentiation of hPSCs into functional and transplantable mDA neurons gives an alternative way for improving therapeutic interventions aimed to restore the impaired motor function in PD patients. Our developed protocol is Wnt signaling-independent, but it will be highly interesting to explore the combinatorial use of patterning signals to improve cell preparations for transplantations, graft composition and mDA neuron functioning *in vivo*.

3.3 PAPER III: A STEM CELL-BASED PLATFORM FOR PHENOTYPIC IDENTIFICATION OF COMPOUNDS MODULATING SEROTONIN SIGNALING

Central 5HTNs constitute a small fraction of cells (~300.000 in the human CNS) located in the raphe nuclei of the HB that exclusively produce 5-HT in the brain since the peripheral 5-HT from the blood and gut does not cross the blood brain barrier (El-Merahbi et al., 2015). Therefore, the study of human 5HTNs *in vivo* is a challenging task due to low accessibility in the brain and their small number. The emergence of hPSC technologies provided the researchers with new tools that made possible to generate the unlimited number of clinically relevant human neurons *in vitro*, including from patients with unique history of neuropsychiatric disorders, genetical background and response to first-line treatments.

The emergence of hPSC technologies lay a path to utilize their potential in drug development and repurposing via screening of small-molecule compounds with known and unknown bioactivity. So far only a limited number of pharmacological agents, namely escitalopram/citalopram alone or escitalopram with tramadol, were used to modulate the 5-HT release and demonstrated the utility of hPSC-derived 5HTNs as a useful screening approach for drug discovery purpose (Lu et al., 2016; Vadodaria et al., 2016; Valiulahi et al., 2021; Z. Xu et al., 2016). In these studies, the authors assessed the drug-induced modulations

of 5-HT release in the culture medium either using high-performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA). Here, we discovered that intracellular 5-HT content could be used as an alternative phenotypic primary readout to detect pharmacologically induced modulations in 5-HT neurotransmission.

In **Paper II** we developed RA-based protocol for derivation of functional mDA neurons and 5HTNs of the HB *in vitro* from hPSCs. In **Paper III** we integrated RA-based protocol for 5HTN derivation into phenotypic drug screening assay and demonstrated that clinically relevant stem cell-derived 5HTNs could be used for medium-throughput screenings (MTS) with thousands of compounds, and, if needed, the developed assay could be easily scaled up to high-throughput screening (HTS) mode.

At the first stage of our phenotypic screening assay, we employed our earlier developed differentiation protocol to efficiently produce mouse 5HTNs within Nkx2.2^{+} temporal differentiation lineage (Dias et al., 2014) and used them as a cellular platform for MTS to perform primary screen. We showed that mESC-derived 5HTNs were the most prevalent subpopulation of neurons (~80%) that expressed classical markers characteristic to 5HTN cell type, including SERT, TPH2, 5-HT1A and could release 5-HT into the medium. With a set of functional assays measuring 5-HT release (ELISA), 5-HT uptake via SERT (ASP^{+} uptake assay) and activity of MAO enzyme (MAO-Glo^{TM} bioluminescent assay) we clearly showed that mESC-derived post-mitotic 5HTNs were responding to drugs sertraline (SSRI), vilazodone (combined SSRI/5-HT1a partial agonist), nialamide and phenelzine (MAO-Is), reserpine (VMAT2-I) (hereinafter called reference compounds) accordingly to their mode of action. Immunocytochemical analysis of reference compound-treated 5HTN cell cultures revealed anticipated alterations in intracellular 5-HT level, that opened a possibility to use 5-HT content as a primary readout parameter for drug screening purpose.

We optimized the developed protocol specifically for phenotypic small-molecule screening in 96- and 384-well plate conditions and developed the assay workflow that included the following steps: production of desired amount of early postmitotic 5HTNs; reseeding of cells into 384- (for primary screen) and 96-well plates (for confirmatory screens) after 8 days in differentiation conditions (DDC); maturation *in vitro* with growth factors BDNF and GDNF; compound's administration at 14 DDC and the following 24-hour overnight treatment (14-15 DDC); fixation, immunostaining and finally image acquisition and analysis (**Fig. 10**).

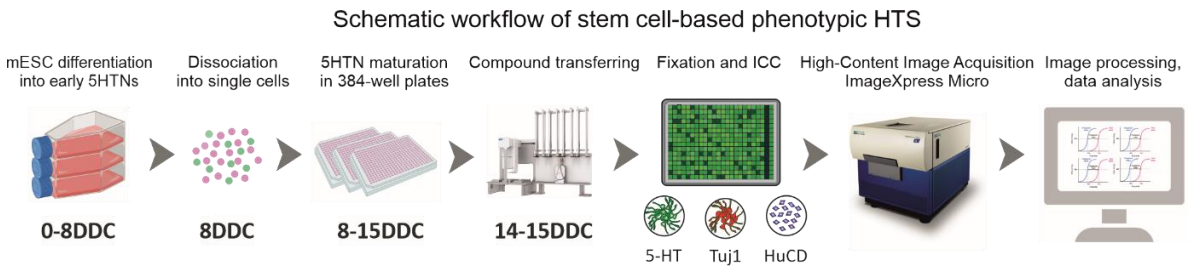

Fig. 10. Schematic workflow of small molecule phenotypic screen in mESC-derived 5HTNs. Some elements are created with BioRender.com.

Under the developed assay conditions, we screened ~5200 compounds with known bioactivity at 10µM in 384-well plates cultured with mouse 5HTNs and identified 287 compounds that significantly changed intracellular 5-HT level: either increased (5-HT UP) or decreased (5-HT DOWN). In the following confirmatory screen, we retested primary hits at 2.5, 10 and 25 µM concentrations and reconfirmed ~60% of compounds (173 hits) based on the same threshold values with the same phenotypic response as in the primary screen.

To validate the previously discovered hits in mouse 5HTN-based primary and confirmatory screens on human 5HTNs, we differentiated HS980 hESCs into 5HTNs that compose ~60% of all neuronal HuC/D^{+} cell population, express pan-neuronal markers (MAP2, SYNAPTOPHYSIN), markers characteristic to 5HTNs (TPH2, SERT) and release 5-HT *in vitro* at 34-35 DDC. The follow-up confirmatory screen on hESC-derived 5HTNs in 96-well plate format reconfirmed ~70% of hits with the same phenotypic response as in the screens on mouse 5HTNs and revealed 95 compounds with unique compound identifier (ID).

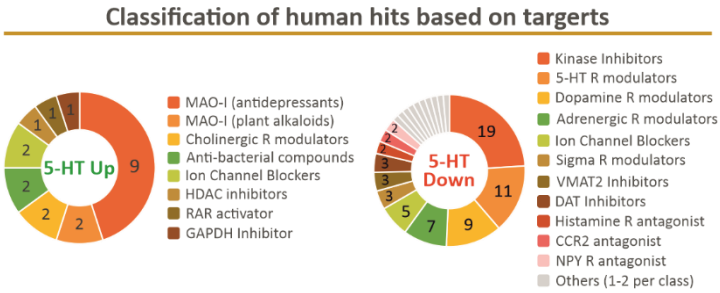

Figure 11. Classification of compounds validated in human follow-up screen, based on the reported target, chemical and/or therapeutic class.

Confirmed hits were classified accordingly to their bioactivity profile where a large number of hits (~45%) exerted their function through modulation of different components of the monoaminergic system (serotonergic, dopaminergic, adrenergic and histaminergic circuitries) such as receptors, transporters, degradation enzymes (**Fig. 11**). Remarkably, we were able to identify 11 out of 20 available MAO-Is which serves as a solid proof-of-concept that our phenotypic approach can be used for screening of compounds with antidepressant-like activity. A set of six compounds were prioritized for further validation through 4 types of secondary assays, including previously used ASP^{+} uptake assay (5-HT uptake), MAO-Glo^{TM} assay (5-HT degradation), 5-HT ELISA (5-HT release) and additional TPH2 assay (5-HT

synthesis). With this limited number of assays, we demonstrated inhibitory activity of selective neuropeptide Y (NPY) Y5 receptor antagonist CGP71683 and isoquinoline alkaloid palmatine on SERT function. Remarkably, we also discovered unknown, at least to our knowledge, significant inhibitory activity of mAChR antagonist oxybutynin on endogenous MAO enzymes in mouse 5HTNs, which was comparable to inhibitory activity of MAO-I phenelzine (served as a positive control). The result was consistent with increased 5-HT release under oxybutynin treatment and verified by incubation of oxybutynin with human recombinant MAO-A enzyme.

In conclusion, the synthesis of scalable stem cell-based protocols of mouse and human 5HTN derivation with phenotypic screening approach where intracellular 5-HT content serve as a primary readout proved to be very fruitful and could be employed for drug discovery purpose at an early pre-clinical stage of drug development.

4 CONCLUSIONS AND FUTURE PROSPECTIVES

The following conclusions can be drawn from the research studies presented, specifically:

1. Temporal patterning of NSCs in the Nkx2.2^{+} temporal lineage in the developing vHB is based on down-regulation of *Gli* genes that results in progressive GliR decay. Removal of GliR provides a switch-like response mediated by upregulation of diffusible and self-activating TGFβ through paracrine TGFβ signaling in the neighboring NSCs, which in turn produce coordinated MN-to-5HTN transition at the population level.
2. Timed and early exposure of hPSC-derived NSCs to RA allows to specify the vMB progenitors towards functional mDA neurons, where the RA-induced patterning is insensitive to concentration of RA within certain range, in contrast to widely used CHIR-based protocols for DA neuron derivation and additionally can be used to generate more caudal and clinically relevant types of neurons such as 5HTNs.
3. Small-molecule phenotypic screen based on automatic quantification of intracellular 5-HT signal in stem cell-derived mouse and human 5HTNs can serve as robust and scalable approach to screen thousands of compounds and reveal small molecules that modulate 5-HT neurotransmission and thus, can be utilized for drug development and repurposing.

Computational modeling of temporal patterning helps to understand how time is encoded in NSCs when they perform transition from one progenitor state to the next one within one temporal lineage. The suggested three-node timer motif in **Paper I** is only a part of more complex network architecture that time the development and itself can't explain the transient expression of *Gli* genes. There is a known negative regulation between Shh-induced Nkx2.2/FoxA2 TFs and *Gli2*/*Gli3* expression, however this repressive node is absent in our developmental timer circuitry. More advanced modeling based on more experiments will be in great need to understand how switch-like responses occur during neurogenesis. There are data showing that TGFβ signaling is a universal pathway that controls correct timing of switch-like responses within different temporal lineages (Sagner et al., 2021). The authors of this study revealed that NSCs use conserved and limited set of global patterning TFs that are induced in a temporally restricted and sequential order. Would be interesting to assess a role of these TFs in our Nkx2.2^{+} temporal lineage. Besides, there are signaling pathways that contribute to spatial patterning in the neural tube such as dorsalizing BMP and Wnt signals secreted by RP cells. Are these pathways influence Shh-dependent patterning and its timing?

Deep understanding of neural patterning helps to advance the differentiation protocols that are tuned to produce the desired type of cells for disease modeling *in vitro*, stem cell-based replacement therapies and for drug development. But it's a challenging task due to the complexity of regulatory networks that control patterning. In 2D culture conditions NSCs inevitably lose their specific positional information that makes impossible to fully reconstruct *in vitro* the microenvironment with all extrinsic cues that direct their specification and differentiation into neurons as *in vivo*. Therefore, one of the future tasks could be to apply the developed in **Paper II** protocol for mDA neuron and 5HTN derivation to the organoid culture. Another future goal is to produce cultures enriched with desired subtype of mDA neurons, for example $SOX6^+$ $AGTR1^+$ mDA neurons, the most vulnerable subpopulation within SNpc that is severely affected in patients with PD (Kamath et al., 2022). To reach this goal, one study suggested sorting mDA progenitors based on expression of two cell surface markers CLSTN2 and PTPRO which presence in cells predict good graft outcome with 90% of donor cells having A9 identity (P. Xu et al., 2022). Authors of another recent study modified the existing CHIR-based protocol for mDA neuron derivation with introducing temporal inhibition of Wnt signaling between day 11 and day 16 which resulted in a robust induction of $Sox6^+$ SNpc-like identity and inhibition of $Otx2^+$ VTA-like identity in mDA neuronal cultures (Oosterveen et al., 2021). I see as a future goal to apply these and other approaches to our RA-based protocol to more efficiently direct NSCs into SNpc-like DA neuronal lineage with the following assessment of the identity *in vivo*, after transplantation into the dorsal striatum of a rodent model of PD.

The central serotonergic system remains a most common target for treatment of various neuropsychiatric disorders. Derivation of cultures enriched with subtype-specific 5HTNs represents a certain interest since the rostral 5HTNs are responsible for high-order brain functions, such as emotional responses. Modification of our RA-based protocol to produce more $EN1^+$ rostral 5HTNs can be one of the possible future goals.

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