Chapter 9

Telencephalic Neurogenesis Versus Telencephalic Differentiation of Pluripotent Stem Cells

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Additional information is available at the end of the chapter

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1. Introduction

The mammalian telencephalon, which comprises the cerebral cortex, olfactory bulb, hippocampus, basal ganglia (striatum and globus pallidum), and amygdala, is a highly complex and evolutionarily advanced brain structure. All higher brain functions including the integration and processing of sensory and motor information, the memory storage and retrieval, and the regulation of emotional and drive states take place at the telencephalic level. In humans, the telencephalon also governs the ability to make rational decisions, to plan for the future and to have the creative impulses [1].

At cellular level, the telencephalon is populated by a large diversity of neurons, including **glutamatergic** projection neurons, **GABA (γ-aminobutyric acid)-ergic** interneurons and projection neurons, as well as **cholinergic** interneurons and projection neurons.

Many neurological pathologies are caused by malfunction of telencephalic neurons, as a result of neurodegenerative processes (e.g. Alzheimer disease), genetic mutations (e.g. Huntington disease), or abnormal development (e.g. autism, schizophrenia and epilepsy), all with devastating consequences for the normal brain function.

During the past ten years much progress has been made in elucidating the mechanisms that orchestrate the generation of different telencephalic neuronal subtypes. A combination of fate-mapping studies with genetic loss-of-function and gain-of-function experiments has been successfully used to uncover important molecular players in the development of the rodent telencephalon.

At early stages of its development, the telencephalon is divided into two main regions: **dorsal** (pallium) and **ventral** (subpallium). The pallium is further subdivided into three longitudinal zones: dorsal, medial, and lateral. The dorsal pallium gives rise to the **neo-**

cortex - the most complex structure of the mammalian brain. The medial pallium develops into the hippocampal formation (archicortex), cortical hem and the choroid field. The lateral pallium matures into the paleocortex (olfactory and some limbic areas). From the **ventral** telencephalon medial, lateral and caudal ganglionic eminences (**MGE, LGE** and **CGE**) emerge giving rise to the **basal ganglia** and parts of the **amygdala**, but also to neurons that migrate into the cortex and olfactory bulbs. The progenitor domains in the embryonic telencephalon generate specific types of neurons which finally form the complex neural networks of the mature telencephalon.

Understanding the developmental ontogeny of the diverse telencephalic neuronal populations provides an essential framework for the design of rational approaches towards pluripotent stem cell differentiation for *cellular models* and *cell replacement therapies* for telencephalic related diseases.

In the first part, we review the stages of the mouse telencephalic development, the morphogens and the transcription factors (TF) that are intimately involved in the telencephalic patterning and neuronal subtype specification (**Sections 2-5**).

In the second part, we present recently reported protocols for differentiation of mouse and human pluripotent stem cells into telencephalic populations, following the development principles and reflecting the *in vivo* signaling pathways; we point on the relevant morphogens and TF in each stage, where the level of expression of relevant sets of TF can be considered as a milestone between each differentiation step *in vitro* [2] (**Sections 6-8**).

Finally, we describe our model system in which the *in vitro* differentiation of human and mouse embryonic stem (ES) cells are temporally aligned to each other and compared with mouse telencephalic neurogenesis *in vivo* [3]. Since the telencephalic development has been extensively studied in animal models, it is important to strengthen the interspecies comparative approaches in order to gain further insights into the human telencephalic development. We provide evidence for differences in the default differentiation of mouse and human pluripotent stem cells that proves the utility of the comparative system for optimizing the directed telencephalic differentiation of human pluripotent stem cells. We also exemplify how Hedgehog (Hh) signaling pathway is implied in telencephalic neuronal fate decision *in vitro* and *in vivo* (**Section 8**).

2. Telencephalic neuronal cell diversity

The neurons populating the mature telencephalon are generally classified based on their intrinsic properties: neurochemical profile, morphology and electrophysiological responses. The understanding of how the telencephalic neuronal subtypes are specified encompasses not only the signaling pathways that act in spatial-temporal sequences to confer positional and molecular identity but also the location of the progenitors early in development and the migration pathways they undertake to reach their final destination in the mature brain. The main neuronal types and telencephalic domains in the adult and embryonic mouse brain are schematically presented in Figure 1.

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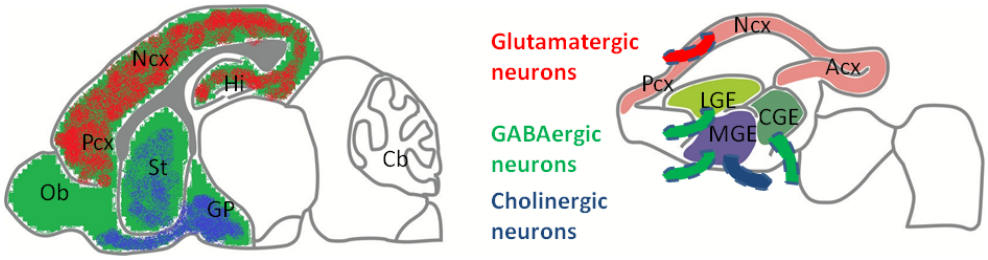

Schematic mouse mature (left side) and embryonic (right) brain; telencephalic subdivision: olfactory bulb (Ob), neocortex (Ncx), palleocortex (Pcx), archicortex (Acx), hippocampus (Hi), striatum (St) and globus pallidum (GP), pallium (pink) medial ganglionic eminence (MGE-violet), lateral ganglion eminence (LGE-light green), caudal ganglionic eminence, (CGE-dark green); domains and origins (as arrows) of telencephalic glutamatergic (red), GABAergic (green) and cholinergic (blue) neurons.

Figure 1. The domains of telencephalic neurons in adult and embryonic mouse brain

Glutamatergic projection (pyramidal) neurons comprise the majority (70-80%) of cortical neurons, they are generated in the dorsal telencephalon; have an excitatory role in the cortical and include many subtypes. Each subtype is characterized by a specific combination of laminar position, morphology, marker expression and connectivity pattern [4;5].

Cajal-Retzius neurons are a transient population expressing reelin and playing a key role in the formation of the cerebral cortex. They die during the first postnatal week [6].

GABAergic neurons are generated in the ventral telencephalon and also include many subtypes of *interneurons* and *projection neurons*.

GABAergic cortical interneurons, comprising 20-30% of the cortical neurons, have an inhibitory role in the cortical circuits; they originate in the ventral telencephalon and subsequently migrate dorsally into the developing cortex.

Other types of GABAergic neurons include the interneurons and projection neurons that populate the striatum, pallidum, olfactory bulb and other forebrain ventral regions.

Different subclasses of GABAergic interneurons arise from different progenitor domains in the subpallium: *somatostatin (Sst)* subclasses of GABAergic interneurons that ultimately reside in the cortex and the basal ganglia are generated in dorsal MGE (dMGE) [7;8]. *Parvalbumin (Pv)* subclasses of GABAergic interneurons, constituting the majority of the cortical interneurons, are generated in ventral MGE (vMGE) [9;10]. *Calretinin (Carl), NPY and reelin*-expressing GABAergic cortical interneurons are produced primarily in CGE [11;12]. Calr expressing GABAergic interneurons, which ultimately reside in the olfactory bulbs and amygdala, arise from the dorsal LGE (dLGE) [13].

GABAergic projection neurons, such as the medium spiny neurons (MSN) which constitute the majority of the striatal neurons, express DARPP32 and Calr and arise from the ventral LGE (vLGE) [13;14].

Cholinergic neurons in the telencephalon (both *interneurons* and *projection neurons*) are generated in the MGE. First, cholinergic projection neurons are produced by vMGE, followed by the production of cholinergic interneurons from dMGE, at later time points. Cholinergic interneurons populate the striatum; cholinergic projection neurons populate the pallidum and the septum and project mainly to neocortex and hippocampus, respectively [15].

3. Stages in telencephalic development

A fundamental feature of the nervous system development is the precise temporal sequence of cell type generation. The first neural cells, **neuroepithelial (NE) cells**, arise from the pluripotent stem cells of the early blastocyst that differentiate from the ectoderm towards the neuroectoderm through a process named **neural induction** [16;17].

Morphologically, NE cells are columnar epithelial cells which form the neural plate and later on the ventricular zone (VZ) of the neural tube. They are considered to be primordial neural stem cells that give rise to various types of neurons, followed by glial cells [18-20]. The elaborated process by which NE cells progress towards telencephalic neurons can be divided into several discrete stages:

1. **Early anterior/posterior (A/P) patterning.** The NE cells in the neural plate acquire an A/P identity; the anterior ones give rise to the telencephalic primordium.
2. **Dorsal/ventral (D/V) patterning.** Once the neural tube is formed and the telencephalic primordium is established, it is subdivided into discrete territories where the NE cells proliferate and transform into neural progenitor (NP) cells that reside in the adjacent newly-formed subventricular zone (SVZ). In the dorsal telencephalon, the NP cells are radial glia and basal (or intermediate) progenitors [5;21-23]. Different progenitor domains are formed in the ventral telencephalon: MGE, LGE and CGE.
3. **Neuronal specification.** Each of the progenitor domains produces specific types of neurons which further develop different neurotransmitter identities and connectivity patterns.

The **signaling pathways** controlling the neural cell fate specification during these stages have been the focus of intense research in the recent years [4;19;24-27]. Extrinsic factors, named **morphogens,** induce two or more different cell fates in a concentration-dependent manner by modulating the expression and activity of specific **TF.** The TF can in turn modulate the secretion of morphogens. The combinatorial expression of these TF instructs each unique NP population to generate progenies that are committed to specific neural fates.

4. Stage-related morphogens in mouse telencephalic neurogenesis

The morphogens known to play a role during telencephalon development are: sonic hedgehog (SHH), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs),

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Wingless/INT proteins (WNTs), transforming growth factors (TGFs) and retinoic acid (RA). They are secreted from specific centers, named organizers, during early stages of development [28].

Genetic evidence based on loss- and gain-of-function studies have indicated that the role of these morphogens can be rather complex. Depending on the developmental stage it ranges from establishment of general patterning characteristics to neuronal specification [5;21;23;26;29-33].

4.1. Early A/P patterning

A/P patterning starts to emerge in parallel with neural induction, prior to and during gastrulation. At embryonic day (E) 8.5, in regions of the embryo protected from the influence of caudalizing factors, such as WNTs, BMPs, and RA, or where their antagonists are secreted, such as Dickkopf1 (DKK1), an inhibitor of the WNT signaling pathway, and Noggin, an inhibitor of the BMP signaling pathway, the NE cells develop an anterior character and form the prospective forebrain (future telencephalon and diencephalon) [28;33-35]. FGFs (e.g. FGF8, FGF15, FGF3) are expressed early on at the anterior tip of the neural plate and then maintained in the anterior limit of the neural tube [32]. Although not a primary inducer of the telencephalic fate, FGF signaling influences the telencephalic gene expression [32;36].

4.2. D/V patterning

With regard to the location and timing of telencephalic progenitor generation, different extrinsic factors are involved in their patterning and self-renewal. WNTs and BMPs pattern the telencephalic progenitors dorsally, while SHH patterns them ventrally. BMPs are expressed dorso-medially and are required for the formation of the choroid plaque and the cortical hem [31;37;38]. WNTs are secreted from the cortical hem and promote the development of the hippocampus [30]. The expression of SHH is first observed at E8.5 in structures adjacent to the ventral telencephalon, and by E9.5 in the MGE and preoptic regions [29]. SHH promotes the formation of all ventral telencephalic subdivisions [29;39-41]. FGFs are involved in both ventral and dorsal patterning [27;30;32;42;43]. Activin, a TGF-related molecule, acts ventrally in the CGE patterning [44]. RA contributes to the patterning of the lateral telencephalon and participates in setting-up the D/V boundary [45-49].

4.3. Neuronal specification

The balance between the signaling inputs that control NP self-renewal and differentiation is critical for the initiation of the terminal differentiation program. FGFs and SHH, in addition to their patterning activities, promote self-renewal and prevent differentiation, while RA promotes neuronal differentiation [1;47]. Notably, it has been shown that the expression of SHH is required during distinct developmental windows for the specification of neuronal identity [29]. FGF signaling may ultimately influence the generation of cell diversity within the ventral telencephalon [30;50]. WNT promotes neuronal differentiation in different late cortical progenitor cell populations [51]. BMPs inhibit neurogenesis but could participate in

late neuronal specification and maturation of different subpopulations [52]. Activin is also a potent neurotrophic factor that induces differentiation of telencephalic neural precursors into calretinin-positive cortical interneurons [44].

5. Stage-related TF in mouse telencephalic neurogenesis

The main TF involved in the early patterning and specification belong to **homeobox domain (HD)** and **basic helix-loop-helix (bHLH)** families, however other TF such as **zinc-finger proteins** also have essential functions.

The main HD containing TF families are: paired-box (e.g. Pax6), forkhead box (e.g. Foxg1), NK2 homeobox (e.g. Nkx2.1, Nkx6.1), orthodenthicle homeobox (e.g. Otx1/2,), sine-oculis homeobox (e.g. Six3), GS homeobox (e.g. Gsh2), distal-less homeobox (e.g. Dlx1-6), LIM homeobox (e.g. Isl1, Lhx2, Lhx6, Lhx8), empty-spiracle homeobox (e.g. Emx1/2), T-box: (e.g. Tbr1/2). Other TF, such as Mash1, Ngn1/2 and Olig2, belong to bHLH class. Gli members are zinc-finger proteins.

5.1. Early A/P patterning

The telencephalic neuroepithelium is first characterized by the expression of **FoxG1** (also named BF1) [53], **Pax6** [54;55] and **Gli3** [56]. The anterior phenotype also expresses **Six3, Otx1** and **Otx2** [57-59].

5.2. Dorsal/ventral patterning

In the mouse **dorsal** embryonic telencephalon, Pax6, Emx1 and Emx2 are specifically expressed in VZ and SVZ progenitor domains (Figure 2A).

Pax6 which is essential for setting up the sharp border between ventral and dorsal telencephalon is mainly expressed in the prospective neocortex, while Emx1 and Emx2 are medially expressed in the archicortex (later hippocampus); Lhx2 is expressed in both regions [30;31;45;60;61], Tbr2 is expressed in the SVZ corresponding to basal progenitor domains [62;63].

Nkx2.1 expression is the hallmark of the MGE development. At E9.5, Nkx2.1 appears within the **ventral** telencephalic domain, defines the MGE at the molecular level and persists in this region throughout development (Figure 2A). Around E10.0 the expression of Gsh2 accompanies the emergence LGE and further to CGE, with a lower expression level in MGE (Figure 2A). Nkx6.2 is expressed along the MGE/LGE sulcus and at high levels in the dMGE [1;64].

The mutual antagonism between Pax6 and Nkx2.1 and later on between Pax6 and Gsh2 is required for the correct positioning of the D/V boundary [65-67]. FoxG1 is involved in both ventral and dorsal patterning [27;30;32;42;43;52].

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The central mechanism that determines NP D/V patterning is the activity of the Gli family of transcriptional regulators–Gli1, Gli2, and Gli3. SHH promotes the formation of a ventral telencephalic subdivision by inhibiting the dorsalizing effects of Gli3 [27;35;56;68]. Gli3 is highly expressed dorsally, with lower expression in the LGE and MGE. Gli1 is expressed ventrally, at high levels in the progenitor domain of the dMGE and vLGE, whereas Gli2 is highly expressed in the progenitor domain of the dorsal telencephalon, with a lower expression in the LGE (Figure 2B).

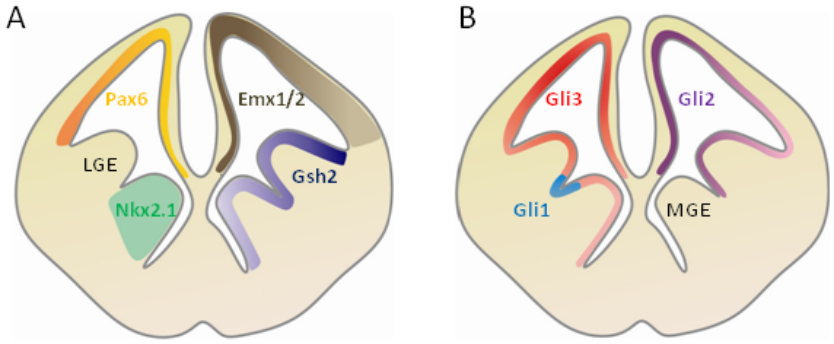

Figure 2. The domains of the main transcription factors implied in dorsal-ventral patterning in mouse embryonic telencephalon.

5.3. Neuronal specification

The mechanisms of neuronal specification in the dorsal telencephalon have been extensively studied in the context of cerebral cortex development. The dorsal progenitors produce neurons, in a tightly controlled temporal order from E10.5 to E17.5. Pax6, Ngn1 and Ngn2 instruct **glutamatergic** identity and inhibit astroglial differentiation [69-72]. The differentiation by Ngns involves the sequential activation of the expression of other TF such as NeuroD, Tbr1 and Tbr2 [69]. NeuroD has been implicated in the terminal differentiation of the hippocampus [73]. The differentiation of specific populations of projection neurons is controlled by neuronal subtype-specific genes, which have only begun to be identified. The timing of cortical neurogenesis is encoded within lineages of individual progenitor cells, with different locations [74].

The earliest born neurons form a layered structure termed the preplate, which is later split into the superficial marginal zone and the deeply located subplate. The cortical plate, which will give rise to six-layered neocortex, begins to develop between these two layers. The later born neurons arriving at the cortical plate migrate past earlier born neurons [5;74]. During development, neurons in different layers are generated in an inside-first, outside-last order, and newly postmitotic neurons are specified to adopt the laminar positions characteristic of their birthdays [5;24;75].

Mash1 is the main neurogenic TF in the ventral telencephalon and is involved also in the neurotransmitter identity specification, being a selective instructor of **GABAergic** identity [70;76-82]. Olig1/2 can promote both neuronal and oligodendroglial fates while inhibiting astrogliogeneis [83].

Dlx genes (Dlx1, 2, 5 and 6) are expressed in ventral progenitors and neurons in MGE, LGE and CGE, and are likely to play a role in neural specification [78;84;85].

Interestingly, MGE, LGE and CGE progenitor domains do not give rise to homogeneous populations of neurons, which is most likely due to a further subdivision of these domains into regions with spatially restricted expression of specific TF.

There is also a temporal control of the specification of various neuronal subtypes; as a general pattern, the earlier- born ventral cells give rise to projection neurons while the more dorsally positioned later-born cells generate interneurons [24;82;86-88].

MGE is characterized by the early production of cholinergic projection neurons from its ventral part, followed by the late production of GABAergic and cholinergic interneurons from the dorsal domains. Two TF are detected at E12.5 exclusively in the MGE: Lhx6 and Lhx8 (or Lhx7) [89], suggesting a role in the specification of MGE-derived neurons. Lhx6-expressing neurons have the characteristics of proto-GABAergic neurons with dual differentiation potential, while Lhx8 seems to be involved in the differentiation of specific cholinergic neurons [15;90]. The differentiation of a common proto-GABAergic precursor into mature subtypes is regulated by the combinatorial activity of the Lhx6, Lhx8 and Isl1. Those proto-GABAergic neurons that maintain the expression of Lhx6 differentiate into mature GABAergic striatal interneurons. By contrast, induction of Isl1 and the combined activity of Lhx7/8 and Isl1 results in down-regulation of Lhx6 and commitment along the cholinergic interneuron sublineage [90-92]. Thus, it appears that a LIM HD transcriptional code determines cell-fate specification and neurotransmitter identity in neuronal subpopulations of the ventral telencephalon.

Ventral LGE generate GABAergic projection neurons that also express Isl1 during early specification, followed by the expression of other striatal-specific TF such as FoxP1, FoxP2, and Ctip2 [93-95]. Later in development, dLGE generates interneurons that migrate to the olfactory bulbs [13;95].

6. Telencephalic neurons generated from mouse ES cells

Mouse ES (mES) cells have emerged as a powerful tool for developmental biology. Several studies have focused on mES-derived telencephalic progenitors and the specific neuronal populations they generate *in vitro* and *in vivo* [3;96-101].

6.1. Early A/P patterning

After the first generation of defined telencephalic precursors from mES cells based on studies of forebrain development [101], significant improvements regarding the success

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of neural induction and telencephalic patterning have been reported during the following years [96;100].

Blocking the WNTs and BMPs pathways by applying the antagonists DKK1 and BMPR1A-FC, respectively, cause neural induction in more than 90% of mES cells and maintained around 35% of the progenitors as telencephalic, expressing FoxG1 [101]. Other following studies has reported a massive FoxG1 expressing telencephalic progenitor generation in serum-free, RA-free conditions [96-98;100], while using a RA treatment, FoxG1 expression has not been detected [98].

6.2. Dorsal/ventral patterning

SHH or a Hh agonist treatment on telencephalic progenitors suppresses the dorsal marker Pax6 and induced the ventral marker Nkx2.1. SHH application does not cause substantial difference in the level of Gsh2 expression [100;101].

The majority of mES cell-derived progenitors exhibit by default a ventral phenotype which has been attributed to the high level of endogenous SHH signaling. Blocking the Hh signaling converts most of the ventral telencephalic precursors into dorsal progenitors, with the majority expressing typical markers of the dorsal telencephalon*:* Pax6 and Emx1/2 [3;97;99].

6.3. Neuronal specification

The telencephalic progenitors derived from mES cells can be directed to neurons that express the excitatory neurotransmitter glutamate and the inhibitory neurotransmitter GABA [3;102-106].

The dorsal progenitors have been shown to produce mature neurons with many features of cortical pyramidal neurons in a temporal manner similar with *in vivo* corticogenesis. The first neurons generated in these cultures are reelin-positive Cajal-Retzius-like and subplate-like neurons expressing Tbr1, followed by the glutamatergic neurons generated in an inside-first, outside-last manner. The majority of the cells generated by Gaspard *et al*. express markers of deep cortical layer V and VI neurons, like Tbr1, Otx1, Ctip2 and FoxP2 [102]. Eiraku *et al.* have also generated deep layer neurons, positive for Ctip2 and Emx1 in the first 9 days of neuronal differentiation of dorsalized progenitors [96].

By transplantation of dorsally patterned progenitors into postnatal murine cerebral cortex, the production of cortical projection neurons with the correct morphology and axonal connectivity has been demonstrated [98]. The pyramidal neurons express Otx1, Emx1, and Ctip2, corresponding to deep layers neurons. They integrate and appropriately project long-distance axons to subcortical targets, without forming tumors [98].

Regarding the protocols for ventral neuronal specification, it has been shown that Nkx2.1 and Gsh2 expressing progenitors give rise both to GABAergic and cholinergic neurons [3;100;101].

Furthermore, the use of Lhx6::GFP ES cells has allowed the isolation of cells with potential for developing into ventral telencephalic subpopulations and their follow-up during transplantation into the postnatal brain. Transplanted Lhx6::GFP cells demonstrate the ability to retain migratory capacity and neuronal commitment without forming tumors and exhibit cortical interneuron characteristics [100].

7. Telencephalic neurons generated from human ES cells

Neural induction takes place in human ES (hES) cells similar to mES cells, however with a different timing. Under serum-free conditions, without adding known morphogens [3;101;105-110], by co-culture with stromal cells [111;112] or by using a recently defined protocol with dual-SMAD-inhibition [113], it has been demonstrated that hES cells differentiate into a synchronized population of NE cells organized into neural-tube-like rosettes within 2 weeks, a time corresponding to the development of the neural plate/tube in a human embryo. Around day 8-10, the primitive NE cells express PAX6, and the neural fate is consolidated by SOX1 expression around day 14 [3;100;105;106;108-110].

7.1. Early anterior/posterior patterning

In the absence of exogenous morphogens, hES cells differentiate into progenitors that uniformly expressed anterior TF, including FOXG1, OTX2, SIX3, LHX2 but not posterior TF [3;107;106;109]. Even when the neural induction is performed by co-culturing with stromal cells, a large population of neural progenitors exhibit anterior characteristics [112]. FOXG1 appears at approximately day 10 and it is still expressed at day 24 and even in postmitotic neurons, 1 month after differentiation. PAX6 is co-expressed in nearly all of the FOXG1 telencephalic progenitors (95% of all cells) one month after differentiation, in the absence of growth factors or morphogens [3;106;114].

7.2. Dorsal/ventral patterning

WNT proteins and their downstream molecules have been found to be highly expressed right after the generation of NE cells. In minimal medium and in the absence of known morphogens, hES cell-derived telencephalic progenitors exhibit a dorsal telencephalic trait, which has been attributed to endogenous WNT signaling [115]. The activation of SHH and/or inhibition of WNT permitts the specification of ventral telencephalic progenitors.

At the lower dosage, SHH reduces the PAX6-expressing cell population and increases the NKX2.1 cell population. At the higher dosage, SHH almost completely eliminates the PAX6-expressing cells, while increasing the NKX2.1 ventral progenitors. The combination of DKK1 and SHH at the lower dosage significantly increases NKX2.1 and decreases PAX6 and GLI3 expression. High concentrations of SHH significantly inhibits GLI3 expression compared with low concentrations of SHH. The specification of dorsal-ventral progenitors by WNT and/or SHH has been partially achieved through differentially regulating the expression of

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active and repressive forms of GLI3. Using of a low concentration of SHH alone results in the differentiation of both LGE and MGE progenitors, whereas additional WNT inhibition (by DKK1) further ventralizes the human neural progenitors, resulting in a predominant population of NKX2.1 expressing MGE progenitors [115]. Aubry *et al*. have patterned the telencephalic progenitors by SHH and DKK1 treatment for 12 days in adherent culture and have found a significant up-regulation of the LGE markers GSH2 and DLX2 [111].

7.3. Neuronal specification

The regionalized dorsal and ventral human telencephalic progenitors further differentiate into functional cortical glutamatergic neurons and telencephalic GABAergic neurons, respectively.

Cortical glutamatergic neurons have been efficiently generated in the absence of morphogens. This indicates the intrinsic tendency of hES cells to generate cortical neural cells [3;109]. Neurons differentiated from dorsal progenitors in the absence of exogenous morphogens for 6 weeks exhibits a pyramidal morphology, with extensive neurite outgrowth, and expressed TBR1 and CTIP2 [96, 115].

Ventral telencephalic GABAergic neurons have been induced with a high concentration of SHH, a low dose of SHH together with WNT inhibitors [115] or by using SHH agonists [3].

Additionally, Aubry *et al*. have focused to the characterization of the striatal-like progenitors, showing that more than a half of the specified neurons were DARPP32 and Calr positive, exhibiting phenotypic features of MSN. Transplantation of the LGE-patterned progenitors in quinolinic acid–lesioned rats (a model for Huntington disease) has confirmed the *in vivo* specification toward striatal MSN [111].

Human ES cell-derived telencephalic glutamatergic and GABAergic neurons have been reported to be electro-physiologically active [111;115;116] and also to integrate in the mature telencephalon after transplantation [117].

8. Modulation of Hh signaling pathway in telencephalic cells generated from mouse and human ES cells

We recently proposed a novel model system in which the *in vitro* differentiation of hES and mES cells are temporally aligned to each other and compared with mouse telencephalic neurogenesis *in vivo*. In this comparative model system, we tested the *in vitro* role of Hh signaling for ES cell-derived telencephalic differentiation (Figure 3) [3].

Neural differentiation of 2 lines of mES cells and 2 lines of hES cells was studied under identical, defined conditions, but following different time-schedules for mouse and human cell cultures. The *in vitro* time schedules were based on data from *in vivo* development as a reference for the stages of neural induction, neural patterning, and neuronal

specification. In addition, we developed a specific profile of marker genes, which was derived from *in vivo* studies.

Our results demonstrated that neural differentiation took place in mES cell-derived cultures resulting in the generation of neural progenitors and neurons in a time-frame which mirrors telencephalic neurogenesis *in vivo*. The expression levels of telencephalic markers were comparable between *in vivo* and *in vitro* differentiated populations. We demonstrated that the neural differentiation in human cells can be temporally aligned with mouse cells in the proposed neurogenic time-windows. Thus, our temporally aligned, comparative cell culture model offered a novel platform for analyzing the effect of signaling molecules on the generation of specific telencephalic populations in mouse and human cell cultures.

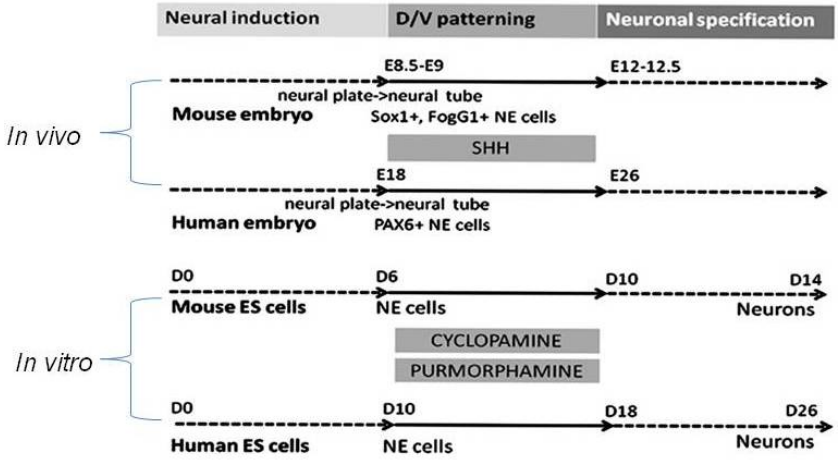

Figure 3. Hh signaling for modulation of dorsal ventral patterning in mouse and human telencephalon in vivo and in vitro, in ES stem cell-derived cell cultures (modified from Nat et al 2012 [3]).

To exemplify the value of this approach we analyzed in greater detail a single process, the step of D/V telencephalic patterning. Thus, we monitored the effect of pharmacological modulators of the Hh signaling pathway, purmorphamine—an agonist and cyclopamine—an antagonist acting on the Smoothened receptor (Smo), regarding the expression of region-specific TFs and signaling molecules relevant for telencephalic development *in vivo*.

Purmorphamine strongly up-regulated the expression of telencephalic ventral markers Nkx2.1, Nkx6.2, Lhx6, and Lhx8 in mouse and human cells, thus reflecting the *in vivo* process of the MGE patterning and specification. Cyclopamine up-regulated the expression of telencephalic dorsal markers, but at lower levels in human compared with mouse cells. Interestingly, the modulation of Smo *in vitro* differentially affected the expression of molecules of the Hh pathway, especially the Gli1 and Gli3 effectors and Ptch receptors, in mouse *vs* human cells.

We additionally examined how the SHH expression itself was modulated by Smo agonist or antagonist treatment. We reported that SHH expression is regulated in a very dynamic way

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by Hh pathway modulation, both in mES and hES cell-derived models. In particular, a very robust up-regulation of SHH by purmorphamine was observed in the human model, where SHH was not expressed in untreated controls. Shh was expressed in both progenitor cells and neurons in our cultures. This might be explained by the non-cell autonomous mechanisms recently described in the mouse embryonic telencephalon, where both Lhx8 and Lhx6 genes controlled the expression of Shh in the mantle zone of the MGE, corresponding to early-born neurons [118]. Thus, Lhx6 and Lhx8 appear to regulate MGE development by promoting Shh expression in MGE neurons, which, in turn, promotes the developmental program of the dMGE.

The activation of Hh signaling *via* Smo with purmorphamine converts the primitive dorsal telencephalic precursors to ventral progenitors. These progenitors differentiate into neuronal subtypes including GABAergic and cholinergic neurons.

Our results provided evidence for the different default telencephalic differentiation of mouse and human ES cells: ventral and dorsal, respectively. Additionally, it proved the utility of the comparative system for optimizing the directed differentiation of human pluripotent stem cells.

9. Conclusion

Recent studies have shown that both mES cells and hES cells differentiate into region specific progenitors, following the same developmental principles that have been identified by studying mouse CNS development. Together with previous findings, our own data support the model in which the human neural progenitors in culture develop a reverse default D/V phenotype compared with mouse. However, early human NE cells can be efficiently differentiated into dorsal and ventral telencephalic progenitors *via* modulating similar molecular pathways as described in rodents.

Therefore, mES and hES cell-derived models, directly compared in parallel experiments and temporally aligned to *in vivo* telencephalic development, offer a platform for testing the effect of morphogens, growth factors, and pharmacological substances for the generation of specific neuronal subtypes.

Additionally, telencephalic progenitors and neurons generated *in vitro* from human pluripotent cells provide a unique paradigm to study the human telencephalic development.

Even more importantly, the telencephalic differentiation of human induced PS (IPS) cells has recently been reported [119;120].

The application of optimized telencephalic differentiation protocols to IPS cell cultures derived from patients with neurodegenerative or neurogenetic diseases will provide unique new opportunities to develop *in vitro* models of human diseases such as Alzheimer's disease, Huntington's disease, epilepsy, and neuropsychiatric disorders. These models, based on human neurons in culture, will critically complement existing animal models, which do not fully reflect important features specific for the normal and pathological human brain.

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