In vitro recapitulation of developmental transitions in human neural stem cells

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1 Introduction

1.1 Neural stem and progenitor cells in the context of human neural development

The brain, arguably the most complex organ of the human body, arises from a small pool of highly plastic neural progenitors, which expand, acquire regional identities and generate specific neuronal subtypes and glial cells. Over the last decades advances in the study of brain development in animal models have offered important insight into the processes guiding neurogenesis. Despite important commonalities the human brain is set apart through species-specific features such as increased size, especially of the neocortex, gyrification and enhanced neuronal complexity as well as gene expression and regulation (Geschwind and Rakic 2013; Boldog et al. 2018; Sun and Hevner 2014; Berg et al. 2021; Vanderhaeghen and Polleux 2023). These differences are in large part due to evolutionary changes in neural stem cell (NSC) biology affecting progenitor diversity and ultimately increasing the neural stem cell pool and neuronal output (Lui et al. 2011; Taverna et al. 2014; Florio et al. 2018; Hansen et al. 2010; Dehay and Huttner 2024; Florio et al. 2015). The intricacy of human neural developmental processes that enable high cognitive function may also result in increased susceptibility to certain neurological diseases that cannot be fully recapitulated in commonly used animal models (Tang et al. 2016; Hodge et al. 2019; Miller et al. 2010). Consequently, reliable models of human specific NSC biology may increase our understanding of species-specific brain development and have considerable therapeutic potential when used to generate specific neuronal subtypes for drug screening approaches or cell-based therapies (Zhao and Bhattacharyya 2018).

1.1.1 Overview on central nervous system development

Human central nervous system (CNS) development follows principles shared among many different species, especially mammals. The study of model organisms, such as zebrafish, frog, chicken and mouse, have provided us with invaluable insights into how our brain forms and how it evolved compared to other mammal species (Tosches et al. 2018; Hodge et al. 2019; Molyneaux et al. 2007; Nord et al. 2015; Bae et al. 2015; Rakic

2009). During gastrulation the embryo is rearranged and divided into 3 germ layers, the mesoderm, endoderm and ectoderm. The mesoderm will later give rise to bones and skeletal muscles as well as the heart, while the endoderm is the origin of the gastrointestinal tract and the respiratory system. The nervous system, as well as the skin, develops from the outermost layer, the ectoderm (Solnica-Krezel and Sepich 2012).

The first step of nervous system development requires the separation of the ectoderm into epidermal and neuroectodermal tissue (primordium). In a pioneering study, Spemann und Mangold showed that neural fate is induced by specialized cells of the dorsal lip of the blastopore, termed the organizer region (Spemann and Mangold 1924). The identification, however, of the secreted factors responsible for neural induction was only made possible in the 1990's with the advent of molecular biology (Kiecker and Lumsden 2012). Without influence from the organizer, BMP's (BMP4, 2 and 7), which are part of the TGFß protein family and secreted by the ectoderm itself as well as the mesoderm, induce epidermal fate. Signaling molecules from the organizer region, including noggin (Smith and Harland 1992), chordin (Sasai et al. 1994) and follistatin (Hemmati-Brivanlou et al. 1994), act as TGFß inhibitors and, by blocking the influence of the other germ layers, lead to the neuronal specification of dorsal ectodermal cells. Accordingly, when ectoderm is isolated in vitro and deprived of its tissue context, it develops into neuroectoderm (Grunz and Tacke 1989; Sato and Sargent 1989). This mechanism was therefore described as the 'default mechanism' of neuronal induction (Reversade et al. 2005; Hemmati-Brivanlou and Melton 1997). However, more players are involved in this process than initially believed, including induction through fibroblast growth factors (FGFs) and Wnt inhibition, making neural induction more complex but also providing stability through redundant mechanisms (Stern 2005; Muñoz-Sanjuán and Brivanlou 2002; Ozair et al. 2013).

The result of neural induction is the development of a sheet of primary neural progenitor cells, the neuroepithelium or neural plate. In a process termed neurulation, the neural plate is then undergoing morphological changes comprised of the formation of a neural groove followed by the closure of the dorsal folds beginning at the center of the neural plate and progressing rostrally and caudally to form of the neural tube. The neural tube gives rise to all the major components of the CNS, while the cavity enclosed by it will later form the ventricles. The peripheral nervous system develops from neural crest precursors migrating away from the dorsal midline of the neural tube (Colas and Schoenwolf 2001).

In the beginning of CNS development regional patterning processes divide the rostral neural tube into three primary brain vesicles, the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain). These three vesicles grow rapidly during development and are further subdivided into the telencephalon and diencephalon (prosencephalon), the mesencephalon and the metencephalon and myelencephalon (rhombencephalon), followed by the spinal cord, which develops from the caudal neural tube (Ishikawa et al. 2012).

The initial phase of high proliferation of neural stem cells is followed by the initiation of neurogenesis in which neurons are generated through asymmetrical division of NSCs. The fetal period of brain development is characterized by extensive growth as well as continued neurogenesis and neuronal migration followed by astro- and oligogenesis, which continues well after birth (Jakovcevski et al. 2009; Sigaard et al. 2016; Miller et al. 2012).

Even though these processes are widely shared in mammalian brain development, the human brain shows species-specific features that result in enhanced cognitive abilities. The human brain differs notably from most other mammals in its gyrencephalic build with a highly folded cortex increasing its surface compared to the lissencephalic, smooth brain of e.g. mice. Notable is also the altogether increased brain size, most pronounced of the neocortex, which undergoes remarkable expansion during evolution (Borrell and Reillo 2012; Smaers et al. 2017). These differences in overall brain shape and size can be traced back to human specific divergent features in brain development, which include neural progenitor cell (NPC) biology and subtype diversity as well as gene expression (Taverna et al. 2014; Bae et al. 2015; Dehay et al. 2015; Florio and Huttner 2014). In addition, human development shows a much slower progression compared to other primates supported by a prolonged gestational period. During childhood and adolescence continued restructuring processes like synaptic maturation, pruning and myelination further refine the advanced structure of the human brain (Silbereis et al. 2016; Miller et al. 2012; Finlay and Huang 2019).

The increased complexity and extended developmental time frame of the human brain as well as the longer life span of humans cause a unique susceptibility to developmental disorders and psychiatric or neurodegenerative diseases, which can only be insufficiently recapitulated in model organisms (Miller et al. 2012; Miller et al. 2010; Doan et al. 2016;

Bae et al. 2015; Shibata et al. 2015). Furthermore, the use of human tissue samples for research is accompanied by practical and ethical difficulties. Thus, the emergence of new techniques, such as the culture and neuronal differentiation of human embryonic stem cells and induced pluripotent stem cells, was necessary to allow rapid progress in revealing specific human biological mechanisms in neurological diseases and enabled the modelling of human brain development *in vitro* (Kelava and Lancaster 2016; Suzuki and Vanderhaeghen 2015).

1.1.2 Different identities of NSCs and NPCs across neurodevelopment

Stem cells

Stem cells in general are undifferentiated cells and represent key players in mammalian development. They share two defining features, namely the ability to self-renew, that is to divide symmetrically to create two identical daughter cells, as well as the ability to give rise to more restricted differentiated progeny (Till and McCulloch 1980; Weissman 2000). During the course of development, however, the potency of stem cells, which describes the range of cell types stem cells can give rise to, becomes gradually more restricted. Totipotent cells with the greatest competence and the ability to generate whole organisms, including the extra embryonic tissue of the placenta and yolk sac (trophoblast cells), are present at the 8-cell stage of the embryo. Subsequently, at the blastocyst stage, cells comprising the center of the inner cell mass have the ability to generate any cell of the body and, when expanded *in vitro*, give rise to human embryonic stem cells. Somatic or adult stem cells are multipotent and only give rise to specific cell lineages. They are responsible for generating and regenerating specific tissues and play an important role in organ homeostasis. The existence of somatic stem cells is well known in the hematopoietic system, the skin and the intestinal tract (Clements and Traver 2013; Alonso and Fuchs 2003; Radtke and Clevers 2005). The role of stem cells in the central nervous system, however, is a relatively recent discovery (Reynolds and Weiss 1992b; Uchida et al. 2000).

Neural stem cells

Neural stem cells represent the primary progenitors of the nervous system and are defined by the ability to self-renew and to generate the three main cell types of the CNS: neurons, astrocytes and oligodendrocytes (Gage 2000). NSCs are present in the embryonic and fetal as well as the neonatal human brain while going through temporal changes during development. The discovery that adult neural stem cells arise in a direct lineage from embryonic stem cells through the gradual increase in specialization and heterogeneity as well as the restriction of potency contradicts the previously prevalent view that NSC populations at different time points are distinct and unrelated (Fig 1.1) (Alvarez-Buylla et al. 2001; Merkle and Alvarez-Buylla 2006; Doetsch 2003). Each step in the lineage progression of neural stem cells is characterized by unique features and the activation of specific sets of transcription factors, which allow the identification of the developmental stage of a neural stem cell (Kriegstein and Alvarez-Buylla 2009). In recent years it has become evident that neural stem cells show greater heterogeneity with many subpopulations contributing to the substantial diversity of specialized neuronal populations in the adult brain. This nuanced view was made possible by the discovery of numerous new transcription factors sub-classifying already established neural stem cell populations (Pollen et al. 2014; Thomsen et al. 2016; Johnson et al. 2015). Characterization of neural stem cells and a thorough study of their biology is a crucial step towards understanding neurodevelopment and developing new strategies for brain repair.

Neuroepithelial stem cells

In line with the concept of progressive specialization, primitive neuroepithelial cells, representing the earliest NSCs to emerge after neural induction, have the competency to give rise to the central as well as the peripheral nervous system (Mujtaba et al. 1998). Following neural tube closure, however, early neuroepithelial cells lose the ability to generate PNS progeny and continue to form only cell populations of the CNS. Initially, the neural tube consists of a single layer of neuroepithelial stem cells (NES cells) that rapidly and symmetrically divide to increase the progenitor pool from which all the diverse brain regions are generated (Fig 1.1a) (Gotz and Huttner 2005; Merkle et al. 2006). During this process neuroepithelial stem cells become regionally patterned through the influence of morphogens released by the surrounding tissue and start to organize into regionally

distinct domains (Kiecker and Lumsden 2005). In addition to being highly competent to react to patterning cues, neuroepithelial stem cells have several specific characteristics. The term ‘neuroepithelial’ accounts for the fact that these cells exhibit multiple epithelial characteristics. For instance, they show a pronounced polarity spanning from the apical to the basal side of the neural tube. As other epithelial tissues, they are anchored through junctional complexes at their apical pole characterized by the expression of the tight-junctions protein ZO1 (Aaku-Saraste et al. 1996). Although throughout their cell cycle neuroepithelial cells remain in contact with the apical (ventricular) surface and the basal (pial) lamina, their nuclei are found to move up and down along this apical-basal axis resulting in a pseudostratified appearance of the neuroepithelium. This movement termed ‘interkinetic nuclear migration’ (INM), in which the mitosis of neuroepithelial cells occurs at the ventricular surface and the S-phase takes place at a more basal location is a remarkable hallmark feature of early stage neural stem cells (Taverna and Huttner 2010; Miyata 2008; Sidman and Rakic 1973). Neuroepithelial stem cells first undergo symmetric, proliferative divisions generating two identical daughter cells. The time frame as well as the rate of symmetrical divisions of NES cells in humans is greater than in other mammals, thereby increasing the stem cell pool (Borrell and Calegari 2014). Eventually NES cells also undergo asymmetric, self-renewing divisions, giving rise to a neural stem cell and a more differentiated cell, such as a more restricted neural progenitor cell (NPC) or a neuron (Haubensak et al. 2004).

As development progresses, newly generated neurons migrate radially outwards and the neuroepithelium expands and becomes a multi-layered structure causing neuroepithelial cells to elongate while still remaining connected to the ventricular and basal laminar. At the onset of neurogenesis neuroepithelial stem cells gradually begin to transform into later-stage, more specialized neural stem cells, the radial glia cells (Fig 1.1b).

Radial glia cells

Radial glia cells are derived from neuroepithelial stem cells and therefore retain many of their characteristic features, including the expression of neural stem cell markers such as NESTIN and SOX2 (Gotz et al. 2005). They also maintain a strong apical-basal polarity and bipolar morphology with long radial processes spanning the entire ventricle, for which they were initially named (Rakic 1971). Radial glia cells were first believed to be mere

guidance tracks as new-born neurons migrate along their fibers to their final position in the cortical plate. In 2000, Magdalena Götz and others were able to show through cell fate analysis that radial glia in the murine cortex generate neurons as well as astrocytes. This landmark discovery demonstrated that radial glia cells are indeed the prime stem cell of the developing CNS (Malatesta et al. 2000; Noctor et al. 2001). In addition to neural stem cell markers they also express glia-associated transcripts such as GLAST, BLBP and GFAP making them distinct from the earlier neuroepithelial cells (Gotz et al. 2005; Hartfuss et al. 2001). Radial glia cells, similarly to neuroepithelial cells, exhibit INM (Matsuzaki and Shitamukai 2015; Gotz et al. 2005; Subramanian et al. 2017). While their processes remain attached to the ventricular and pial surface during the entire cell cycle, the cell nucleus is moved up and down in a similar fashion, yet only within the ventricular zone and not further basally to the pial surface (Hartfuss et al. 2001; Noctor et al. 2002). As with neuroepithelial cells, cell division initially only occurs at the ventricular surface that forms an epithelial niche. An important function of INM is therefore thought to be the fact that the space for divisions at the ventricular surface is limited and nuclei moving basally during S-phase create room for more cells to divide.

Radial glia cells are present throughout the CNS and during neurogenesis give rise to all the diverse neuronal subtypes (Anthony et al. 2004). In order to create this great diversity of neurons, neural stem cells early in development acquire a regional identity, which is subsequently consolidated in radial glia cells. Thus, a major hallmark of radial glia is their fixed regional identity (Onorati et al. 2011). In contrast to neuroepithelial cells, they are no longer competent to react to patterning morphogens. Consequently, they are only able to generate neurons of a specific region and form distinct progenitor populations in different parts of the brain (Merkle et al. 2007). This characteristic was brought to light through studies in mice in which radial glia cells were heterotopically transplanted to different brain regions. Following their development, it was evident that radial glia continued to generate progeny of their region of origin without being influenced by the new environment (Grimaldi et al. 2005; McCarthy et al. 2001; Carletti et al. 2002; Yang et al. 2000; Na et al. 1998). In contrast, earlier neuroepithelial cells are able to adapt and generate progeny appropriate for the new location (Olsson et al. 1997; Temple 2001; Grimaldi et al. 2005). This shows the increased specialization and reduced potency from neuroepithelial to radial glia cells, which are no longer able to react to environmental cues by changing their regional identity

and generate a progressively restricted repertoire of neuronal cell types (Shen et al. 2006; Pinto and Götz 2007; Kohwi and Doe 2013).

These recent insights into the role and properties of radial glia were mostly obtained through animal experiments. Brain size, however, varies greatly between species and especially the evolutionary increase in size and surface area of the primate and human neocortex is considered to be key in enhancing cognitive function and intelligence. Mice, for example, have a much smaller cortex and show no gyrification of the brain surface (lissencepalic brain). Specific evolutionary changes to neural stem and progenitor cell behavior and molecular function play a substantial role in increasing the neuronal output in humans. New research on primate and human neurogenesis has been able to uncover several unique characteristics of human radial glia (Hansen et al. 2010; Pilz et al. 2013).

The 6-layers of the cortical plate are generated in an inside-out manner with deep-layer neurons generated first, followed by upper layer neurons. Through self-renewing asymmetrical divisions, radial glia generate an identical daughter cell as well as a more restricted basal progenitor (BP, indirect neurogenesis). In mice, these BP are mostly Tbr2-positive intermediate progenitors (IP), which no longer express neural stem cell markers. IP lose contact to the VZ and migrate basally where they settle in a second germinal layer, the subventricular zone (SVZ). There they undergo a final symmetrical division generating two neurons, which migrate radially outward along radial glia fibers to their final position (Noctor et al. 2004; Miyata et al. 2004; Englund et al. 2005).

Human radial glia, in contrast, have been shown to generate a much greater variety of basal progenitor cells, which are virtually absent in mice. This increase in progenitor cells leads to a massive enlargement of the SVZ, which in humans is sub-divided into two parts: an inner and an outer SVZ (Smart 2002; Nowakowski et al. 2016). In the outer SVZ of primates, a high number of Tbr2-positive IP was observed, which undergo multiple rounds of division. Their potentiation of neuronal output is believed to account in part for the increased cortical size (Kriegstein et al. 2006; Martínez-Cerdeño et al. 2006).

In addition, in a landmark study Hansen and others identified basal progenitors with neural stem cell properties, which reside in the outer subventricular zone (Hansen et al. 2010). These outer radial glia (oRG) express, similar to ventricular radial glia (vRG), neural stem cell and glia markers. However, in contrast to vRG they do not have an apical process connecting them to the epithelial stem cell niche of the ventricular surface and therefore

lose several apical domain properties, like the expression of prominin-1 (CD133) (Fietz et al. 2010). In contrast to IP they are able to undergo multiple rounds of self-renewing asymmetrical divisions and thereby again increase neuronal output. ORG do not undergo interkinetic nuclear migration. However, just prior to dividing oRG exhibit a characteristic rapid basal movement of the nucleus termed mitotic somal translocation (MST) (Ostrem et al. 2014; Ostrem et al. 2017). This basal movement of oRG is believed to extend the SVZ outward and increase the germinal zone in humans.

In order to characterize oRG cells further and identify genes uniquely expressed in these progenitor cells, follow-up studies used various strategies on fetal human cortical tissue to isolate oRG from vRG and other committed cell types. This included microdissection of samples, sorting for cells expressing specific surface markers such as prominin-1 and classification of single cells by RNA sequencing. Genes identified in these studies include ANXA1 and CRYAB for vRG and TNC, PTPRZ1, FAM107A, HOPX and LIFR for oRG (Johnson et al. 2015; Pollen et al. 2015; Thomsen et al. 2016). Interestingly, some of the transcripts exclusively up-regulated in human vRG and oRG, like ARHGAP11B and TRNP1 or exclusively in oRG like TMEM14B when overexpressed in mouse RG cells were able to induce the generation of basal progenitors and even introduce gyrification into parts of the lissencephalic mouse brain (Stahl et al. 2013; Florio et al. 2015; Liu et al. 2017). Together these new revelations provide deeper insight into the differences in neural stem cell biology contributing to the advanced structure and size of the primate and human brain.

As brain development proceeds, the number of terminally differentiated cells greatly increases and synaptic connections are formed. In a second wave following neurogenesis, radial glia cells initiate gliogenesis and start to generate astrocytes and oligodendrocytes (Fig. 1.1c) (Beattie and Hippenmeyer 2017; Rowitch and Kriegstein 2010). By the end of this developmental period, the majority of ventricular radial glia cells delaminate from the ventricular surface and transform into multipolar astrocytes (Alvarez-Buylla et al. 2001; Kriegstein et al. 2009). The number of neural stem cells declines and in the neonatal brain only a small fraction of progenitor cells remains present in restricted regions of the brain (Merkle et al. 2004) (Fig. 1.1d).

Adult neural stem cells

In mammals, astrocyte-like adult neural stem cells have been found in two main regions of the brain: the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus in the hippocampus (Nottebohm 1985). While adult neurogenesis was observed in some vertebrate species, it was long believed that no new neurons are born in the adult mammalian brain. In the 1990's, however, several studies started to challenge this premise. Reynolds and Weiss were able to isolate an astrocyte-like population from the mouse striatum with the ability to give rise to neurons and astrocytes *in vitro* and with this changed the conviction that neurogenesis ceases shortly after birth (Reynolds et al. 1992b; Reynolds et al. 1992a). Interestingly, these adult NSCs are found to reside in a specialized stem cell niche *in vivo*, occupying the same space as before radial glia cells and share many common properties with radial glia, including glial markers (BLBP, GFAP) and neural stem cell markers (SOX2, NESTIN) (Garcia et al. 2004; Suh et al. 2007; Kempermann et al. 2015). One study applied specific labeling of radial glia and their progeny using a Cre-lox-based system and labeling dividing cells with BrdU to confirm the lineage relationship between fetal radial glia and adult NSCs (Merkle et al. 2004). This transition constitutes a further step of progressive specialization in neural stem cells.

Similar to radial glia, adult NSCs have apical processes attached to the ventricular surface and often retain long basal processes connecting them, however, not to the apical brain surface, but the basal lamina of blood vessels (Fig. 1.1d) (Mirzadeh et al. 2008; Shen et al. 2008). In the mouse SVZ adult NSCs, also referred to as type B cells, are almost quiescent and slow dividing. They give rise to type C cells, transient amplifying intermediate progenitors, which generate immature neuroblasts. Subsequently, these neuroblasts migrate tangentially to the olfactory bulb and terminally differentiate into local interneurons (Lois and Alvarez-Buylla 1994; Doetsch et al. 1999; Carleton et al. 2003). Adult NSCs in the dentate gyrus also show radial glia properties and generate intermediate progenitors, which migrate radially outward into the hippocampus to give rise to new granule neurons (Seri et al. 2001; Kee et al. 2007). Even though neurogenesis continues in these specific regions throughout life, adult NSCs are not able to generate other region-specific neuronal subtypes and loose stem cell capacity when transplanted away from their stem cell niche to different brain regions (Herrera et al. 1999). Since the adult mammalian brain in general undergoes little if any neuronal regeneration it is unable

to react to either acute trauma or slowly acting neurodevelopmental disorders with adequate repair strategies.

In humans the use of BrdU staining and carbon-14 dating approaches indicate that new neurons are being generated in the hippocampus and therefore a similar mechanism of adult neurogenesis might be active (Eriksson et al. 1998; Spalding et al. 2013). However, many questions still remain unanswered in great part due to the difficulty to access the human brain and the fact that samples are often obtained from individuals of old age and/or suffering from severe diseases. In addition, there appear to be inter-individual differences in neurogenesis caused by genetic and lifestyle factors and significant inter-species differences that call for adequate human models of neural stem cell biology and neurogenesis (Sorrells et al. 2018; Kempermann et al. 2018; Terreros-Roncal et al. 2021).

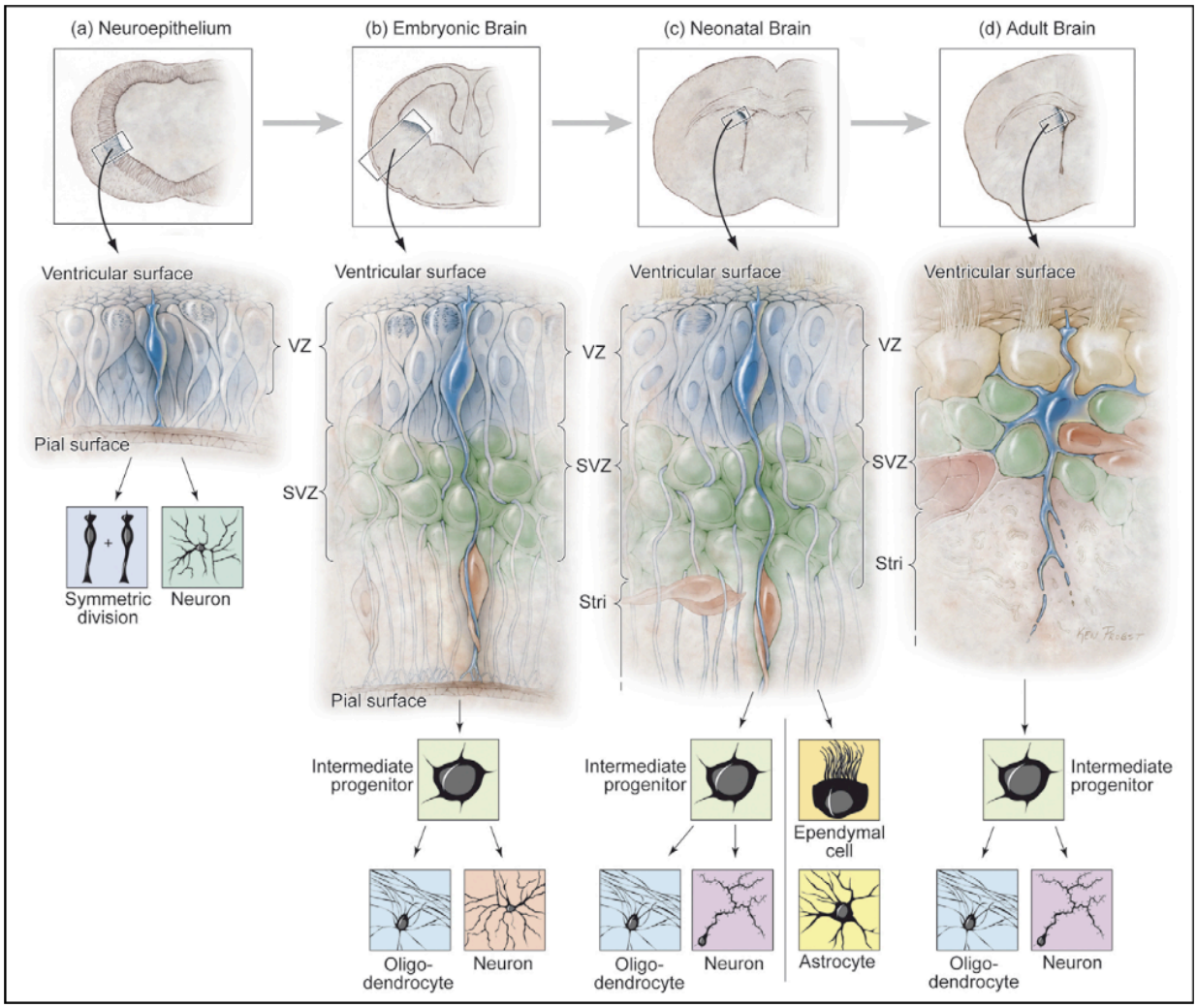


Figure 1.1: Neural stem cell progression in forebrain development

Neural stem cells (blue) progress during brain development into more restricted progenitor cells which generate different progeny either directly or via intermediate progenitors (green). **(a)** Early neuroepithelial stem cells divide most often symmetrically to increase the stem cell pool but also generate some early born neurons. **(b)** NES cells transform at the onset of neurogenesis into radial glia stem cells, which are regionally specified and divide preferentially asymmetrically to generate neurons and glia subtypes. **(c)** In the neonatal brain some radial glia stem cells persist to generate late borne neurons, astrocytes, oligodendrocytes and ependymal cells. **(d)** A fraction of radial glia stem cells transforms into astrocyte-like adult stem cells, which retain contact with the ventricle and the basal lamina of blood vessels through radial processes and generate specific interneurons as well as oligodendrocytes in the adult brain. VZ: ventricular zone; SVZ: subventricular zone; Stri: striatum. Illustrations adapted from Merkle and Alvarez-Buylla (2006).

1.1.3 Acquisition of regional identity in neural stem cells

To create the great diversity of cell types described earlier from the primitive neuroepithelium, developmental processes direct early neural stem cells to self-organize and generate specialized brain regions (Wilson and Houart 2004). During development neural stem cells gradually specialize and acquire a defined regional and temporal identity that determines the repertoire of progeny they are able to give rise to. Neuronal subtypes generated in discrete anatomical locations therefore already have encoded the morphology and function they will assume in the adult brain (Kohwi et al. 2013).

Regional identity is induced in NSCs through a process termed neuronal patterning, in which organizer regions instruct neural precursors to specialize and form boundaries defining different regions of the brain. Neuronal patterning occurs progressively, starting from the primitive neuroepithelium where broad domains are defined which are continuously refined during and after neurulation.

The organizers are specialized mesodermal and endodermal cells in close proximity to the neural plate (Kiecker et al. 2012; Martinez Arias and Steventon 2018). First, they establish a polarity within the neuroectoderm along the anterior-posterior and the medial-lateral axis. Morphogens, signaling molecules released by these organizers, create a gradient, and thereby a specific local environment for each cell, depending on their position in the tissue. The different environmental cues then activate or repress region-specific transcription factors within each cell. Slight differences in the starting concentration of these transcription factors are amplified by their reciprocal repression,

which determine the identity of each cell and help to define strict boundaries between brain regions (Briscoe and Small 2015; Labalette et al. 2015).

When the neuroectoderm is induced, it has by default an anterior, forebrain identity and only through patterning factors from the surrounding tissue are the midbrain, hindbrain and spinal cord further defined (Levine and Brivanlou 2007; Stern 2001). Important morphogens that posteriorize the neural tube in a dose-dependent manner include proteins of the Wnt family, FGFs and retinoic acid (RA) (Gouti et al. 2015; Ciani and Salinas 2005; Mason 2007; Maden 2007). The anterior identity of the forebrain is preserved through different inhibitors of those factors (BMP inhibitors: chordin, noggin, cerebrus; Wnt inhibitors: cerebrus, dickkopf, FRZB1; RA degrading enzyme: Cyp26), which create an anterior low – posterior high gradient of posteriorizing morphogens (Wilson et al. 2004; Beccari et al. 2013). The dorsal-ventral axis of the neural tube is defined by two opposing gradients: BMPs secreted by the dorsal mesoderm and sonic hedgehog (Shh) signaling from the ventral notochord, formed by the endoderm (Wilson and Maden 2005; Le Dréau and Martí 2012) (Fig 1.2). Following neural tube closure, the positional identity of neural stem cells is further refined by secondary organizers such as the anterior neural ridge, the zona limitans intrathalamica, the floor plate and the isthmic organizer/midbrain-hindbrain boundary, which form within the neural tissue itself (Echevarría et al. 2003; Vieira et al. 2010).

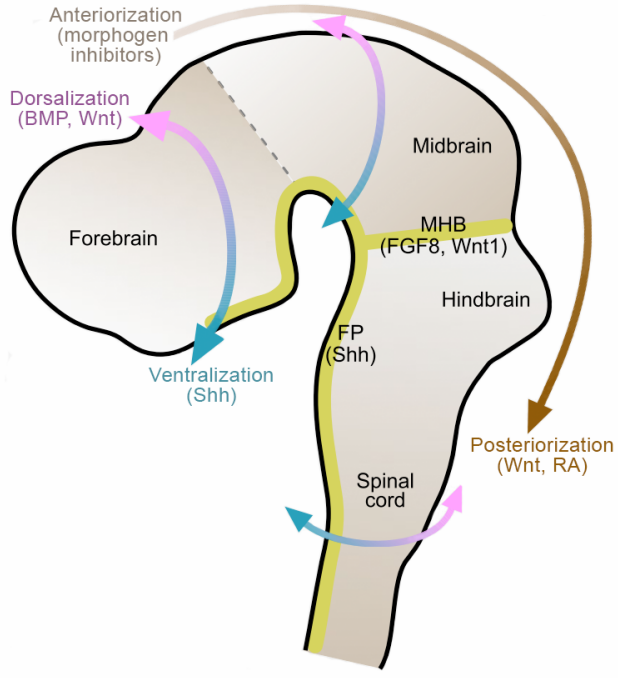


Figure 1.2: Regional patterning of the CNS *in vivo*

The regional identities within the CNS are determined along the anterior-posterior and dorsal-ventral axes through the action of morphogens released from various organizers (yellow). FP: floor plate; MHB: midbrain-hindbrain boundary.
Illustration adapted from Suzuki and Vanderhaeghen (Suzuki et al. 2015).

The increasingly complex regionalization of the fetal brain is encoded in neural stem cells through the expression of a combination of regionally specific transcription factors (TF). In the anterior-posterior axis, forebrain identity is defined by the expression of, among others, TFs like FoxG1, Otx2 and Six3, the midbrain by Otx2 and Irx3 (EN1/2) and the hindbrain and spinal cord by Irx3 and Gbx2 (Wurst and Bally-Cuif 2001; Kobayashi et al. 2002; Hébert and Fishell 2008). Important for the patterning of the hindbrain and spinal cord is the HOX gene complex, a highly conserved system of genes that are expressed in overlapping domains along the anterior-posterior axis (Philippidou and Dasen 2013; Nolte et al. 2019; Mallo et al. 2010). The same principle applies to the dorsal-ventral axis in which, for example, in the forebrain the dorsal part, that will form the cerebral cortex, is characterized by the expression of Pax6 and Lhx2, while the ventral forebrain expresses Nkx2.1, Lhx6 and Dlx1/2 and develops into the ganglionic eminences (Sussel et al. 1999; Leung et al. 2022; Al-Jaberi et al. 2015). The dorsal spinal cord expresses Irx3 and Gbx2, while the ventral spinal cord is characterized by the transcription factors Nkx2.2 and Olig2 (Shi et al. 2024; Rayon et al. 2021).

In parallel to the patterning of the nervous system the primitive neuroepithelial stem cells of the neural plate and early neural tube, which have a high competence to respond to morphogens, progress into the more specialized and less competent radial glia cells. When this transition is complete, radial glia cells can no longer respond to regionalizing factors and are fixed in their positional identity (Onorati et al. 2011). They will now only generate neuronal and glial subtypes for a specific brain region.

1.2 Experimental access to human neural development

The study of the human brain faces mayor challenges due to the limited access to human neural tissue for research purposes. In addition, the study of brain development carries severe ethical and technical implications when applying human fetal tissue. An attractive solution is therefore the generation of neural cells types from pluripotent stem cells (PSC) *in vitro*. The advent of *in vitro* embryonic stem cell (ESC) culture, and later the discovery of induced pluripotent stem cells (iPSCs), and their ability to generate a multitude of defined somatic cell populations such as heart, intestinal and neural cells has given researchers unprecedented experimental access to early embryonic development.

Moreover, these technologies offer new possibilities for disease modeling, genetic manipulation and therapeutic applications in particular for neurological disorders (Sterneckert et al. 2014; Doi et al. 2020; Balusu et al. 2023). For these purposes, however, it is of great interest to what extent the differentiation of PSCs into the desired cell type *in vitro* indeed faithfully recapitulates the developmental steps undergone *in vivo* and identify possible deviations caused by the artificial *in vitro* environment (Conti and Cattaneo 2010).

1.2.1 Pluripotent stem cells *in vitro*

Embryonic stem cells (ESCs) are isolated from the inner cell mass of the blastocyst and can be maintained *in vitro* in a pluripotent state. The first ESC lines were derived in the early 1980s by Evans et al. from mice and cultured in medium containing fetal bovine serum (FBS) as well as leukemia inhibiting factor (LIF) and co-cultured with feeder cells (Kaufman and Evans 1981). However, due to the significant differences in mouse and human cell biology it took another decade to adapt this technology to the human system, even though their potential for research was evident (Bongso et al. 1994). In 1998 Thompson et al. were able to establish the first ESC line from the inner cell mass of the human blastocyst and subsequently significant progress has been made in further improving the derivation as well as the culture conditions of hESCs (Thomson et al. 1998; Kim et al. 2005; Marteyn et al. 2011; Strelchenko et al. 2004).

ESCs represent a potentially unlimited source for virtually any cell type of the human body, which can be utilized for research and cell replacement therapies. However, several hurdles have to be overcome in order to obtain reliable experimental results from embryonic stem cells and their derivatives as well as to make them suitable for possible therapies in humans. For example, protocols for the derivation and maintenance of ESCs need to be well defined and reproducible to avoid batch-to-batch variations in experimental outcomes and should safeguard form epigenetic and cytogenetic instability and spontaneous differentiation (Garitaonandia et al. 2015; Tompkins et al. 2012).

To achieve the potential of ESCs as a source for cell replacement therapies, it is necessary to develop processes that are easily amenable to scale-up production. Importantly, cell therapies need to comply with Good manufacturing guidelines for medical

products (cGMP), which include xenon-free culture conditions for the delivery to humans (Unger et al. 2008). Improved culture conditions for human ESCs therefore involve the use of xenon-free media, basic fibroblast growth factor (bFGF) and the use of serum replacement instead of FBS (Amit et al. 2000). In addition, the use of Rho-associated kinase inhibitor (ROCK) identified by Watanabe et al. has greatly improved the survival of ESCs and has enabled the use of single cell suspensions during passaging (Watanabe et al. 2007b). The development of several chemically defined and semi-defined culture media, systematically tested by the International Stem Cell Initiative (ISCI) now allow feeder free maintenance of ES cells and greatly increase the homogeneity of *in vitro* cultures (Adewumi et al. 2007). Together these developments have significantly improved the applicability of ES cells in research and clinical therapy. However, ethical and legal implications pose a substantial drawback for the use of ESCs (McLaren 2001). Especially legal circumstances in Germany involving the Stammzellgesetz (StZG) posed a challenge that slowed progress towards fully realizing their potential.

Efforts in recent years to study the molecular characteristics and pathways active in ESCs have led to the uncovering of important mechanisms maintaining pluripotency and self-renewal (Cartwright et al. 2005; Chambers et al. 2003; Li et al. 2005; Rodda et al. 2005). This research was a defining step and cumulated in 2006 in the landmark discovery of induced pluripotency. Yamanaka et al. were able to show that 4 key factors (Klf4, Sox2, Oct4 and Myc), previously implicated in maintaining pluripotency, are able to reset somatic cells to an embryonic stage, when expressed through viral vectors (Takahashi et al. 2007; Takahashi and Yamanaka 2006). These induced pluripotent stem cells (iPSCs) have since further revolutionized the field. Follow up studies have shown that iPSCs appear mostly indistinguishable from ESCs regarding their properties and they have therefore since played an increasingly important role (Gore et al. 2011; Hussein et al. 2011; Lister et al. 2011). In addition to alleviating ethical concerns connected to ESCs, iPSCs potentially allow the generation of any cell type of an individual, opening great possibilities for personalized medicine and research (Tabar and Studer 2014). The generation of disease specific iPSCs has enabled significant progress in disease modeling and has helped in understanding especially human-specific and multifactorial disorders (Brennand et al. 2011; Carvajal-Vergara et al. 2010; Lee et al. 2009; Zhang et al. 2011). One particular advantage of iPSCs for cell replacement therapies is the potential to avoid the

immune rejection of allogeneic transplantation. The development of safer methods is an important step towards clinical applications in addition to improved efficiency and speed of the reprogramming process. Examples of enhanced methods include reducing the number of transcription factors and developing integration free techniques using a non-integrative Sendai virus, protein transduction or mRNA and microRNA transfection (Anokye-Danso et al. 2011; Kim et al. 2009; Nakagawa et al. 2008; Warren et al. 2010; Zhu et al. 2010; Fusaki et al. 2009). A further advance in standardizing iPSCs generation and culture is the development of cell banking facilities that use consistent methods for generation and evaluation such as the human iPSC initiative (HipSci) in the UK and the European Bank for induced Pluripotent Stem Cells (EBiSC) (Morrison et al. 2015; De Sousa et al. 2017). In addition, the possibility of designing iPSC lines with matching haplotypes for transplantation is an attractive way of speeding up the generation of cell replacement therapies without the necessity for immunosuppression (Turner et al. 2013; Yoshida et al. 2023). Together these recent advances in stem cell technology have let to first clinical trials using iPSCs derived somatic cell types for diseases such as macular degeneration and Parkinson's (Mandai et al. 2017; Barker et al. 2017; Schweitzer et al. 2020).

For the future progress of stem cell research, it will be crucial to define and improve protocols for the controlled and efficient differentiation of PSCs into authentic somatic cell types to be used in the modeling of human specific features of development and disease as well as cell-based therapies.

1.2.2 Differentiation of PSCs towards neuronal fates

Neural induction

Pluripotent stem cells have the potential to generate any cell type of the human body in nearly unlimited quantities (Thomson et al. 1998; Amit et al. 2000; Reubinoff et al. 2000). Early *in vitro* experiments showed that mouse and human ES cells spontaneously differentiate upon withdrawal of the self-renewal supporting environment into a mix of cells from all three germ layers (Wobus et al. 1984; Doetschman et al. 1985). In recent years, defined protocols for the derivation of important disease relevant cell types, such as cardiomyocytes (Rao et al. 2016), blood cells (Choi et al. 2009; Wang and Ye 2009),

hepatocytes (Hay et al. 2008), pancreatic β-cells (Nair et al. 2019) and neurons (Perrier et al. 2004; Zhang et al. 2001) have been established, giving scientists and clinicians unprecedented access to previously difficult to obtain cell populations. For the development of suitable protocols, insights from embryonic development gained from animal models are translated to guide the process of *in vitro* differentiation into authentic somatic cell types (Keller 2005; Suzuki et al. 2015).

Accordingly, the first steps of the neural differentiation of PSCs *in vitro* mimic neural induction *in vivo* and include passing a state of intermediate multipotent progenitors. To drive this process, different strategies for the specification of neural lineages from PSCs have been applied over the years. In traditional neural induction protocols, PSCs are cultured as aggregates in suspension and spontaneously differentiate into so called embryoid bodies (EBs) when mitogens are removed. Serum-free medium conditions favor neuronal differentiation and reduce meso- and endodermal cell types leading to the assumption of a 'neuro-by-default' mechanism as observed *in vivo* (Reubinoff et al. 2001; Muñoz-Sanjuán et al. 2002; Tropepe et al. 2001; Vallier et al. 2004; Ozair et al. 2013). The addition of FGF2 further enhances a neuronal fate decision and under these conditions a considerable fraction of cells form neural tube-like rosette structures within the EBs (Zhang et al. 2001). Intriguingly, the organization of neural rosettes arising during neural differentiation *in vitro* is strongly reminiscent of the early neural tube during embryonic development. Corresponding to neuroepithelial stem cells *in vivo*, progenitors show a highly polarized morphology with apical tight- and adherens junction expression (Lazzari et al. 2006; Elkabetz et al. 2008; Colleoni et al. 2010; Li et al. 2005). Subsequent protocols used various methods to enrich for neuronal precursors following plating of the EBs including manual selection, immuno- or enzyme-based selection and selective media conditions (Li and Zhang 2006). Rosette structures, consisting of neuroepithelial-like stem cells, can be differentiated into the three cell types of the CNS, neurons, astrocytes and oligodendrocytes offering first access to these difficult to obtain cell types (Reubinoff et al. 2001; Zhang et al. 2001). However, a considerable downside of the use of EB-based protocols is their great variability depending on the starting-size and cell density of the EBs as well as the homogeneity of the starting population of PSCs (Bauwens et al. 2008). Consequently, the generated neuronal populations notably differ in their subtype composition and are often contaminated with meso-endoderm cells lacking the necessary

purity despite enrichment strategies (Hsu et al. 2007; Muotri et al. 2005; Reubinoff et al. 2001; Vallier et al. 2004).

In order to address these issues, more elegant strategies for direct neuronal induction preventing the unwanted differentiation into other germ layers were recently developed. The differentiation of PSCs in feeder-free monolayer culture without the use of EBs not only reduces the variability known from EB formation but also enables better monitoring and synchronization of the induction process. By inhibiting transforming growth factor β (TGFβ)/activin and bone morphogenetic protein (BMP) signaling, the efficacy of the neural induction process can be greatly improved (Chambers et al. 2009; Kim et al. 2010; Zhou et al. 2010). Combined treatment of PSCs with Noggin, blocking the TGFβ pathway, and the small molecule SB431542, which effectively inhibits Lefty/Activin/TGFβ signaling by blocking ALK receptor phosphorylation yields a homogeneous monolayer of neuroepithelial-like stem cells within 6 days. Since SMAD proteins are downstream effectors of both pathways this induction method was termed "dual SMAD inhibition" and variations of this protocol are now widely used for the derivation of early neural progenitors (Chambers et al. 2009).

Indeed, independent of the strategy applied for neural induction, a key challenge remains to develop protocols that faithfully recapitulate early neural patterning processes *in vitro* to generate specific neuronal and glial subtypes in adequate quality and quantity for research purposes and translational therapies.

Neuronal subtype specification

A major requirement for the use of PSC-derived neurons in disease modeling and cell replacement therapies is the development of successful strategies to derive very specific neuronal subtypes. In the initial stages of most congenital, psychiatric or neurodegenerative disorders, a particular subpopulation is often prominently affected by dysfunction, damage or loss (Surmeier et al. 2017; Alegre-Abarrategui et al. 2019; Saxena and Caroni 2011; Fu et al. 2018). However, in many cases the cause for this selective vulnerability is not fully understood and further research is needed to illuminate specific disease mechanisms. In recent years, substantial progress has been made in developing appropriate differentiation protocols for the generation of respective disease-relevant neuronal subtypes.

In general, these protocols follow a common principle by aiming to recapitulate the neural patterning processes taking place in early neurodevelopment *in vitro* (Suzuki et al. 2015). During neural patterning the neuroepithelial stem cells of the neural tube acquire a specific regional identity along the anterior-posterior and dorsal-ventral axis of the brain through the coordinated exposure to different morphogenic gradients. Neural progenitors with a defined regional code subsequently generate specific neuronal subtypes for the different regions of the brain.

To replicate this process, it is necessary to decipher the exact conditions that induce the desired regional transcriptional code in neuroepithelial-like stem cells *in vitro*. Interestingly, when the neural induction medium lacks any regional cues, NE-like stem cells acquire an anterior-dorsal code, expressing PAX6, OTX2 and FOXG1, suggesting that this commitment represents the default identity of neural stem cells (Chambers et al. 2009; Gaspard et al. 2008; Li et al. 2009; Tropepe et al. 2001; Vallier et al. 2004; Watanabe et al. 2005; Ozair et al. 2013). These early anterior NE stem cells, however, are a transient population *in vivo* and can likewise not be kept in culture *in vitro* for prolonged periods of time. Following neural induction this population can either be directly differentiated to give rise to cortical projection neurons or their positional identity can be further defined by adding specific combinations of morphogens, such as WNTs, RA and FGFs to shift the anterior-posterior identity or SHH, BMPs and WNTs to specify the dorsal-ventral identity (Maury et al. 2015; Le Dréau et al. 2012). In general, these patterning factors act in a in a dose-dependent manner and induce for example forebrain, midbrain, hindbrain or spinal cord identity depending on the applied concentrations. This mechanism makes it considerably easier to define progenitor identities at the outer most boundary of each axis.

Following this principle, ventral forebrain progenitors can be generated by ventralizing the early NE-like stem cells through high activation of SHH to a medial ganglionic eminence (MGE) fate expressing the ventral marker NKX2.1. When these ventral progenitors are differentiated, they give rise to functionally mature cortical interneurons expressing parvalbumin, somatostatin or calbindin (Liu et al. 2013; Maroof et al. 2013; Nicholas et al. 2013). Likewise, more dorsal lateral ganglionic eminence (LGE) identity can be induced through lower concentrations of SHH (Kohtz et al. 1998; Carri et al. 2013). Midbrain and hindbrain identity can be induced through exposure to WNTs or FGFs, which *in vivo* are

secreted by the isthmic organizer at the midbrain-hindbrain boundary (Gibbs et al. 2017; Koch et al. 2009b). Through combination with SHH activation it is possible to generate midbrain dopaminergic neurons in high purity (Kirkeby et al. 2012; Kriks et al. 2011). Neurons with spinal cord identity can be generated by posteriorizing NE-like stem cells applying RA (Li et al. 2005; Kirino et al. 2018; Koch et al. 2009b). In combination with SHH or BMP agonists the transcription code of particular domains along the dorsal-ventral axis of the spinal cord can be induced to obtain either dorsal spinal interneurons or ventral motor neurons (Li et al. 2005; Ogura et al. 2018; Gupta et al. 2018; Davis-Dusenbery et al. 2014).

Through this basic strategy it has become possible to derive a great variety of important neuronal subpopulations including cortical neurons, midbrain dopaminergic neurons, medium spiny neurons, motor neurons and hindbrain serotonergic neurons (Shi et al. 2012a; Kirkeby et al. 2012; Carri et al. 2013; Maury et al. 2015; Lu et al. 2016; Kriks et al. 2011).

However, a key challenge has been to improve the efficacy of the patterning process and thereby to increase the purity of the intended neuronal subtype. To this end, differentiation protocols often include lineage selection approaches or optimize directed differentiation by applying reporter lines, which indicate the expression of a defining marker gene and can be used to analyze the ideal conditions and time windows for effective induction (Maroof et al. 2013; Kirino et al. 2018).

What these protocols have in common is, that they constitute so-called ‘run-through’ protocols, in which human PSCs are directly differentiated into mature neuronal cultures without a defined intermediate progenitor stage. Before final differentiation can be induced, protocols often need to include steps for validation of the correct marker expression and purity of the neural progenitor population. Subsequently PSC-derived neurons often require several weeks of *in vitro* culture to exhibit electrophysiological properties associated with functional maturation (Maroof et al. 2013; Nicholas et al. 2013). Due to this complex and lengthy differentiation process and inherent individual differences ‘run through’ protocols often result in batch-to-batch variation and decreased homogeneity of the resulting neuronal cultures (Kim et al. 2011; Keller et al. 2018). These drawbacks could be amended by proliferating neural stem cell intermediates, either before or following a regional patterning step. Intermediate progenitor populations can be validated

and subsequently proliferated as stable starting populations for the generation of region-specific neuronal subtypes with minimal variability. An added benefit of this method is the economization of cost-intensive materials involved in the culture of PSCs and the neural induction and regional patterning process.

1.2.3 Properties of different *in vitro* cultured neural stem cell populations

Human pluripotent stem cell-derived neural stem cells represent a key tool to access important aspects of early neural development, especially with regards to human specific features. However, to study the molecular and cellular properties of neural precursors during development, NSCs generated *in vitro* need to adequately reflect characteristics of existing NSC populations *in vivo*.

Unlike classical 'run through' protocols, NSCs as well-defined stable intermediates potentially allow the generation of specific neuronal subpopulations with minimal variability for disease modeling, drug-screening approaches or cell replacement therapies. A key challenge in the derivation of NSCs *in vitro* is therefore the isolation of a population that allows stable *in vitro* expansion while retaining full responsiveness to patterning cues.

Reynolds and Weiss first published protocols for the culture of neural stem cells isolated from the fetal or adult mouse brain using propagation as neurospheres in the presence of FGF2 and EGF (Reynolds et al. 1992a; Reynolds and Weiss 1996). Subsequently, research groups adapted protocols for the derivation of human neural precursors (Buc-Caron 1995; Svendsen et al. 1996; Chalmers-Redman et al. 1997; Murray and Dubois-Dalcq 1997). Even though these neurosphere-based protocols are highly variable, allow only short-term propagation or showed glial bias after multiple passages they represent the first steps to derive *bona fide* neural stem cells *in vitro*.

Since then, numerous protocols for the derivation of rodent and human neural stem cells have been developed, either through isolation from neuronal tissue or differentiation of PSCs. *In vitro* cultured NSC populations show great differences with respect to morphology, regional marker expression, responsiveness to regionalization and proliferation as well as differentiation potential (Conti et al. 2010). Similar to neural development *in vivo* a progressive lineage restriction can be observed during the neural specification of PSCs. By comparing features of different NSC models *in vitro* to *in vivo*

arising neural progenitor populations developmental correlates and potential relationships between different *in vitro* NSC populations can be identified (Fig. 1.3).

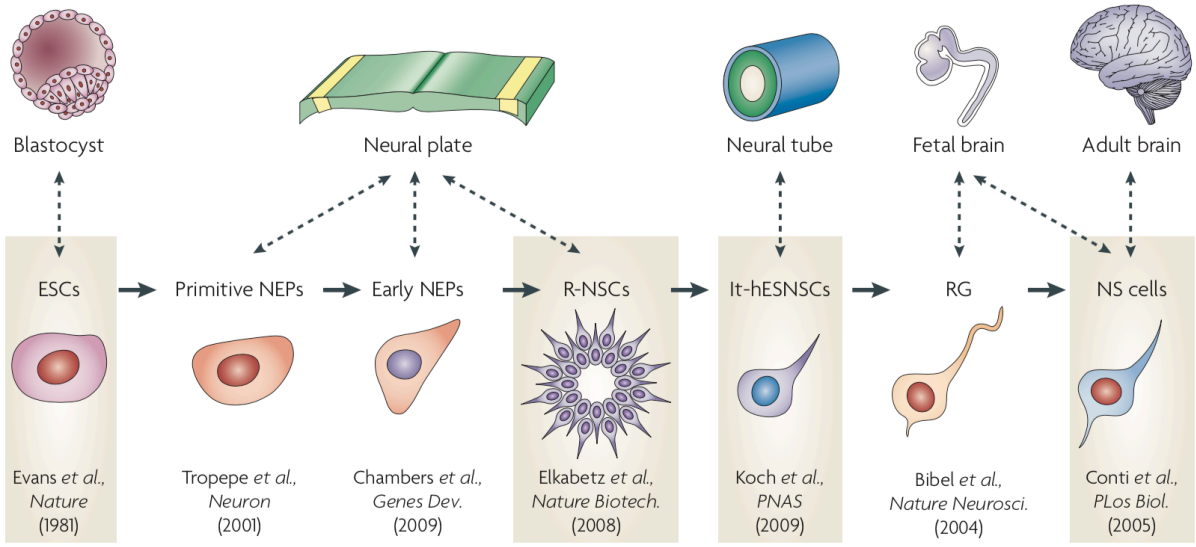


Figure 1.3: Correlation of *in vitro* cultured NSC populations to developmental stages *in vivo*

This schematic shows a selection of neural stem cell populations derived from murine (Tropepe et al. 2001; Bibel et al. 2004; Conti et al. 2005) and human (Chambers et al. 2009; Elkabetz et al. 2008; Koch et al. 2009b) ESCs. Based on their characteristics, NSC systems can be aliened to corresponding neurodevelopmental stages *in vivo*. Primitive and early NEPs have broad differentiation potential towards CNS and PNS fates whereas neural tube-stage NEPs are restricted to CNS phenotypes. Later RG-like NSC populations are fixed in their regional identity and can only give rise to specific neuronal and glial subtypes. Shaded boxes indicate populations capable of *in vitro* long- or short-term propagation. NEPs: Neuroepithelial progenitor cells; R-NSCs: rosette neuroepithelial stem cells; It-hESNSC (It-NES): long-term self-renewing human ESC-derived neuroepithelial stem cells; RG and NS cells: radial glia and neural stem cells (both radial glia-like stem cell). Illustrations adapted from Conti et al. (Conti et al. 2010).

One way of classifying NSCs is by looking at their differentiation potential and responsiveness to morphogens, which declines progressively during the transition from primitive NSCs to NEPs and later to RG cells. Other important distinctions can be made by considering morphological characteristics and gene expression profiles.

For example, one frequently observed feature of early NSC cultures is the arrangement of cells in so called ‘neural rosettes’ with pronounced apical-basal cell polarity. These structures strongly resemble the neural tube *in vivo* (Zhang et al. 2001; Elkabetz et al. 2008; Michael Karus 2014; Ziv et al. 2015). NSC populations with this architecture have

therefore been correlated to neural plate- and neural-tube stage neuroepithelial stem cells.

Primitive neural precursors with incomplete neuronal commitment and the ability to generate neuronal as well as non-neuronal tissues can be derived from mouse ESCs through a 'neuro-by-default' mechanism. These early neural precursors are a transient population, depend on the presence of FGF2 and LIF and rapidly lose their broad differentiation potential in culture (Tropepe et al. 2001) (Fig. 1.3).

The efficiency of neural induction from hPSCs has been greatly improved by targeted 'dual-SMAD' inhibition (Chambers et al. 2009; Kim et al. 2010; Zhu et al. 2010). Using this method, a fully neutralized, homogeneous early neuroepithelial-like population with broad differentiation potential towards CNS and PNS fates can be derived. Early NEPs exhibited a default forebrain regional identity expressing Otx2 and Foxg1 and are highly responsive to regionalization. However, this population is again not stable in culture and converts after passaging to a rosette-like neural stem cell (R-NSC).

In 2008, Elkabetz et al. published the derivation of a primitive rosette-forming NSC population (R-NSCs) from human and mouse ESCs. R-NSCs are characterized by the expression of early NSC markers such as SOX1, PAX6 and BF1 (FOXG1) and exhibit an anterior forebrain identity (Elkabetz et al. 2008). Their responsiveness to regionalizing factors is similar to early NEPs, which gives them the ability to generate various CNS subpopulations including motor neurons and dopaminergic neurons in addition to PNS fates. Interestingly, rosettes formed by R-NSCs exhibit pronounced polarization with apical expression of ZO1, N-Cadherin and the glycoprotein prominin-1 (CD133) and show interkinetic nuclear migration strongly reminiscent of neuroepithelial progenitors *in vivo*. Together with the differentiation potential towards CNS and PNS fates, R-NCSs show high correlation to neural plate stage NES cells. Importantly, NSCs with similar characteristics to R-NSCs can be isolated from murine neural plate tissue (Elkabetz et al. 2008). *In vitro* R-NSC are maintained using SHH and Notch agonists for only limited passage numbers. To further proliferate these early progenitors, the medium is switched to classic neural expansion medium containing FGF2 and EGF. However, under these conditions R-NSCs lose their rosette architecture and convert to a later-stage neural stem cell population termed $NSC^{FGF2/EGF}$. The morphology and expression profile of $NSC^{FGF2/EGF}$ corresponds to previously reported radial glia-like fetal derived murine and

human NSCs (Pollard et al. 2006; Conti et al. 2005) while they are no longer responsive to regionalizing cues. By comparing these two distinct populations, R-NSCs and $NSC^{FGF2/EGF}$, Elkabetz et al. were able to identify important differentially expressed transcripts that characterize rosette-stage NSCs in contrast to RG-like NSC-specific transcripts. Interestingly, in line with developmental patterning mechanisms, R-NSCs in transition to $NSC^{FGF2/EGF}$ assume a posterior regional identity under the influence of FGF2 and are no longer able to generate cortical neuronal subtypes. This phenomenon has been described in a number of studies and strongly limits the possibility to expand anterior neural precursors *in vitro* (Stern 2001; Cox and Hemmati-Brivanlou 1995; Mason 1996).
In 2009 Koch et al. reported the derivation of a later stage rosette forming neuroepithelial-like stem cell population from hESCs with the capacity for long-term self-renewal (lt-hESNSCs or lt-NES cells) (Koch et al. 2009b). This population expresses PAX6, SOX2, and NESTIN together with several, but not all, rosette-specific markers previously identified by Elkabetz et al., including PLZF, DACH1 and PLAGL1. The rosettes in lt-hESNSCs cultures are significantly smaller than described in R-NSC populations but are characterized by the same apical expression of ZO1, N-Cadherin and CD133 at the center of the rosette. This epithelial cytoarchitecture is retained together with a stable neuro- and gliogenic capacity, even after extensive proliferation in the presence of FGF2 and EGF for up to 100 passages. lt-hESNSCs likely correlate to neural tube stage NES cells because of their rosette formation and restriction to CNS fates. Indeed, in 2013 Tailor et al were able to directly derive primary hindbrain NES cells from human fetal tissue (hbNES cells), which correspond to lt-hESNSCs in morphology and expression profile (Tailor et al. 2013). Interestingly, when lt-hESNSCs are generated from ESCs, they exhibit a default anterior phenotype. However, after few passages under the influence of FGF2 they undergo a pronounced restriction of regional identity exhibiting a posterior phenotype compatible with a ventral anterior hindbrain fate. From this position, lt-hESNSCs can be shifted by ventralizing SHH/agonists and FGF8 to generate dopaminergic neurons, whereas regionalization with SHH/agonists in combination with posterization through RA induces spinal cord phenotypes including spinal motor neurons (Fasano et al. 2010; Kriks et al. 2011; Li et al. 2005; Maury et al. 2015; Koch et al. 2009b). Therefore lt-hESNSCs, other than previously reported rosette-like NSCs, are able to retain some responsiveness to patterning cues, which together with long-term stability makes them an attractive tool for

the study of hindbrain and spinal cord neural subpopulations. A hurdle for the application of NSCs in cell transplantation therapies is the overgrowth that can be observed in some of the early NES-line populations when transplanted *in vivo*. Importantly, late stage NES like lt-hESNSCs can be transplanted into the mouse brain without detection of overgrowth and have been shown to integrate into the *in vivo* neuronal network (Koch et al. 2009b).

One of the first homogenously expandable NSC populations (NS cells) was derived by Conti et al 2005 through neural induction of murine ESCs in serum-free adherent monoculture and subsequent re-plating in the presence of either FGF2 alone or FGF2 and EGF. NS cells express neural stem cell markers like NESTIN and SOX2 in addition to BLBP, Vimentin and RC2, a combination previously shown to be expressed by radial glial cells in the murine brain (Hartfuss et al. 2001; Chanas-Sacre et al. 2000; Barry and McDermott 2005). NS cells have an elongated bipolar morphology and do not form neural rosettes. In time-laps recordings, interkinetic nuclear migration, a characteristic of neuroepithelial and radial glia cells, could be observed in these cultures. Together these findings correlate NS cells to radial glia stage neural progenitors *in vivo* (Conti et al. 2005; LaMonica et al. 2013). A similar NSC population was previously derived from mouse ESCs through retinoic acid treatment, however, without describing any proliferation step (Liour and Yu 2003; Bibel et al. 2004).

Radial glia *in vivo* show great spatiotemporal diversity with regionally distinct fixed identity and differentiation potential biased towards neurons or glia depending on the developmental time point. These characteristics are recapitulated in NSCs isolated from murine and human fetal brain (Conti et al. 2005; Pollard et al. 2006; Sun et al. 2008). RG-like cells from different regions of the developing brain retain their regional identity *in vitro* independent of the presence of morphogens and show a preference towards glial differentiation depending on the time of isolation (Miller and Gauthier 2007; Edri et al. 2015).

Together, these various different NSC models illustrate the challenge to generate a population that is physiological relevant, homogenously expandable and amenable to regional patterning cues. With improved understanding of the neural induction process in ESCs it has become possible to generate NSCs *in vitro* that strongly correlate to their *in vivo* counterpart. However, many of the early arising neural progenitor populations are extremely transient and their plasticity makes it difficult to maintain a default anterior

phenotype under proliferating conditions. FGF2 and EGF, the classical mitogens deployed for NSC expansion, act as morphogens strongly affecting regional identity towards a posterior phenotype. This also applies to a recently developed protocol using small molecules for the extensive propagation of smNPCs (Reinhardt et al. 2013; Li et al. 2011a). Especially the stable expansion of an early NSC population with anterior phenotype for the generation of highly relevant cortical neuronal subtypes, which are affected for example in Alzheimer's or other forms of dementia, remains challenging.
In contrast, later RG-like NSCs can be homogenously proliferated *in vitro* while retaining a stable regional identity. However, they lose capacity for regionalization and the ability to generate various neuronal subtypes. Consequently, the combination of effective regionalization of neuroepithelial-like stem cells with the regional stability of radial glia stage cells in a neural induction protocol might facilitate the propagation of stable region-specific neural progenitor populations.

1.3 Aim of the study

Neural stem cells derived from PSCs have great significance for modeling neurodevelopment and disease as well as for the development of cell-based therapies. In the last two decades, a variety of protocols for the derivation and *in vitro* cultivation of NSCs have been developed. However, the physiological relevance of some of these *in vitro* models is still unclear and expansion of regionally stable yet broadly patternable NSC populations remains a challenge. This study therefore aims to explore whether it is possible to recapitulate the progression of NSC, specifically of NES cells to radial glia cells, *in vitro* and to generate populations of committed subtype-specific progenitors that have the capacity for long term *in vitro* expansion.

Can the developmental transition from neuroepithelial to radial glia neural stem cells be recapitulated *in vitro*?
In the past, long-term propagating neuroepithelial stem cells (lt-NES cells) have been developed from ES and iPS cells, which correspond to NES cells of the neural tube with regards to morphology and marker expression (Koch et al. 2009b). This population clearly differs from primary NSCs established from the fetal brain in preceding studies, which

exhibit an expression profile strongly reminiscent of radial glia cells (Conti et al. 2005; Pollard et al. 2006; Sun et al. 2008; Sun et al. 2009), known to represent the main source of neurons in the developing brain (Gotz et al. 2005; Kriegstein et al. 2009).
If lt-NES cells indeed correspond to neuroepithelial stem cells *in vivo,* it should be possible to recapitulate neurodevelopment *in vitro* and establish a human radial glia-like neural stem cell from lt-NES cells. A comparative analysis of PSC-derived radial glia-like cells with primary fetal derived RG cells should illuminate the similarities between both populations and possible differences caused by the lack of an intact *in vivo* environment.

Are *in vitro* generated radial glia-like neural stem cells endowed with a stable and defined regional identity?

In contrast to neuroepithelial stem cells, the regional identity of radial glia cells *in vivo* is stable and not influenced by their environment, e.g. through heterotopic transplantation or exposure to morphogens *in vitro* (Merkle et al. 2007). *In vitro* generated lt-NES cells have by default an anterior identity directly after generation from PSCs and over time acquire a ventral hindbrain phenotype. This posteriorization during the course of cultivation can be attributed to the continued exposure to FGF2 and its known posteriorizing properties. Through exposure to various morphogens, lt-NES cells can be shifted to different regional phenotypes such as mesencephalic and spinal cord neurons, not, however, back to an anterior phenotype. In the present study, RG-like cells are generated from NES cells with different regional identities, including early passage NES cells with anterior identity and lt-NES cells regionalized through different morphogens. If RG-like cells generated from PSC-derived NES cells share the characteristics of *in vivo* RG cells, they should have the ability to acquire and stably retain this regional identity and generate specific neuronal subpopulations corresponding to their regional phenotype. In light of recent studies, which further explore the molecular and cellular differences of regionally and temporarily distinct RG cell populations *in vivo,* it is particularly relevant, if some of these characteristics can be modeled by correlated regionally specified RG-like cells *in vitro* (Hansen et al. 2010; Pollen et al. 2014).

2 Materials

2.1 Technical equipment

Device	Name	Manufacturer	Registered office
Autoclave	D-150	Systec	Wettenberg, Germany
Balance	BL610	Sartorius	Göttingen, Germany
Balance	LA310S	Sartorius	Göttingen, Germany
Block heater	Thermomixer compact	Eppendorf	Hamburg, Germany
Centrifuge (cell culture)	Megafuge 1.0R	Sorvall	Hanau, Germany
Centrifuge (table top)	5415D	Eppendorf	Hamburg, Germany
Counting chamber	Fuchs-Rosenthal	Faust	Halle, Germany
Digital camera	Canon Power Shot G5	Canon	Krefeld, Germany
FACS® analyzer	FACS® Calibur	BD Biosciences	San Jose, USA
FACS® sorter	FACS® DiVa	BD Biosciences	San Jose, USA
Fluorescence lamp	HAL100	Carl Zeiss	Jena, Germany
Fluorescence microscope	Axioskop 2	Carl Zeiss	Jena, Germany
Freezer -80°C	HERAfreeze	Kendro	Hanau, Germany
Gel electrophoresis chamber	Agagel	Biometra	Göttingen, Germany
Imaging system	Chemidoc 2000	Bio-Rad	München, Germany
Imaging system	Geldoc EZ	Bio-Rad	München, Germany
Incubator	HERAcell	Kendro	Hanau, Germany
Inverse light microscope (Cell culture)	Axiovert 25	Carl Zeiss	Jena, Germany
LED light source	Colibri 2	Carl Zeiss	Jena, Germany
Liquid nitrogen store	MVE 611	Chart Industries	Burnsville, USA
Microscope (cell culture)	Axiovert 40 CFL	Carl Zeiss	Jena, Germany
Microscope	Axiovert 200M	Carl Zeiss	Jena, Germany
Microscope	Axio Imager Z1	Carl Zeiss	Jena, Germany
Microscope	IX 81 Confocal	Olympus	Hamburg, Germany
Microscope	DMI6000 B	Leica Microsystems	Wetzlar, Germany
Microscope camera	Axiocam MRM	Carl Zeiss	Jena, Germany
Microscope slides	Superfrost plus	Menzel-Gläser	Braunschweig, Germany
Microspectrophotometer	Nanodrop ND-1000	Thermo Fisher Scientific	Wilmington, USA
Micropipettes	Labmate L2-L1000	Labmate	Langenfeld, Germany
Plastic coverslips	Thermanox®plastic coverslips	Nunc	Wiesbaden, Germany
PCR cycler	T3000 Termocycler	Biometra	Göttingen, Germany
pH-meter	CG840	Schott	Mainz, Germany
Pipette-boy	Accu-Jet	Brand	Wertheim, Germany

Polyester membrane	Transwell-Clear	Corning	Bodenheim, Germany
Real-time qPCR machine	Mastercycler realplex	Eppendorf	Hamburg, Germany
Refrigerator 4°C/-20°C	G 2013 Comfort	Liebherr	Lindau, Germany
Shaker	Bühler Schüttler WS 10	Johanna Otto	Hechingen, Germany
Sterile laminar flow hood	HERAsafe	Kendro	Hanau, Germany
Stereo microscope	STEMI 2000-C	Carl Zeiss	Göttingen, Germany
Thermocycler	T3 Thermocycler	Biometra	Göttingen, Germany
Table centrifuge	Centrifuge 5415R	Eppendorf	Hamburg, Germany
Ultracentrifuge	Sorvall	Discovery 90SE	Hanau, Germany
Vacuum pump	Vacuubrand	Brand	Wertheim, Germany
Vortexer	Vortex Genie 2	Scientific Industries	New York, USA
Water bath	1008	GFL	Burgwedel, Germany

2.2 Cell culture and molecular biology consumables

Consumable	Manufactureur	Registered office
6-well culture dishes	Corning	Corning, USA
6-well culture dishes	PAA	Pasching, Austria PAA
12-well culture dishes	Corning	Corning, USA
24-well culture dishes	Corning	Corning, USA
96-well culture dishes	Corning	Corning, USA
96-well culture dishes	PAA	Pasching, Austria PAA
Cellsieve Nylon 40 µm	BD Falcon	Bedford, USA
Cellstar ™ 175 cm^2 flasks	Greiner Bio-One	Solingen, Germany
Coverslips	Menzel Gläser	Braunschweig, Germany
Cryovials 1 ml	Nunc	Wiesbaden, Germany
Cryovials 1.8 ml	Nunc	Wiesbaden, Germany
PCR strip tubes 0.2 ml	Peqlab	Erlangen, Germany
Petri dishes ∅ 10 cm	BD Biosciences	Heidelberg, Germany
Petri dishes ∅ 3.5 cm	BD Biosciences	Heidelberg, Germany
Petri dishes ∅ 6 cm	BD Biosciences	Heidelberg, Germany
Round bottom tubes - 12x75 mm	BD Biosciences	Heidelberg, Germany
Serological pipettes 1 ml	BD Biosciences	Heidelberg, Germany
Serological pipettes 10 ml	BD Biosciences	Heidelberg, Germany
Serological pipettes 2 ml	BD Biosciences	Heidelberg, Germany
Serological pipettes 25 ml	BD Biosciences	Heidelberg, Germany
Serological pipettes 5 ml	BD Biosciences	Heidelberg, Germany
Syringe filter 0.2 µm	PALL	Dreieich, Germany
Syringe filter 0.45 µm	Sarstedt	Nuembrecht, Germany

Syringes 20 ml	BD Biosciences	Heidelberg, Germany
Syringes 5 ml	BD Biosciences	Heidelberg, Germany
TC dishes ∅ 10 cm	FALCON, Corning	Corning, USA
TC dishes ∅ 3.5 cm	FALCON, Corning	Corning, USA
TC dishes ∅ 6 cm	FALCON, Corning	Corning, USA
Tubes 0.5 ml	Axygen, CorningBio-One	Corning, USA
Tubes 1.5 ml	Axygen, CorningBio-One	Corning, USA
Tubes 15 ml	FALCON, Corning	Corning, USA
Tubes 2 ml	Axygen, CorningBio-One	Corning, USA
Tubes 50 ml	FALCON, Corning	Corning, USA

2.3 Chemicals and reagents

Substance	Manufactureur	Registered office
2-Mercaptoethanol	Invitrogen	Karsruhe, Germany
A83-01	Miltenyi Biotec	B.Gladbach, Germany
Agarose	PeqLab	Erlangen, Germany
Ampuwa	Fresenius Kabi©	Bad Homburg, Germany
Ascorbic acid (L-)	Sigma-Aldrich	Deisenhofen, Germany
Azide	Sigma-Aldrich	Deisenhofen, Germany
B27 supplement	Invitrogen	Karlsruhe, Germany
BDNF	Cell guidance systems	Cambridge, UK
Bromphenol blue	Sigma-Aldrich	Deisenhofen, Germany
BSA solution (7.5%)	Sigma-Aldrich	Deisenhofen, Germany
CaCl2	Sigma-Aldrich	Deisenhofen, Germany
cAMP	Sigma-Aldrich	Deisenhofen, Germany
Collagenase IV	Sigma-Aldrich	Deisenhofen, Germany
CytocoonTM Buffer II	Evotec Technologies	Hamburg, Germany
D-Glucose	Sigma Aldrich	Deisenhofen, Germany
DAPI	Sigma-Aldrich	Deisenhofen, Germany
DMEM	Invitrogen	Karlsruhe, Germany
DMEM/F12 (1:1)	Invitrogen	Karlsruhe, Germany
DMSO	Sigma-Aldrich	Deisenhofen, Germany
DNA ladder (100bp)	PeqLab	Erlangen, Germany
DNase (cell culture)	Cell Systems	St. Katharinen, Germany
DnaseI (mol. Bio.)	Invitrogen	Karlsruhe, Germany
dNTPs	PeqLab	Erlangen, Germany
dNTPs	PeqLab	Erlangen, Germany
DPBS	Invitrogen	Karlsruhe, Germany

EDTA	Sigma Aldrich	Deisenhofen, Germany
EGF	R&D Systems	Wiesbaden, Germany
Ethanol	Sigma Aldrich	Deisenhofen, Germany
Ethidium bromide	Sigma Aldrich	Deisenhofen, Germany
FCS	Invitrogen	Karlsruhe, Germany
FGF2	R&D Systems	Wiesbaden, Germany
Forskolin	Sigma Aldrich	Deisenhofen, Germany
Gelatine	Sigma Aldrich	Deisenhofen, Germany
Geltrex	Gibco by Life Technologies	Waltham, USA
HCl	Sigma Aldrich	Deisenhofen, Germany
Human brain RNA	Aligent Technologies	Santa Clara, USA
Insulin	Sigma Aldrich	Deisenhofen, Germany
Knockout-DMEM	Invitrogen	Karlsruhe, Germany
KnockOut™Serum Replacement	Gibco by Life Technologies	Waltham, USA
L-glutamine (10x)	Invitrogen	Karlsruhe, Germany
Laminin (Ln)	Sigma Aldrich	Deisenhofen, Germany
LDN-193189	Miltenyi Biotec	B.Gladbach, Germany
Matrigel (MG)	BD Biosciences	Heidelberg, Germany
Mowiol	Carl Roth	Karlsruhe, Germany
N2 supplement (100x)	Invitrogen	Karlsruhe, Germany
NaCl	Sigma Aldrich	Deisenhofen, Germany
Neurobasal medium	Invitrogen	Karlsruhe, Germany
Non-essential amino acids (10x)	Invitrogen	Karlsruhe, Germany
Paraformaldehyde	Sigma Aldrich	Deisenhofen, Germany
PDGF-AA	R&D Systems	Wiesbaden, Germany
Penicillin Streptomycin	Invitrogen	Karlsruhe, Germany
PFA	Sigma Aldrich	Deisenhofen, Germany
Pluripro medium	Cell guidance systems	Cambridge, UK
Poly-L-ornithin	Sigma Aldrich	Deisenhofen, Germany
Purmorphamine	Merck Millipore	Darmstadt, Germany
Rho-Kinase-Inhibitor Y-27632	Cell guidance systems	Cambridge, UK
SAG	Calbiochem	Läufelfingen, Germany
Serum Replacement	Invitrogen	Karlsruhe, Germany
Taq DNA polymerase	Invitrogen	Karlsruhe, Germany
Trijod-L-Thyronin (T3)	Sigma Aldrich	Deisenhofen, Germany
Triton-X-100 Trypanblue	Sigma Aldrich	Deisenhofen, Germany
Trypanblue	Invitrogen	Karlsruhe, Germany
Trypsin inhibitor (TI)	Invitrogen	Karlsruhe, Germany
Trypsin-EDTA	Invitrogen	Karlsruhe, Germany
XAV 939	Enzo	Lörrach, Germany

2.4 Cell lines

Cell line	Source
hESC line H9.2	Haifa, Israel (Amit et al. 2000)
hESC line I3	Haifa, Israel (Amit et al. 2000)
iPSC line #69	Bonn, Germany (Iefremova et al. 2017)
iPSC line #80	Bonn, Germany (Iefremova et al. 2017)
H9.2 lt-NES cell line	Bonn, Germany (Koch et al. 2009b)
I3 lt-NES cell line	Bonn, Germany (Koch et al. 2009b)
iPSC PK1 lt-NES cell line	Bonn, Germany (Falk et al. 2012)
hbNES cell line Sai1	Cambridge, UK (Tailor et al. 2013)
hbNES cell line Sai2	Cambridge, UK (Tailor et al. 2013)
primary NS cell line	Cambridge, UK (Sun et al. 2008)

2.5 Cell culture media

All basal media were ordered from Invitrogen (Karlsruhe). All compounds were mixed and sterile-filtered (0.2 µm filter), stored at 4°C and used within 4 weeks.

PSC medium (iPSCs and ESCs)	
99 %	PluriPro
1x	Penicillin/Streptomycin

Neural stem cell (N2) medium	
98 %	DMEM/F12
1 %	N2 Supplement
20 µ/ml	Insulin
1 %	Penicillin/Streptomycin
1,6 mg/ml	D-Glucose solution

Neural stem cell maintenance medium	
99,9 %	Neural stem cell (N2) medium
0,1 %	B27 supplement
10 ng/mL	FGF2
10 ng/mL	EGF

Neural induction medium	
50 %	Neural stem cell (N2) medium
50 %	Neurobasal medium
1 %	B27 supplement
0.21 µM	A83-01
0,5 µM	LDN-193189
2 µM	XAV939
300 ng/ml	cAMP
1 %	Non-essential amino acids
1 %	Glutamax
1 %	Penicillin/Streptomycin
1.6 mg/ml	D-Glucose

Neural differentiation medium (NGMC)	
50 %	N2 medium
46 %	Neurobasal medium
2 %	B27 supplement
1 %	Non-essential amino acids
1 %	Penicillin/Streptomycin
100 ng/ml	cAMP

Astrocyte differentiation medium (MEF)	
86 %	DMEM-high-glucose
10 %	FCS, heat inactivated
1 %	Sodium pyruvate
1 %	L-Glutamine
1 %	Non-essential amino acids
1 %	Penicillin/Streptomycin

Oligodendrocyte differentiation medium I	
98 %	DMEM/F12
0.5 %	N2 Supplement
10 ng/mL	EGF
10 ng/mL	PDGF
10 µM	Forskolin
1 %	Penicillin/Streptomycin
1.6 mg/ml	D-Glucose solution

Oligodendrocyte differentiation medium II	
98 %	DMEM/F12
0.5 %	N2 Supplement
0,5 µM	LDN-193189
10 ng/mL	PDGF
200 µM	Ascorbic acid
30 ng/ml	T3
1 µg/ml	Laminin
1 %	Penicillin/Streptomycin
1.6 mg/ml	D-Glucose solution

Oligodendrocyte differentiation medium III	
98 %	DMEM/F12
0.5 %	N2 Supplement
200 µM	Ascorbic acid
30 ng/ml	T3
1 µg/ml	Laminin
1 %	Penicillin/Streptomycin
1.6 mg/ml	D-Glucose solution

Neural stem cell freezing medium	
70 %	KnockOut™ Serum Replacement
20 %	Cytocoont™ Buffer II
10 %	DMSO

PSC freezing medium	
70 %	KnockOut™ Serum Replacement
20 %	PluriPro
10 %	DMSO
10 µM	Rho-Kinase-Inhibitor Y-27632

2.6 Cell culture additives

Reagent	Concentration (stock)	Solvent
A83-01	500 nM	DMSO / Ethanol
All-trans retinoic acid	10 mM	DMSO
Ascorbic acid	200 mM	H_2O

B27	1:100	supplement mix
Cyclic(c)AMP	300 µg/ml	H_2O
EGF	10 µg/mL	PBS
FGF2	10 µg/mL	PBS + 0,1% BSA
Forskolin	10 mM	DMSO
Glucose	160 mg/ml	H_2O
Insulin	5 mg/ml	10 mM NaOH
LDN-193189	200 µM	DMSO
N2	1:200	supplement mix
PDGF-AA	10 µg/mL	4 mM HCL in H_2O
Purmorphamine	5 mM	H_2O
ROCK inhibitor Y-27632	5 mM	H_2O
SAG	1 µM	H_2O
Triiod-L-Thyronin (T3)	30 µg/ml	NaOH
XAV939	10 µg/mL	DMSO

2.7 Cell culture solutions

1x Trypsin/EDTA (TE)	
90 %	PBS
10 %	Trypsin/EDTA (TE) 10x

Trypsin inhibitor (TI)	
100 %	PBS
0.25 mg/ml	Trypsin inhibitor (>700 units/mg) mixed in PBS, sterile filtered and stored at 4°C
TI was mixed in PBS, sterile filtered and stored at 4°C	

Poly-L-ornithine (PO)	
100 %	H_2O
0.25 mg/ml	Poly-L-ornithine (PO)
The mixture was sterile filtered and stored at 4°C	

Laminin (Ln) coating solution	
100 %	H_2O
1 µg/ml	Laminin (Ln) in PBS

Geltrex	
98 %	DMEM/F12
2 %	Geltrex

Matrigel	
98 %	DMEM/F12
2 %	Geltrex

Cyto buffer for cell freezing	
43.25 g	Myo-Inositol (in 800ml distilled water)
200 ml	PBS
5 g	Polyvinylalcohol
Polyvinylalcohol was added in small portions and stir continuously. The mixture was sterile-filtered and stored at 4°C	

Glucose solution (0.4 g/ml)	
100 %	Ampuwa H_2O
0.4 g/ml	L-glucose
The solution was sterile filtered and stored at 4°C	

2.8 Primers

Gen	Forward	Reverse
18S	ttccttggaccggcgcaag	gccgcatcgccggtcgg
AQP4	ggaatttctggccatgctta	agacttggcgatgctgatct
ASCL1	cggccaacaagaagatgagt	tggagtagttgggggagatg
CD44	ggctttcaatagcaccttgc	acacccctgtgttgtttgct
CTIP2	gacgaagatgaccacctgct	gctccaggtagatgcggaag
DLX2	ctccctcagctctctcctca	tgtgtccaagtccaggctaa
FEZF2	tatccacacccaggaaaagc	gtgggtcagcttgtggttct
FOXG1	ccctcccatttctgtacgttt	ctggcggctcttagagat
GAD67	gctggggctgcacttggctt	cacctccccaggcagcatcg
GAPDH	acgacccttcattgacctcaact	atatttctcgtggttcacacccat
GPM6A	tgagatggcaagaactgctg	ccaggccaacatgaaaagat
HES5	gcccggggttctatgatatt,	gagttcggccttcacaaaag
HEY1	cgaggtggagaaggagagtg	ctgggtaccagccttctcag
HOP	gcattgacagcttcactcca	ggaaatgctagccacaccat

HOXB2	tttagccgttcgcttagagg	cggatagctggagacaggag
HOXB4	acacccgctaacaaatgagg	gcacgaaagatgagggagag
HOXB6	gcggatgaattcgtgcaaca	ttcatgcgtcggttctggaa
LIM3	gcaggacactgaggacagaa	accctgggatctggaaactc
MMRN1	cagggagcatcactcagaca	ttgaggccatcttccatttc
NOTCH1	actgtgaggacctggtggac	ttgtaggtgttggggaggtc
NR2F1	acaggaactgtcccatcgac	gatgtagccggacaggtagc
OTX2	tgcaggggttcttctgtgat	agggtcagagcaattgacca
PAX6	aataacctgcctatgcaaccc	aacttgaactggaactgacacac
PLAGL1	gcctcagtcacctcaaaagc	cttaacctgtggggcaaaga
PLZF	ctatgggcgagaggagagtg	tcaatacagcgtcagccttg
RFX4	tctgagacggcaaacatcac	gactcgatgggagactgctc
S100β	aaagagcaggaggttgtgga	aggaaaggtttggctgcttt
SIM1	ttgccaacacttcaccatgt	tggtctcctgctgtctgatg
SOX1	caatgcggggaggagaagtc	ctctggaccaaactgtggcg
SOX9	tacgactacaccgaccacca	tcaaggtcgagtgagctgtg
SPACL1	caactgctgaaacggtagca	gaactcttgccctgttctgc
vGlut1	ctgccgcagctggacgttct	gccgaagcctccgcagttca

2.9 Antibodies

Primary Antibodies

Antibody	Host/Isotyp	Dilution	Company
3CB2 (vimentin)	Mouse IgG	1:200	Hybridoma Bank
βIII-tubulin	Mouse IgG	1:3000	Covance
βIII-tubulin	Rabbit polyclonal	1:3000	Covance
BLBP	Rabbit polyclonal	1:100	Abcam
Brn2	Goat polyclonal	1:500	Santa Cruz Biotec
Calbindin	Rabbit IgG	1:1000	Cell Signaling
CD133	Mouse IgG	1:100	Miltenyi Biotec
CD44	Mouse IgG	1:3000	eBioscience
Ctip2	Rat IgG	1:300	Abcam
Dach1	Rabbit polyclonal	1:100	Proteintech Group
DARPP32	Rabbit IgG	1:1000	Cell Signaling
Doublecortin	Rabbit polyclonal	1:500	Cell Signaling
FoxG1	Rabbit polyclonal	1:300	Abcam
GABA	Rabbit polyclonal	1:800	Sigma-Aldrich
GAD67	Mouse IgG	1:1000	Merck Millipore
GFAP	Rabbit polyclonal	1:500	DAKO

GLAST	Rat IgG	1:100	Miltenyi Biotec
HoxB4	Rat polyclonal	1:250	Hybridoma Bank
ISL1	Rat IgG	1:300	Hybridoma Bank
LIM1/2	Mouse IgG	1:250	Hybridoma Bank
Lim3	Mouse IgG	1:250	Hybridoma Bank
MAP2ab	Mouse IgG	1:500	Sigma-Aldich
N-Cadherin	Mouse IgG	1:500	Sigma-Aldrich
Nestin (hum)	Mouse IgG	1:500	R&D Systems
NeuN	Mouse IgG	1:100	Merck Millipore
Nkx2.1	Mouse IgG	1:500	Zytomed
Nkx2.2	Mouse IgG	1:300	Hybridoma Bank
Olig2	Rabbit IgG	1:500	Merck Millipore
Otx2	Goat polyclonal	1:300	R&D Systems
Parvalbumin	Rabbit IgG	1:5000	Swant
Pax6	Rabbit polyclonal	1:300	Covance/Hiss
PLZF	Mouse IgG	1:50	Calbiochem
Satb2	Mouse IgG	1:400	Abcam
Sox2	Mouse IgG	1:300	R&D Systems
Sox9	Goat polyclonal	1:300	R&D Systems
Tbr2	Rabbit polyclonal	1:500	Abcam
vGlut1	Rabbit polyclonal	1:1000	Synaptic Systems
ZO1	Rabbit polyclonal	1:100	Invitrogen
β-Catenin	Rabbit IgG	1:300	Cell Signaling

Secondary antibodies (conjugated)

Antibody	Host/Isotyp	Dilution	Company
Alexa 488 anti-mouse IgG	Goat IgG	1:1000	Invitrogen
Alexa 555 anti-mouse IgG	Goat IgG	1:1000	Invitrogen
Alexa 488 anti-mouse IgM	Goat IgG	1:1000	Invitrogen
Alexa 555 anti-mouse IgM	Goat IgG	1:1000	Invitrogen
Alexa 488 anti-rabbit IgG	Goat IgG	1:1000	Invitrogen
Alexa 555 anti-rabbit IgG	Goat IgG	1:1000	Invitrogen
Alexa 488 anti-rat IgG	Goat IgG	1:1000	Invitrogen
Alexa 555 anti-rat IgG	Goat IgG	1:1000	Invitrogen
Cy3 anti-mouse	Goat polyclonal	1:250	Jackson / Dianova
Cy3 anti-rabbit	Goat polyclonal	1:250	Jackson / Dianova
Cy3 anti-rat	Goat polyclonal	1:250	Jackson / Dianova
Cy5 anti-mouse	Goat polyclonal	1:250	Jackson / Dianova

Cy5 anti-rat	Goat polyclonal	1:250	Jackson / Dianova
FITC anti-mouse	Goat polyclonal	1:250	Jackson / Dianova
FITC anti-rabbit	Goat polyclonal	1:250	Jackson / Dianova

2.10 Immunohistology and molecular biology reagents and kits

Reagents for Immunohistology

PFA fixation solution (4%)	
40 g	PFA
1000 ml	H_2O
The solution was heated until PFA dissolved completely, pH adjusted to 7.4 and sterile filtered.	

Immuno-blocking solution	
89.9 %	PBS
10 %	FCS
0.1 %	Azid
0.1 % or 0.5 %	Triton x100 (only for intracellular epitopes)

Moviol / DABCO	
12 ml	Tris solution (0.2 M; pH 8.5)
6 ml	H_2O
6 g	Glycerol
2.6 g	Moviol
0.1 g	DABCO

Molecular biology reagents for PCR analysis

PCR master mix (10 ml)	
1 ml	10x reaction buffer
600 µl	$MgCl_2$ (50 mM)
40 µl each	dNTPs
8.24 ml	Ampuwa H_2O

6x DNA loading buffer	
2 ml	EDTA (0.5M; pH 8.5)

6 g	Sucrose
0.2 ml	2% Bromphenol-blue-solution 2%
0.2 ml	Xylene-cyanol-solution
0.2 g	Ficoll
3.8 ml	Aqua bidest.
The solution was mixed well, prepared in 1 ml aliquots and stored at 4°C.	

50x Tris-acetate-EDTA-buffer (TAE)	
242 g	Tris
100 ml	EDTA solution (0.5M; pH 8.5)
57.1 ml	Water-free acetic acid (100%)
Add H2O to 1000 ml	

Molecular biology kits

Name	Company	Office
iScript cDNA Synthesis Kit	Bio-Rad	München, Germany
RNeasy Kit	Qiagen	Hilden, Germany

2.11 Software

Name	Application	Producer
AxioVision 40 4.5.0.0	Fluorescence microscopy	Carl Zeiss
Bioconductor	Gene expression analysis	Bioconductor
EnrichR	Gene expression analysis	Kuleshov et al., 2016
Excel 2008	Data analysis	Microsoft
Excel 2008 Image	Data analysis	Microsoft
FlowJo 6.8	Flow cytometry analysis	Tree Star
GenomeStudio 2010	Gene expression analysis	Illumina
Image J 1.42q	Quantifications	NIH
Microsoft Office 2008	Figures and text processing	Microsoft
PermutMatrix	Gene expression analysis visualization	ATGC
Photoshop CS3	Figures processing	Adobe
Primer3 v0.4.0	Primer picking	Rozen and Skaletsky (2000)
Prism 6	Data analysis and statistics	GraphPad
Quantity One 4.6.8	Electrophoresis gel documentation	Bio Rad
Word 2008	Writing documents	Microsoft

3 Methods

3.1 Cell culture

All cell culture experiments were performed under sterile conditions in a laminar flow hood with sterile media solutions and glass and plastic instruments. Cells were cultivated in an incubator at 37°C, 5 % CO2 and saturated air humidity. Cell cultures were monitored daily for proliferative capacity and morphological integrity and regularly checked via PCR and confirmed negative for mycoplasma.

3.1.1 Cell culture dish coating

Poly-L-ornithine (PO)

For the culture of lt-NES cells and RG-like NS cells, cell culture plates were coated with Poly-L-ornithine and incubated at 37 % for at least 2 hours. Subsequently the plates were rinsed twice with PBS and coated with the laminin diluted 1:100 (10 µg/ml) in PBS. After an incubation at 4°C for a minimum of 5 hours, plates were either used or sealed with Parafilm and stored for a maximum of 7 days at 4°C.

Matrigel/Geltrex

Matrigel/Geltrex was thawed overnight on ice within the fridge and diluted in ice-cold Knockout DMEM 1:30 using precooled pipets. Cell culture plates were then coated with the Matrigel/Geltrex solution and incubated over night at 4°C. For longer storage at 4°C, plates were sealed with Parafilm.

3.1.2 Culture of lt-NES cells

Long-term proliferating neuroepithelial stem (lt-NES) cells from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) were provided by Philipp Koch (Koch et al. 2009b). Primary hindbrain derived NES cells were kindly provided by the Austin Smith Lab. Both, PSC- and primary-derived lt-NES cells were maintained in neural stem cell medium containing DMEM/F12, N2 supplement (1:100; both Life Technologies), 1,6

g/L glucose, 10 ng/mL FGF2 and EGF (R&D Systems), 1:1000 B27 supplement and penicillin-streptomycin (1:100, Invitrogen) on poly-L-ornithine/laminin (both Sigma Aldrich) precoated plastic dishes. Seeding densities were 5-8x10^5 cells/cm^2 and cells were passaged using trypsin and trypsin inhibitor every 2-3 days. Lt-NES cells were stored following trypsinization and resuspension in freezing medium in 1 ml cryovials and transferred immediately to an -80°C freezer or a liquid nitrogen storage tank.

3.1.3 Culture of pluripotent stem cells

Two (male and female) iPS cell lines were previously generated by non-integrative Sendai viral vectors and supplied by the group of Julia Ladewig at the Institute. PSCs (the iPS cell lines #69 and #80 and the ES cell lines H9 and I3) were maintained on Geltrex-coated cell culture plates and cultured in PluriPro medium with daily medium changes. When PSCs reached confluency, the cultures were dissociated to single cells using TrypL Express and split at a 1:2 ratio. Following dissociation to single cells the suspension was centrifuged at 160g for 3 min at 4 °C and resuspended in PluriPro medium. 5 µM ROCK inhibitor Y-27632 was added to the cell suspension to promote single cell survival. For freezing the cells were resuspended in PSC-freezing medium containing 5 µM ROCK inhibitor Y-27632 and knockout serum replacement. Cryovials were first stored in a freezing container at -80°C before being transferred to liquid nitrogen tanks. For thawing PSCs vials were first partially thawed in a 37°C water bath before being transferred to 10 volumes of 37°C PluriPro medium with 5 µM ROCK inhibitor Y-27632 and plated on Geltrex-coated dishes.

3.1.4 Generation of radial glia-like neural stem cells from lt-NES cells

RG-like NS cells were generated through a sequential differentiation protocol depicted in Figure 4.1 B. NES cells were plated on matrigel or geltrex coated dishes and, after reaching confluency following 1-2 days of cultivation, were induced to differentiate by growth factor withdrawal in neural differentiation medium (NGMC) containing DMEM/12 (N2 supplement; 1:50) and Neurobasal (B27 supplement; 1:50) mixed at a 1:1 ratio. Medium was changed every other day. Although radial glia-like NS cells emerge as soon

as neurogenesis starts, it was found that neuroepithelial stem cells persist in the differentiated culture for up to 3 weeks and tend to overgrow the culture after re-initiation of self-renewal due to their faster proliferation time. Therefore, a differentiation time of at least 4-6 weeks was determined to allow for the complete conversion of NES cells into radial glia-like cells. Consequently, cultures were treated with trypsin (0.05%) at 37°C for 5 minutes, followed by addition of trypsin-inhibitor (Life Technologies) in equal volume. Cells were washed down with DMEM/F12 medium, carefully triturated to single cells using a plastic pipette and centrifuged at 300 g for 5 minutes at 4°C in a Megafuge 1.0R (Heraeus). Cultures were plated on poly-L-ornithine/laminin coated dishes at a 1:3 ratio, and medium was switched back to neural stem cell medium. Medium was changed every other day with addition of 10 ng/ml EGF and FGF2 to the cultures in between medium changes. Within 5 passages a homogeneous culture of RG-like NS cells was obtained, which was propagated under the same conditions for more than 25 passages. For storage RG-like NS cells were treated in the same manner as for passaging and, following centrifugation and removal of the supernatant, resuspended in ice cold freezing medium. The cells were transferred to 1 ml aliquots in freezing containers and cooled at -80 before being stored in liquid nitrogen storage tanks. In order to defreeze RG-like NS cells the aliquots were heated in a water bath at 37°C until only a small rest of ice remained. The cells were then immediately resuspended in preheated neural stem cell medium and plated on poly-L-ornithine/laminin coated dishes.

3.1.5 Generation of spinal cord-like NS cells form posterior lt-NES cells

For the generation of spinal cord-like NS cells, lt-NES cells were first pre-patterned to a spinal cord identity by treatment with retinoic acid (10 µM) for 14 days with media changes every other day. In order to ensure the regional identity of posteriorized lt-NES cells prior to differentiation the cultures were stained for HoxB4 following 14 days of treatment. Consequently, the cells were pated on matrigel coated dishes and retinoic acid was removed from the culture. To induce the generation of radial glia-like cells from spinal cord-like lt-NES cells, differentiation was initiated and maintained for 4 to 6 weeks before subsequently growth factors were reintroduced to proliferate the residual neural stem cell

population. During the course of 5 passages a homogeneous radial glia-like cell population emerged.

3.1.6 Generation of forebrain-like NS cells by differentiating PSCs via cortical rosettes

Cortical rosettes were induced from PSCs as previously described by Shi et al. including slight adaptations (Shi et al. 2012a). When PSCs cultured in PluriPro medium reached ~100% confluence the cells were washed once with PBS and neural induction was initiated by changing the medium to neural induction medium containing DMEM/F12 (N2 supplement; 1:50), Neurobasal (B27 supplement; 1:50) mixed at a 1:1 ratio and 300 ng/ml cAMP, 0.5 µM LDN-193189, 0.21 µM A83-01 and 2 µM XAV939. The cells were maintained in this condition on Geltrex-coated dishes for 8-12 days while the neural induction medium was replaced every day. The PSCs were closely monitored for morphological changes during differentiation. Subsequently the cells were dissociated with TrypL Express and replated on Geltrex-coated dishes. After plating, a uniform neuroepithelial sheet appeared. At this point, neural rosettes were analyzed after splitting with TrypL Express at a 1:3 ratio for the expression of neural rosette and forebrain markers. After successful neural induction and regional identity was confirmed, neural differentiation was initiated. When cells reached confluence, the medium was switched to neural differentiation media containing DMEM/F12 (N2 supplement; 1:50), Neurobasal (B27 supplement; 1:50) mixed at a 1:1 ratio supplemented with 300 ng/ml cAMP. Cortical rosettes were maintained in differentiation medium for up to 6 weeks while morphological changes were monitored. The differentiated cultures were dissociated to single cells using TrypL Express and plated at a 1:3 ratio. The medium was switched to neural stem cell medium containing DMEM/F12 (N2 supplement; 1:50) supplemented with 10 ng/mL FGF2 and EGF and the cells were passaged every 5-6 days using Trypsin and split at a 1:2 ratio. After 5 passages a uniform culture of cells with radial glia morphology appeared.

3.1.7 Generation of ventral forebrain-like NS cells from cortical rosettes

Default dorsal patterned cortical rosettes were generated as described before. Following the 10-day neural induction the PSC-derived rosettes were split at a 1:2 ratio on Geltrex coated plates using TrypL Express. To ventralize the dorsal forebrain rosettes the protocol previously developed by Maroof et al. was adapted (Maroof et al. 2013). The neural induction medium was supplemented with the small-molecule agonists of Smothened, 1 µM Purmorphamine and 1 µM SAG for additional 10 days (day 10-20) (Stanton and Peng 2009). Following ventralization through SHH pathway activation the cells were dissociated using TrypL Express and plated at a 1:2 ration on Geltrex coated dishes and stained for rosette markers and the ventral forebrain marker Nkx2.1and the dorsal marker Pax6. When ventral forebrain regional identity was confirmed and the cells reached confluence the medium was switched neural differentiation media containing DMEM/F12 (N2 supplement; 1:50), Neurobasal (B27 supplement; 1:50) mixed at a 1:1 ratio supplemented with 300 ng/ml cAMP for 6 weeks. Following differentiation, the cells were dissociated using TrypL Express and split at a 1:3 ratio on laminin-coated plates. The medium was switched to neural stem cell medium containing 10 ng/mL FGF2 and EGF. Subsequently appearing radial glia-like cells were cultured as described previously.

3.1.8 Neuronal and glial differentiation of radial glia-like NS cells

For terminal neuronal differentiation of RG-like NS cells the cells were plated on Matrigel-coated dishes. When the cultures reached confluence EGF was removed from the neural stem cell medium for 7 days. Subsequently the medium was switched to neural differentiation medium (NGMC), which was exchanged every other day. The cells were maintained in this condition for 4-6 weeks and morphological changes during differentiation monitored. Cells with fine extended processes appeared, which stained positive for the neuronal markers βIII-tubulin, microtubule associated protein-2 (MAP2), and NeuN.

Astrocyte differentiation was initiated by plating RG-like NS cells on Matrigel-coated dishes and switching the medium to MEF medium containing 10% fetal bovine serum. Within 2-3 days the cells adopt astrocyte morphology and uniformly expressed GFAP. The cells were maintained in this condition for 4 weeks before further analysis.

For oligodendrocyte differentiation cells were plated on poly-L-ornithine/laminin coated dishes and cultured in DMEM/F12 containing N2 supplement (1:50). For the first two weeks the medium was supplemented with 10 ng/ml PDGF-AA, 10 ng/mL EGF, and 10 mM forskolin (medium I), for the third week PDGF-AA, 30 ng/ml T3, 200 mM ascorbic acid and 0.5 µM LDN-193189 were added (medium II). In week fore and five only T3 and ascorbic acid remained in the culture. Laminin (1µg/ml) was added for improved cell adhesion (medium III). Cell cultures were analyzed after 5-6 weeks for the expression of O4.

3.2 Immunocytochemical analysis

Cells were fixed with 4% neutral-buffered paraformaldehyde (PFA) for 15 minutes at room temperature and then washed with PBS. For blocking and permeabilization specimens were treated with 0.1% Triton X-100, 10% fetal calf serum (FCS) in PBS for 1 hour, followed by incubation with the primary antibody in PBS, 0.1% Triton X-100 and 10% FCS for 16 hours at 4°C. Cells were rinsed twice with PBS before incubation with the secondary antibody solution for 45 minutes, further washing in PBS and counterstaning with DAPI. For surface markers 10% FCS in PBS without Triton was used for blocking and incubation with antibodies.

3.3 Fluorescence activated cell-sorting (FACS) analysis

Flow cytometry analysis was performed using single cells prepared by trypsinization. Cells were centrifuged and subsequently incubated with 10% fetal calf serum in PBS for 10 minutes followed by exposure with the primary antibody for 30 minutes at 4°C. Cells were then washed twice with PBS and incubated with the secondary antibody for 30 minutes. Analysis was performed with the FACSCalibur® cytometer equipped with an argon laser (488 nm). Forward scatter (FSC), side scatter (SSC) and PE or FITC fluorescence were recorded. Data analysis was performed using the FlowJo software.

3.4 RNA preparation and semi-quantitative reverse transcriptase PCR analysis

Total mRNA samples were extracted using RNAeasy extraction kit. The RNA concentration was determined with Nanodrop-1000, and 1 µg of total RNA was used for reverse transcription with the iScript cDNA synthesis kit. Genes were amplified using Taq DNA Polymerase, 10x PCR reaction Buffer (-$MgCl_2$) and 50 mM $MgCl_2$ and dNTPs. To compare the expression level of different genes, probes were normalized to GAPDH by performing 15, 20, and 25 cycles. PCR conditions and cycle numbers were established by using commercially available human fetal (single donor, female, 19 weeks of gestation) or adult brain probes. The selected numbers of cycles varied from 30 to 40 cycles depending on the particular mRNA abundance with denaturation at 94 °C for 1 min., annealing temperatures at 58 °C to 63 °C for 60 seconds and elongation at 72 °C for 2 min. Omission of cDNA sample during PCR served as negative controls. All reactions were performed on a T3 Thermocylcer. Primer sequences used are listed in the materials section.

3.5 Statistical analysis

Quantitative data was generated in biological replicates and collected and processed randomly. Means and standard deviations (SD) were computed using Microsoft EXCEL and GraphPad Prism software. All results presented as bar graphs show mean ± SD. Student's t test was performed to determine whether a significant difference exists between groups (*p <0.05; **p<0.01; ***p <0.001).

3.6 Gene expression study and bioinformatics

Gene expression analysis was performed in cooperation with Franz-Josef Müller. Total RNA was processed for Illumina HT12v4 microarray analysis according to the protocols provided by the manufacturer (www.illumina.com). PluriTest analysis was performed as previously described (Müller et al. 2011). Raw probe level expression data was extracted from the microarray scan files with GenomeStudio 2010. The raw probe expression values were loaded into R/Bioconductor, transformed with the variance-stabilizing transformation

(VST) and normalized with the robust spline normalization (RSN) provided by the lumi Bioconductor package as previously described (Du et al. 2008).

The resulting values were used with the GeoDE Bioconductor package to determine the “Characteristic Direction” and significantly differentially regulated genes based on the Characteristic Direction algorithm with the shrinkage parameter set to 1 (Clark et al. 2014). The Characteristic Direction algorithm is a multivariate approach to differential gene expression analysis, which first defines a separating hyper-plane between two sample groups (e.g. between lt-NES and NS cells) in the high dimensional, genome wide gene expression space and then identifies the “Characteristic Direction” between the sample groups as the direction normal to the separating hyper-plane. Next the algorithm identifies those genes, which contribute to the total differential expression along the “Characteristic Direction”. The relative contribution to the characteristic direction can then be used to rank the genes in order of relevance. To determine the significance cutoff for this gene list, the algorithm compares the measured contribution of each given gene to the “Characteristic Direction” to those observed in random perturbations of the same data set. Consequently, those genes are identified, whose contributions to the “Characteristic Direction” cannot be explained by random noise as null hypothesis.

Probe to gene mapping was performed with BioMart (Moreews et al. 2011), and the values displaying the highest differential expression differences as determined by GeoDE were chosen for heatmap visualization. Gene Set Enrichment analysis was performed with the identified lists of gene level identifiers with the EnrichR web tool (http://amp.pharm.mssm.edu/Enrichr/) (Chen et al. 2013). Heatmap visualization, clustering and serration was performed using the PermutMatrix software based on the Euclidian distance, McQuitty’s criteria and the Multi-Fragment Heuristic (Caraux and Pinloche 2005).

Hierarchical clustering using average linkage as metric was performed in the lumi package, principal component analysis was performed using the prcomp command in R. Visualization of the sample relationships based first three components was performed with the rgl-package in R. Labeling and coloring of the resulting plots was performed with Illustrator CS5.

3.7 Electrophysiological recordings

Electrophysiology was performed by standard procedures of the Institute of Reconstructive Neurobiology in cooperation with Jadeep Kesavan. Whole cell current-clamp and voltage-clamp recordings were carried out with an Axopatch- 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) interfaced by an A/D-converter (Digidata 1440, Molecular Devices, Sunnyvale, CA, USA) to a PC running Pclamp software (Version 10, Molecular Devices, Sunnyvale, CA, USA). Pipette electrodes (GB150F-8P, Science Products, Hofheim, Germany) were fabricated using a vertical puller (PC-10, Narishige, Tokyo, Japan) and filled with a solution containing (in mM): 110 potassium gluconate ($C_6H_{11}O_7K$), 20 KCl, 10 NaCl, 10 EGTA, 1 $CaCl_2$, 4 Mg ATP, 0.4 Na GTP, 10 Hepes (pH 7.2; 310–320 mOsm). In addition, the pipette solution also contained 0.1% biocytin and neurons were filled by passive diffusion of biocytin from the patch pipette during electrophysiological recording. The signals were low-pass filtered at 10 kHz and sampled at 50 kHz. All recordings were performed at room temperature (22 – 25°C) in a bath solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 $MgCl_2$, 10 Hepes and 15 D-glucose (pH 7.4; 320–330 mOsm).

4 Results

4.1 Sequential differentiation of pluripotent stem cells generates a defined multipotent RG-like neural stem cell population

During CNS development *in vivo*, embryonic stem cells differentiate into early neuroepithelial stem (NES) cells that at the onset of neurogenesis give rise to radial glia (RG) cells. RG cells represent the major source of neurons and glia in the embryonic and fetal brain (Götz and Barde 2005). The first part of the presented work explores, if a protocol of sequential differentiation can be established, that mimics this process and allows for the generation of a stable multipotent radial glia-like neural stem (NS) cell intermediate from pluripotent stem cells.

4.1.1 Lt-NES cells give rise to RG-like NS cells under differentiating conditions

Previous studies have shown that NES cells can be generated from hPSCs, however, stable *in vitro* culture of early rosette NSCs has presented a challenge (Perrier et al. 2004; Zhang et al. 2001; Elkabetz et al. 2008). Long-term self-renewing (lt-) NES cells applied in this study can stably proliferated in the presence of the mitogens fibroblast growth factor 2 (FGF2) and epithelial growth factor (EGF) (Koch et al. 2009b; Falk et al. 2012). In contrast to previously published rosette NSCs such as R-NSCs by Elkabetz et al.2008, lt-NES cells represent a stable intermediate neural stem cell population that can be propagated for more than 100 passages. Upon omission of growth factors, lt-NES cells consistently give rise to βIII-tubulin-positive neurons with a strong bias towards a GABAergic neurotransmitter phenotype and a small fraction of GFAP-positive astrocytes. Even after extensive passaging, lt-NES cells continue to grow in dense rosette-like structures reminiscent of the neural tube.

Lt-NES cells express typical neural stem cell markers, like the intermediate filament protein Nestin, and the transcription factors Sox2 and Pax6 (Zhang et al. 2001). They also express dachshund 1 (DACH1) and promyelocytic leukemia zinc finger protein (PLZF), markers that have previously been described in early neural rosettes (Elkabetz et al. 2008; Koch et al. 2009b). Especially prominent is the expression of the tight-junctions marker

zona occludens 1 (ZO1) at the apical membrane, the center of the rosette, highlighting the epithelial features of lt-NES cells. Sox9 is known to be an astrocyte marker, which is also characteristically expressed in radial glia cells (Scott et al. 2010; Stolt et al. 2003). When stained in lt-NES cells, Sox9 could not be detected (Fig. 4.1A). Lt-NES cells are therefore believed to be neural stem cells corresponding to the neural tube stage of development.

It was now hypothesized that, if lt-NES cells truly represent neuroepithelial stem cells, they should also be able to follow the *in vivo* progression of neural development and generate radial glia-like NS cells. Since this process takes place at the onset of neurogenesis, these cells should arise during the differentiation of lt-NES cells and remain present within the culture as long as neurogenesis continues (Merkle et al. 2006).

Lt-NES cells spontaneously initiate differentiation and generate neurons upon withdrawal of FGF2 and EGF. To test the hypothesis a protocol was developed in which lt-NES cells, generated from the hESC line I3, were used as a starting population and cultured for 4 weeks under differentiating conditions. Subsequently, the cells were dissociated, re-plated on laminin-coated dishes at low density and FGF2 and EGF were reintroduced into the culture (Fig 4.1B). Any neural stem cells remaining in the differentiating culture should be responsive to the growth factors and reinitiate proliferation. Since earlier introduction of growth factors resulted in the overgrowth of the culture by remaining NES cells, 4 weeks were determined as the length of differentiation necessary for the transition of all lt-NES cells to a later stem cell stage.

Indeed, at passage 1 these cultures consisted of a mix of differentiated cells as well as precursors. In order to select for proliferating cells, the culture was passaged several times and at passage 5 a homogenous population of cells with bipolar morphology emerged and could subsequently be propagated for more than 30 passages. (Fig. 4.1B)

This new population shows clear differences to lt-NES cells in growth pattern and morphology. Most cells have an elongated cell body with long bipolar processes whereas some appear rounded. These cells do not grow in rosettes and can be cultured at significantly lower density compared to lt-NES cells (Fig. 4.1). They also have a slower doubling time compared to the faster proliferating lt-NES cells and were split 1:2 approximately every 5–7 days.

Immunocytochemical analysis showed that the cells continue to homogeneously express the neural stem cell markers Nestin, Sox2 and Pax6. However, the typical rosette-associated transcription factors Dach1 and PLZF are no longer present. ZO1, while still being expressed, does not show the previously observed localization to the apical membrane but is enriched at cell-cell contacts. The radial glia-associated transcription factor Sox9 is now uniformly expressed (Fig. 4.1C). Importantly, this shows characteristics similar to the transition from neuroepithelial stem cells to radial glia cells during development, as some epithelial features have been down-regulated following the sequential differentiation protocol, whereas glial cell markers show a strong increase in expression (Gotz et al. 2005).

These findings indicate that it is possible to generate a cell population with characteristics of radial glia cells (radial glia-like neural stem cells) from hES cells through an intermediate NES cell stage.

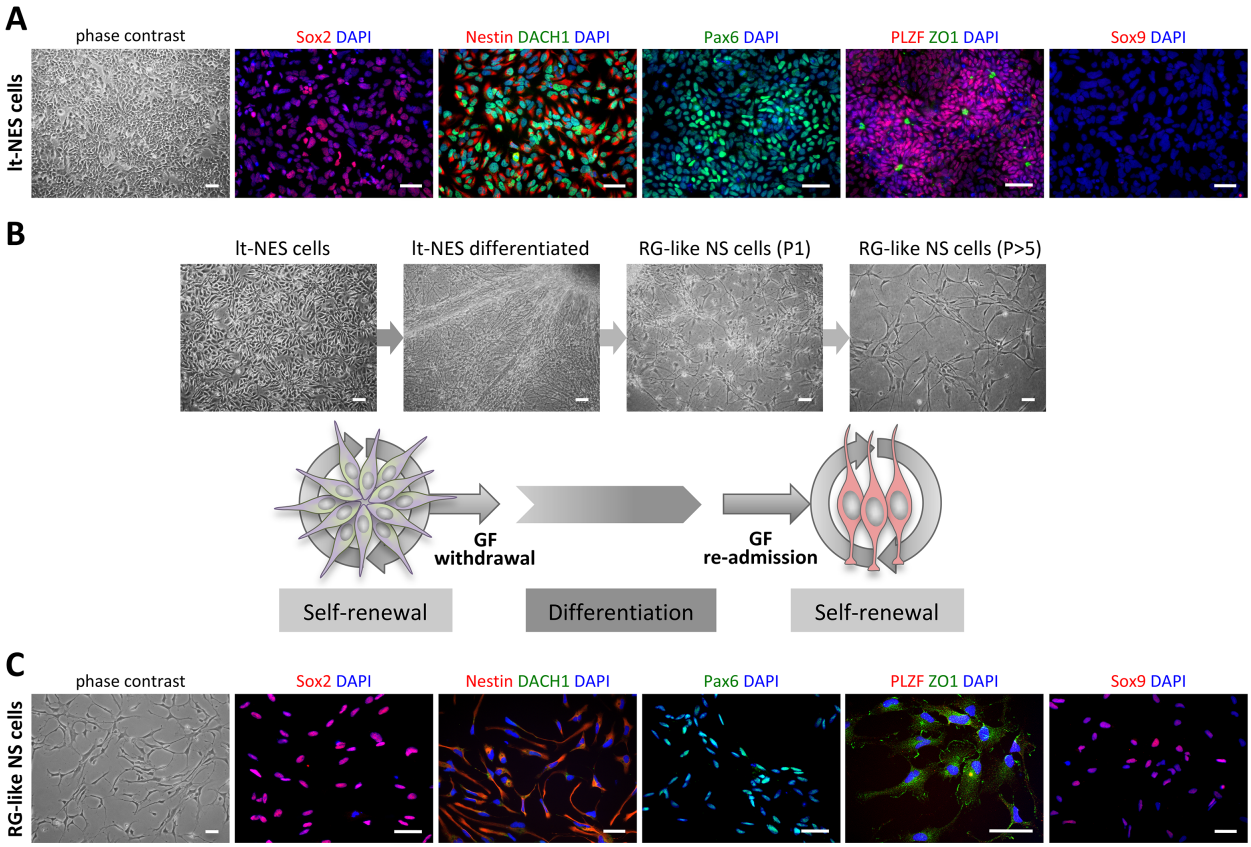


Fig. 4.1: Generation and characterization of radial glia-like NS cells from neuroepithelial (NES) cells.

(A) lt-NES cells grow in rosette-like patterns and express Sox2, Nestin, Pax6, DACH1 as well as PLZF but not Sox9. At their apical pole, rosettes form tight junctions as identified by ZO1. **(B)** Experimental outline: Lt-NES cells self-renew in the presence of FGF2 and EGF. Following growth factor withdrawal for 4 weeks, cultures form dense neuronal networks with prominent neurites. Trypsinization and re-admission of growth factors gives rise to an inhomogeneous cell population (P1). Within 5 passages, a homogeneous population of radial glia-like NS cells develops. **(C)** lt-NES cell-derived NS cells (RG-like NS cells) show radial glia like morphology and marker expression. Sox2, Nestin, Pax6 and Sox9 are homogeneously express, whereas DACH1 and PLZF are absent. Scale bars: 50 µm.

4.1.2 RG-like NS cells show radial glia marker expression

To characterize the newly obtained population further, the cells were analyzed for additional known radial glia cell markers and the expression compared to the lt-NES cell population they were derived from. Immunocytochemical staining revealed that RG-like NS cells express the radial glia associated marker brain lipid binding protein (BLBP), which was not detected in the original lt-NES cell population. Vimentin (3CB2), a marker expressed in radial glia cells, was strongly expressed in the RG-like NS cell culture, whereas lt-NES cells only showed a weak staining (Fig. 4.2A).

In addition, neuroepithelial cells and radial glia cells reportedly differ with respect to adhesion proteins and tight junction marker expression. As previously described, lt-NES cells typically grow at high density and form rosette-like structures with typical polar expression of tight junction proteins like ZO1 as well as N-cadherin and β-catenin marking the center of the rosette (Fig. 4.1A, 2B). This structure is thought to correspond to the ventricular lumen of the neural tube *in vivo*. In contrast, radial glia cells do not form tight-junctions and re-localize ZO1 to adherens junctions, which play a major role in forming and maintaining the radial glia scaffolding (Aaku-Saraste et al. 1996; Miyamoto et al. 2015; Ferent et al. 2020). In RG-like NS cell populations, which typically grow at low density and exhibit an elongated bipolar morphology, ZO1, N-cadherin and β-catenin were expressed independently of cell density. Only a subset of the cells, however, exhibits a polar accentuated expression of these markers, showing that changes to adhesion proteins were also apparent in the lt-NES to RG-like NS cell transition (Fig. 4.2B).

By using flow cytometry, the expression of CD133 (Prominin-1) was examined, a marker commonly used to identify and isolate neural stem cells (Gotz et al. 2005; Lee et al. 2005).

Both lt-NES and radial glia-like NS cells showed comparable expression of CD133. The radial glia cell specific surface marker CD44 was present on RG-like NS cells, whereas it was virtually absent from lt-NES cells (Falk et al. 2012). The glutamate aspartate transporter GLAST, described in radial glia cells, showed a strong up-regulation in radial glia-like NS cells compared to lt-NES cells (Fig. 4.2C).

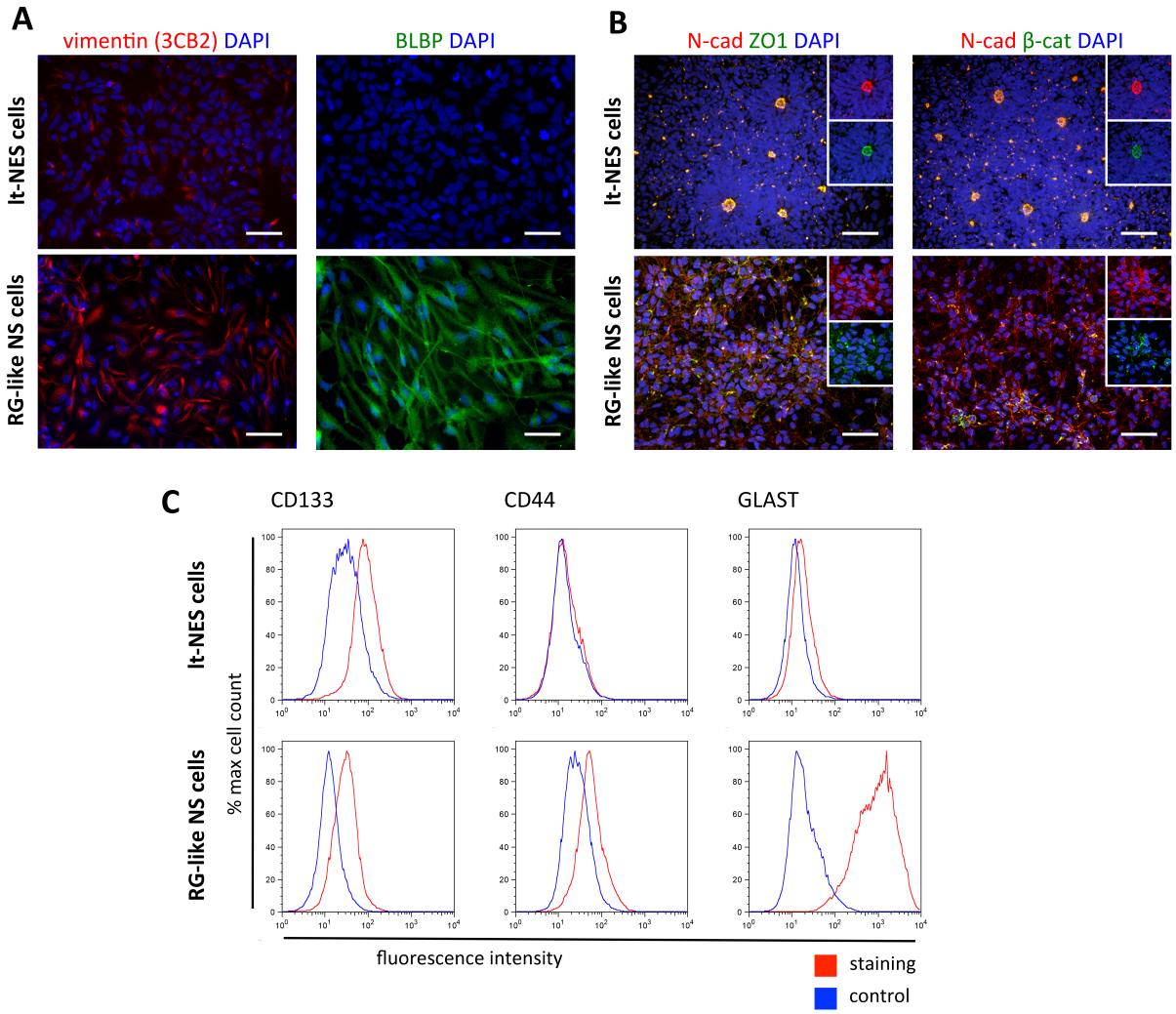


Figure 4.2: RG-like NS cells express prominent radial glia markers in addition to adhesion molecules and tight junction-associated markers but lack typical polarity of lt-NES cells.

(A) Lt-NES cell-derived RG-like NS cells express the radial glia markers vimentin (3CB2) and BLBP, which are near absent in lt-NES cells. **(B)** Lt-NES cells form neural rosettes, which at the center stain positive for the tight junction marker ZO1, N-cadherin and ß-catenin. RG-like NS cells exhibit a bipolar morphology with accentuated expression of these markers in a subset of the cells. **(C)** Flow cytometric analysis of the neural stem cell marker CD133 shows comparable expression in lt-NES and RG-like NS cells. The radial

glia markers CD44 and GLAST are prominently expressed in RG-like NS cells compared to lt-NES cells. Scale bars: 50 µm

4.1.3 Similarity of primary radial glia cells to hPSC-derived RG-like NS cells

Several protocols have been described that allow the generation of neural stem cells of different developmental stages from hPSCs. However, it is currently in dispute whether these *in vitro* cell populations have a correlate *in vivo*.

To assess the developmental relevance of hPSC-derived RG-like NS cells, this cell culture-generated population was compared to primary radial glia cells, isolated from human fetal tissue (primary NS cells), kindly provided by the Austin Smith Laboratory (Sun et al. 2008; Tailor et al. 2013). Interestingly, when comparing the morphology of RG-like NS cells to that of primary NS cells, the two populations exhibit great similarities. Both show an elongated, bipolar cell shape, grow at low density and do not show rosette formation. In immunocytochemical staining, primary NS cells as well as RG-like NS cells express the neural stem cell markers Sox2, Nestin and Pax6 homogeneously and both populations are missing the rosette markers DACH1 and PLZF. ZO1 is not polarized and accentuated at the apical membrane as in NES cells. However, if examined in greater magnification, weak ZO1 immunoreactivity can be detected. Additionally, primary NS cells as well as hPSC-derived RG-like cells were positive for the radial glia cell marker Sox9 (Fig. 4.3A).

Comparative semi-quantitative RT-PCR analysis of lt-NES, RG-like NS derived thereof, and primary NS cells demonstrated that all three populations express genes described to characterize neural stem and progenitor cells such as SOX1, PAX6, NOTCH1 and its downstream effector genes HES5 and HEY1. Among the neural stem cell markers expressed were also RFX4, GPM6A and ASCL1 (MASH1). In contrast, known genes for neuroepithelial stem cells, such as PLZF, MMRN1, PLAGL1, and NR2F1 were only detected in lt-NES cells, whereas in RG-like NS cells as well as primary NS cells these makers were strongly down-regulated or absent. Screening for markers described in radial glia cells, like the water channel AQP4 as well as HOP, CD44, the glia marker SOX9 and S100beta and the matricellular protein SPARCL1, found these markers were highly up-

regulated in RG-like NS cells and primary NS cells while absent from the original lt-NES cell population (Elkabetz et al. 2008; Koch et al. 2009b) (Fig. 4.3B).

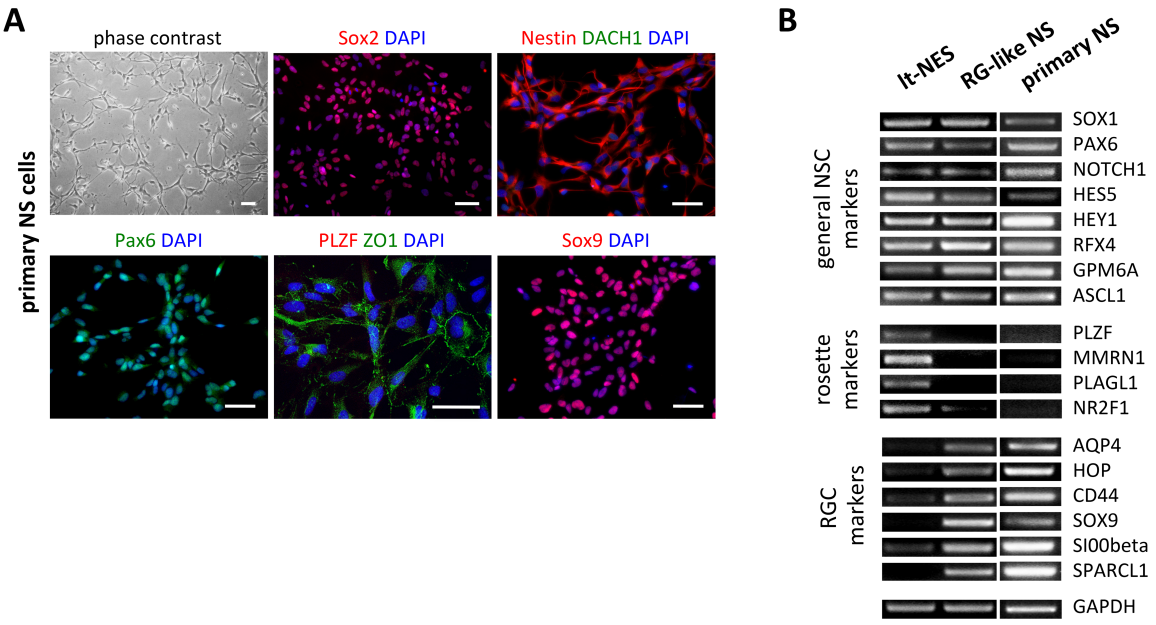


Figure 4.3: Comparison of primary NS cell with radial glia-like NS cells shows significant similarity in morphology and marker expression.

(A) Primary NS and lt-NES cell-derived RG-like NS cells show comparable morphology and marker expression. Both populations homogeneously express Sox2, Nestin, Pax6 and Sox9, whereas DACH1 and PLZF are absent (compare Fig. 4.1). **(B)** Lt-NES cells, RG-like NS cells derived thereof and primary NS cells share expression of the neural stem/progenitor markers SOX1, PAX6, NOTCH1, HES5, HEY1, RFX4, GPM6A and ASCL1. The typical rosette-associated markers, PLZF, MMRN1 PLAGL1 und NR2F1 are expressed by lt-NES cells but not by the NS cell populations. These share expression of the typical radial glia cell (RGC)-associated genes, AQP4, HOP, CD44, SOX9, S100beta and SPARCL1 which are absent in lt-NES cells. Scale bars: 50 µm

4.1.4 Comparative transcriptome analysis of RG-like NS and lt-NES cells highlights their distinct developmental stage

To better define transcriptional differences between cell populations, microarrays were used to allow for comparative global gene expression analysis of cDNA samples of the original lt-NES cell population and lt-NES-derived RG-like NS cells (referred to here also as NS cells). Applying the characteristic direction algorithm, large-scale global differences between the two neural stem cell classes could be confirmed (Clark et al. 2014).

Consequently, the samples were subjected to PluriTest, an unbiased classifier identifying the pluripotent state (Müller et al. 2011). This test confirmed that both cell population had exited the pluripotent state, interestingly, however, it also already separates NES from NS cells, placing NES cells closer to the pluripotent state and RG-like NS cells closer to differentiated cells (Fig. 4.4A).

In a principal component analysis, this differential gene expression was described by a direction vector between NES and NS cells in expression space. Applying the characteristic expression approach, the vector differentiating between NES and NS cells can be used to identify the most significantly up-regulated transcripts expressed by the two populations (Clark et al. 2014). This analysis revealed close to 1700 genes significantly up-regulated in NES cells compared to NS cells. Conversely, over 1900 gene probes showed significant up-regulation in NS vs. NES cells (Fig. 4.4B, C, Table 1). Other algorithms for the detection of differentially expressed genes revealed even larger numbers of differentially expressed transcripts between the stem cell classes (data not shown).

A scatter plot depiction of the gene expression data shows specific genes exhibiting significant differential expression between NES and NS cells as well as genes shared by both stem cell classes. In the NES cell sample, genetic markers for neuroepithelial stem cells such as NMYC, LIN28B, MMRN1, ZBTB16 (PLZF) and NR2F1 are highly expressed. Notably, the newly generated RG-like NS cells (NS cells) show a significant up-regulation of radial glia marker genes. This includes SOX9, FABP7 (BLBP), HOPX, SPARCL1, CD44 or GFAP. Among the genes shared by both populations are in particular markers characterizing neural stem and progenitor cells, namely SOX2, NES (NESTIN), BMI1, CITED2 or ASCL1 (Fig. 4.4C).

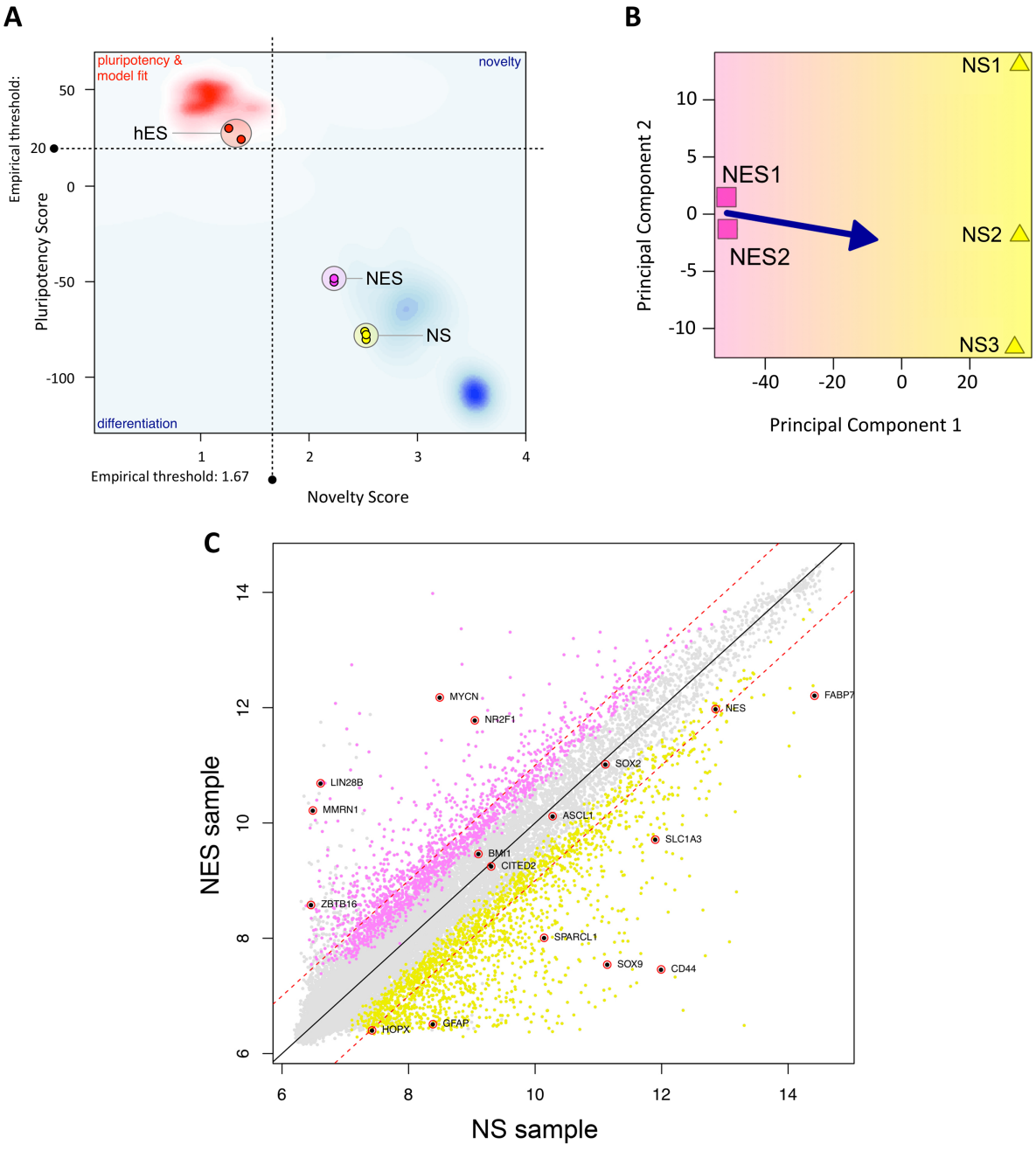


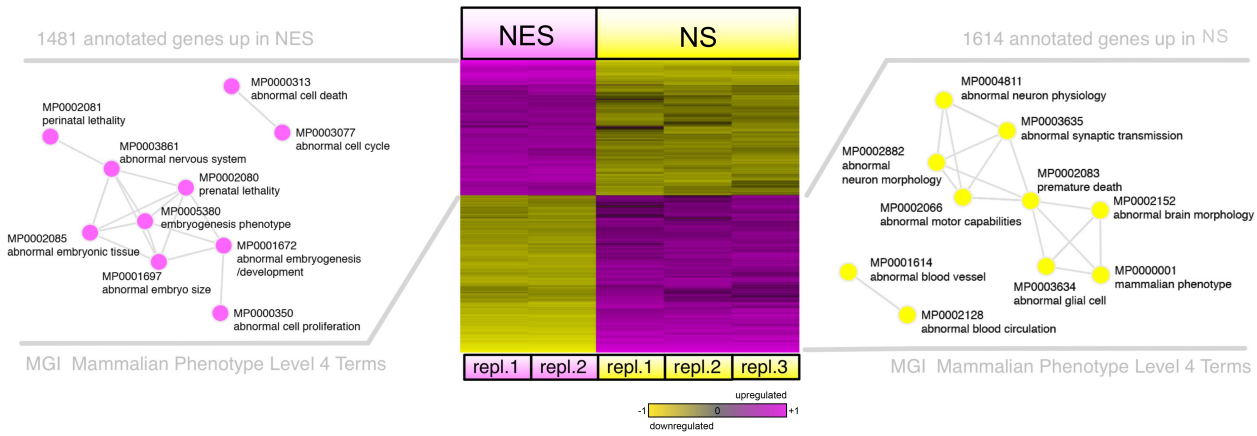
Figure 4.4: Comparative transcriptome analysis of NES cells and NS cells derived thereof

(A) Lt-NES and NS cells characterized by Pluripotency (Y) and Novelty Scores (X) clearly differ from those of the undifferentiated pluripotent stem cells from which they were differentiated. The PluriTest plot puts the resulting Pluripotency and Novelty Scores into the context of the empirical distribution of pluripotent and somatic samples. Briefly, the background red and blue clouds are colour coded empirical density distributions derived from all undifferentiated samples (red) and differentiated samples (blue) used for constructing and validating the PluriTest classifier (Müller et al. 2011). Two exemplary samples from the hESC line H9.2 (red circles) fall clearly in the range of the

undifferentiated, pluripotent stem cells (as indicated by the empirical red density distribution of pluripotent stem cells). lt-NES (pink circles) and NS cells (yellow circles) differentiated from the H9.2 hESC line are in regard to their PluriTest scores far apart from the undifferentiated cells and fall closer to the differentiated samples (as indicated by the blue empirical density distribution of differentiated cells). Interestingly, NS cells (yellow circles) are also set apart by their PluriTest scores from the lt-NES cells and fall closer to most differentiated cell types (as indicated by the blue empirical density distribution of differentiated cells in the lower right corner). **(B)** 2D principal component projection of the characteristic direction between the two sample classes (blue arrow) based on gene expression data. The characteristic direction vector differentiates between NES cells (magenta squares) and NS cells (yellow triangles) and is used by the characteristic direction approach (Clark et al. 2014) for the identification of differentially expressed genes. **(C)** Scatterplot of Illumina HT12v4 gene expression of one exemplary NES cells and one exemplary NS cell sample with the gene probes identified as significantly differentially up-regulated in lt-NES cells (magenta dots) and NS cells (yellow dots) as determined by the characteristic direction algorithm. Probes detecting known marker genes for neural stem cells (BMI1, ASCL, SOX2, NES), for rosette-type cells (LIN28B, ZBTB16, MMRN, MYCN, NRF2) and for radial glia fates (HOPX, GFAP, SPARCL1, SOX9, CD44, SLC1A3) are highlighted by black dots and red circles. Transcriptome data analysis was performed in collaboration with Franz-Josef Müller (University of Kiel).

In order to link biological function to expression data EnrichR gene ontology analysis (Chen et al. 2013) with the MGI Mammalian Phenotype Terms level 4 was performed on the basis of the significantly differentially regulated transcripts, represented here in a heatmap. This analysis associated transcripts up-regulated in lt-NES cells with MGI Level 4 Phenotype Terms implicating cell proliferation and embryonic development (Fig. 4.5A left). Conversely, genes overexpressed in NS cells relate to brain morphology and neuronal and glial function, again reflecting a later developmental stage (Fig. 4.5A right). Extracting the top 20 up-regulated genes in NES cells revealed developmentally earlier processes such as stem cell proliferation (MYCN, ID3, ZIC2, LIN28B) and neural differentiation (DLK1, FZD9, ZFHX3), as well as embryonic neural morphogenesis (CNTNAP2, CRABP1, SFRP2). In contrast, the top 20 expressed transcripts in NS cells highlight genes implicated in later processes including glia progenitor identity and function (CD44, CAV1, NFIX), neural function (GABBR2, KCNMB1, NFIB, SCG2, MOXD1, GAP43) and cell-cell and cell-matrix interactions and NSC proliferation (ANXA2, TNC, TAGLN, LGALS1, IGFBP7). These findings are consistent with the premise that NS cells represent a developmentally later and more defined neural stem cell class (Fig. 4.5B).

A



B

NES	top 20 genes up		NS
CRABP1	GNPDA1	C1orf61	CAV1
UBB	ZIC2	IGFBP7	HLA-DRA
LOC648390	ZFHX3	LGALS1	ANXA2
RPRML	SFRP2	TNC	GAP43
CNTNAP2	FABP5L2	SCG2	TAGLN2
ID3	MYCN	CD44	C1QL1
LIN28B	IGDCC3	NFIB	KCNMB1
CAMKV	DLK1	NFIX	MAGEC2
ALPL	CHST15	TAGLN	GABBR2
FZD9	BTBD17	MOXD1	ARHGEF6

Figure 4.5: Comparative gene ontology analysis and top differently up-regulated genes in NES vs. RG-like NS (NS cells)

(A) Gene list enrichment analysis based on the 3095 significantly differentially up-regulated and annotated genes (middle panel in a heatmap view) in NES cells (left) and NS cells (right), respectively, using the EnrichR-tool (Chen et al. 2013). For each class the 10 most significantly enriched gene sets from the Mouse Genome Informatics Mammalian Phenotype Level 4 Terms are displayed. Each term represented by a node is shown in context in a force-directed network visualization. Edges denote overlap among the genes in each term-specific gene list as detailed in **(B).** Top 20 up-regulated genes in NES cells and NS cells, respectively of the up-regulated genes identified by the characteristic direction approach. Transcriptome data analysis was performed in cooperation with Franz-Josef Müller (University of Kiel).

4.1.5 RG-like NS cells differentiate into functional neurons and glia

Neural stem cells are defined by their capacity for self-renewal and the ability to differentiate into multiple cell types of the nervous system, neurons as well as glia. Therefore, radial glia-like NS cells were next examined for their differentiation potential upon exposure to appropriate instructive cues.

In order to differentiate radial glia-like NS cells into neurons, the mitogen EGF was first omitted from the culture for 10 days followed by withdrawal of FGF and change of the

medium to neural induction medium. 4 weeks after the initiation of spontaneous differentiation, cells with neuronal morphology positive for the neuron-specific marker βIII-tubulin as well as glial fibrillary acidic protein (GFAP)-positive astrocytes could be detected. Neurons detected in the culture also co-expressed the microtubule-associated marker Map2ab. In addition, some βIII-tubulin-positive cells co-expressed the early neuronal marker doublecortin (DCX), while others had already up-regulated the mature neuronal marker NeuN. Some cells in the culture continued to express Nestin, which can either indicate remaining neural stem cells and ongoing neurogenesis or mark immature astrocytes.

When radial glia-like NS cells were exposed to medium containing 10% fetal calf serum for only 2 weeks, almost all of the cells became GFAP-positive, showing an enhanced differentiation potential towards astrocytes, a behavior previously described for (primary mouse) radial glia cells (Sun et al. 2008).

For the differentiation of RG-like NS cell into oligodendrocytes a two-step protocol adapted form Glaser et al. was applied (Glaser et al. 2007). First the cells were differentiated under the influence of platelet-derived growth factor AA (PDGF-AA), EGF and forskolin for 2 weeks, in order to induce glial cell types. Next, cells were cultured for 1 week in medium containing PDGF-AA, T3, LDN and ascorbic acid (AA) to support the proliferation of oligodendroglial cells. For terminal maturation cells were consequently cultured for 3 weeks under the influence of T3, AA and laminin. Following this protocol some O4-positive oligodendrocytes could be detected in the cultures (Fig. 4.6A).

The capacity of radial glia-like NS cells to differentiate was quantified at early (5-10) and late (20-25) passages. After 4 weeks of differentiation 20-25% of the cells expressed βIII-tubulin and approximately 40% were positive for GFAP. Most of the remaining cells (30%) continued to express Nestin. The potential of radial glia-like NS cells to differentiation into neurons and glia stayed consistent in early and late passages with similar numbers of neurons and glia generated over multiple passages (Fig. 4.6B).

Following the confirmation that RG-like NS cells are able to generate neurons expressing mature neuronal markers, the next important step was to assess their electrophysiological properties. For that purpose, NS cells were differentiated for a prolonged period of 8 weeks. The generated neurons were then analyzed, using patch-clamp recordings, for their ability to display mature electrophysiological functions, such as the generation of

action potentials and spontaneously active synaptic circuitries. Whole-cell current clamp recordings could show that RG-like NS cell derived neurons generate single or multiple action potentials following the injection of a depolarizing current (Fig. 4.6C, D; n=6). In addition, a fast and transient inward current, sensitive to TTX application, as well as sustained outward currents could be observed (Fig. 4.6E). Importantly, using whole-cell voltage clamp measurements, spontaneous postsynaptic currents could be recorded, in line with the neurons' capacity to form functional synapses (Fig. 4.6F). Therefore RG-like NS cell have the ability to generate functional neurons and glia.

Taken together, these results indicate that it is possible, through sequential differentiation of ES cells, to recapitulate the formation of radial glia cells from neuroepithelial cells in the fetal brain and generate a radial glia-like neural stem cell *in vitro*.

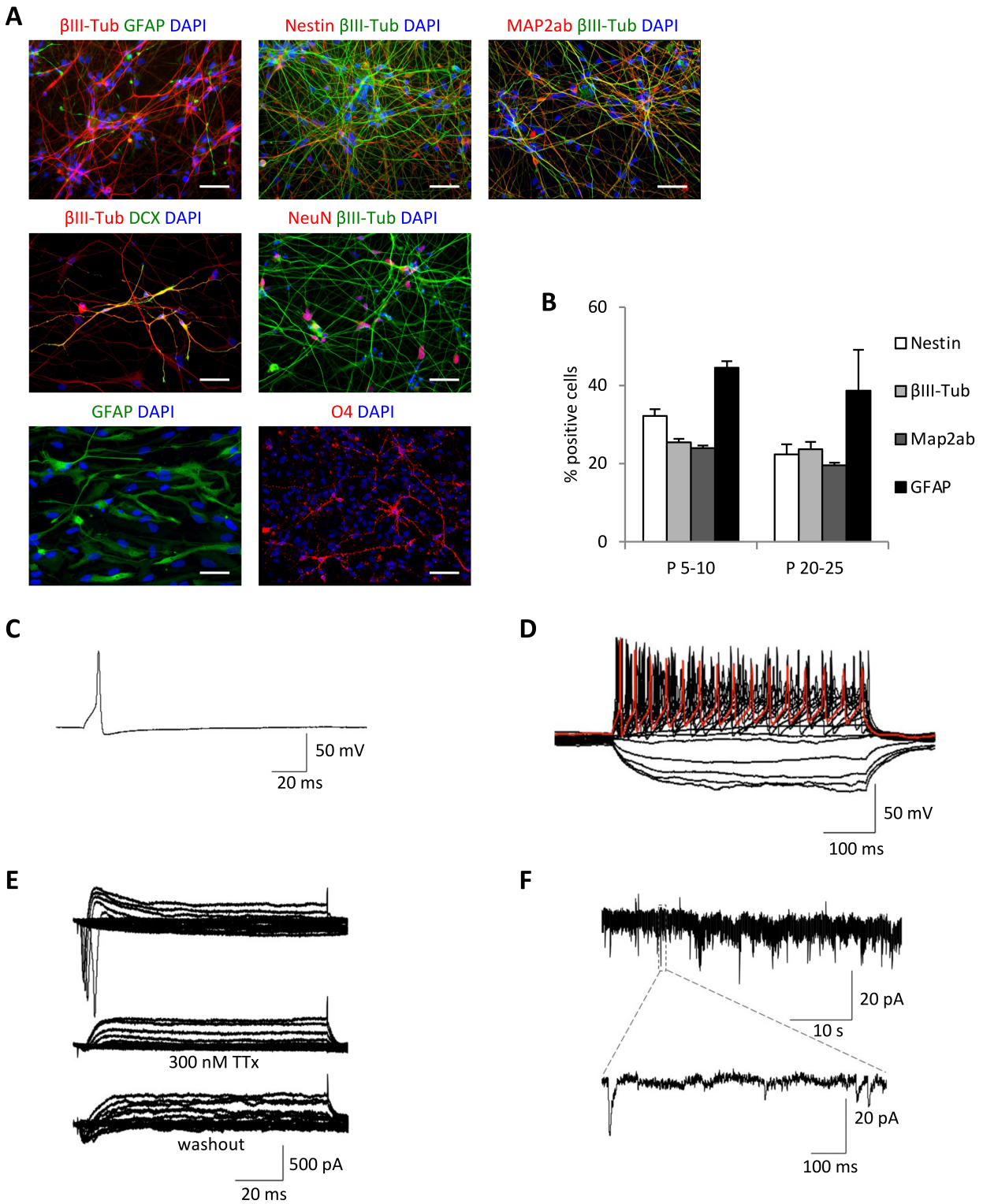


Figure 4.6: Lt-NES cell-derived RG-like NS cells differentiate into functional neurons and glia

(A) Upon growth factor withdrawal, lt-NES cell-derived RG-like NS cells give rise to βIII-tubulin-positive neurons and GFAP-positive astrocytes. In addition, the cultures contain some Nestin-positive cells. Neurons co-express MAP2ab. Few neurons express doublecortin after 4 weeks of growth factor withdrawal while most of them up-regulate the

mature neuronal marker NeuN. Addition of serum (10% FCS) induces homogeneous differentiation into GFAP-expressing astrocytes. Following appropriate inductive cues some O4-positive oligodendrocytes could be observed. **(B)** Despite continuous passaging, RG-like NS cells retain a stable neurogenic differentiation potential (bar graph depicts % of nestin-, βIII-tubulin-, MAP2ab-, and GFAP-immunoreactive cells in passages 5-10 and 20-25 after 4 weeks of growth factor withdrawal. Shown are means ± s.d. **(C)** Whole-cell current-clamp recording of a single action potential elicited by 100 pA current injection step for 10 ms (n=6). **(D)** Representative traces of multiple action potentials evoked by 500 ms current injection steps from -50 pA to +120 pA in 10 pA increments in neurons differentiated for eight weeks. Membrane potential was maintained at approximately -60 mV (n=6). **(E)** Representative traces of whole-cell currents induced by 10 mV depolarizing voltage steps from a holding potential of −80 mV to +60 mV before and after TTX (300 nM) application (n=6). **(F)** A representative trace demonstrating spontaneous postsynaptic currents recorded at a holding potential of -70 mV (n=6). The boxed region is shown at a higher magnification below. Scale bars: 50 µm. Electrophysiology was performed by Jaideep Kesavan (University of Dublin).

4.2 RG-like NS cells can be derived from different pluripotent sources as well as primary NES cells

In recent years various protocols for the generation of neural stem cells with different properties *in vitro* have been established, making the comparison of research results more challenging (Conti et al. 2010). It is therefore important to evaluate if the developed protocol is consistent and applicable to cells from different genetic backgrounds as well as comparing generated neural stem cells to populations *in vivo*.

4.2.1 RG-like NS cells can be generated from different genetic backgrounds

In order to establish the reproducibility of the newly established protocol, the temporal withdrawal of growth factors was applied to cell lines derived from different genetic backgrounds including the ES cell lines I3 and H9 (Falk et al. 2012; Koch et al. 2009b) as well as induced pluripotent stem cells (IPS cell line #69) (Takahashi et al. 2006; Mertens et al. 2013b). The resulting RG-like NS cell lines showed bipolar radial glia morphology while lacking rosette formation and the typical apical localization of ZO1. They expressed neural stem cell markers (Sox2, Nestin and Pax6) and the radial glia marker Sox9, whereas the neuroepithelial markers (Dach1, PLZF) were down-regulated. These cell

lines could be propagated more than 30 passages without changes in morphology and upon growth factor withdrawal were able to differentiate into βIII-tubulin-positive neurons and GFAP-positive astrocytes (Fig. 4.7).

Neurogenesis in humans takes place over a prolonged period of time compared to other species (Finlay et al. 2019). It is therefore conclusive to hypothesize that human neural stem cells persist in differentiating cultures for a comparable timespan and remain able to respond to growth factors. To test this, It-NES cells were differentiated for a prolonged period of 6 months prior to the re-admission of growths factors. Indeed, it was possible to drive a proliferating neural stem cell line exhibiting hallmark morphology and maker expression of radial glia cells and able to differentiate into neurons, indicating that neural stem cells are present in differentiating cultures for extended periods of time (Fig. 4.7C).

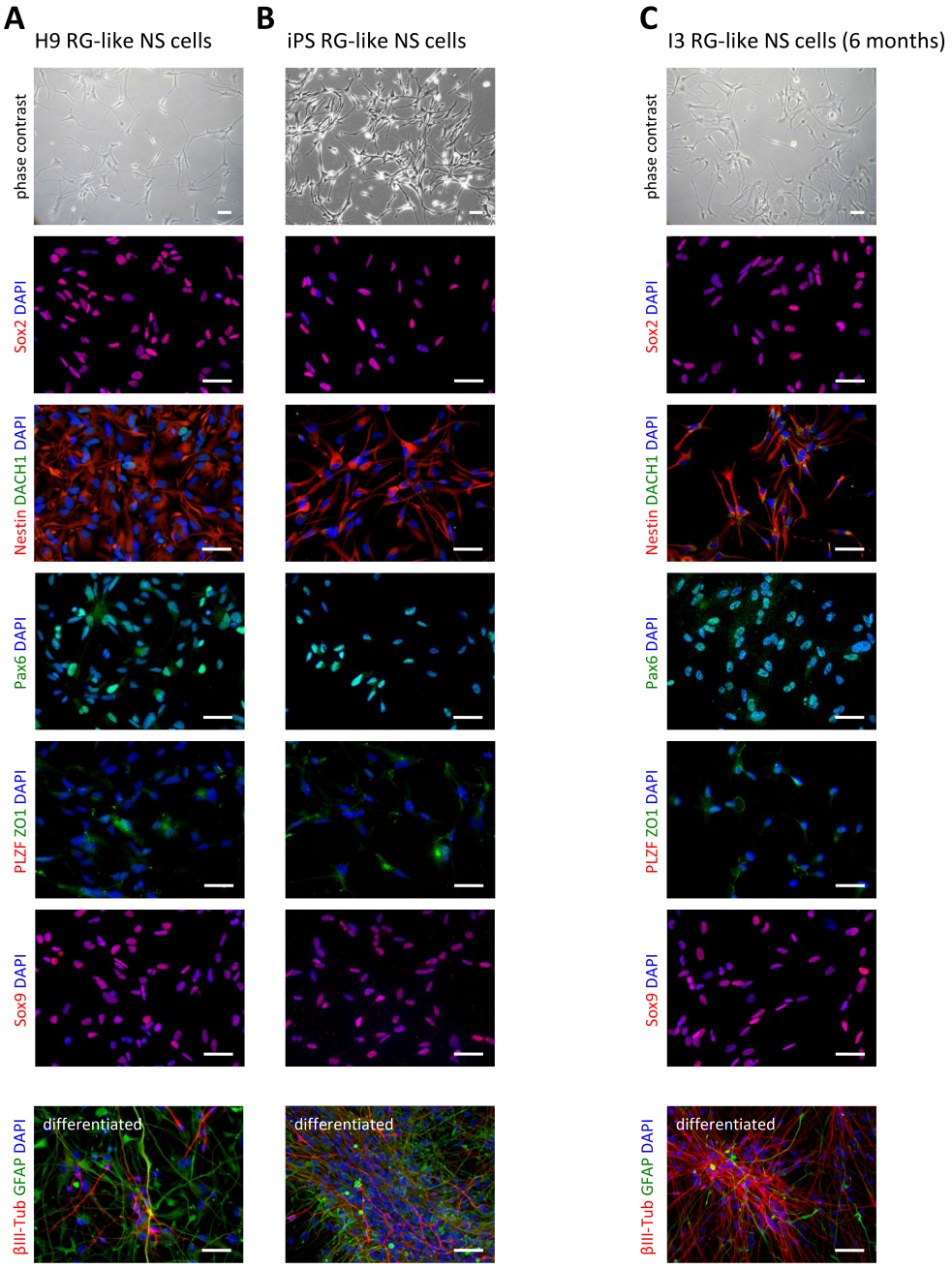


Figure 4.7: Radial glia-like NS cells generated from neuroepithelial stem (NES) cells of different origin

(A) RG-like NS cells generated from lt-NES cells derived from the hESC line H9.2 after 4 weeks of growth factor withdrawal. These H9.2. RG-like NS cells differentiate into βIII-tubulin-positive neurons as well as GFAP-positive astrocytes following consecutive differentiation (last panel). **(B)** RG-like NS cells generated from lt-NES cells derived from human induced pluripotent stem cells after 4 weeks of growth factor withdrawal. These IPS RG-like NS cells likewise generate βIII-tubulin-positive neurons and GFAP-positive astrocytes after differentiation (last panel). **(C)** RG-like NS cells retrieved from I3 hESC-derived lt-NES cell cultures following growth factor withdrawal for up to 6 months. Upon

differentiation this I3 RG-like cell line likewise generated βIII-tubulin-positive neurons and GFAP-positive astrocytes (last panel). Note that all RG-like NS cells exhibit a bipolar morphology and a prominent expression of the neural stem cell marker Sox2 and Nestin as well as the radial glia marker Sox9 while down-regulating the rosette markers DACH1 and PLZF. As previously shown, ZO1 changes localization compared to lt-NES rosette. Scale bars: 50 µm

4.2.2 Primary hindbrain-derived NES cells give rise to RG-like NS cells

An important validation of the concept that *in vitro* generated neural stem cells indeed correspond to cells arising *in vivo*, is to subject primary NES cells to the newly established temporal differentiation protocol. To this end, primary NES cells isolated from human fetal tissue with hindbrain identity (hbNES cells, provided by the Smith laboratory) were used as a starting population (Tailor et al. 2013). These cells correspond to embryonic stem cell derived lt-NES cells, and it is of great interest if they can likewise be converted to RG-like NS cells (Fig. 4.8A).

As a first step, primary NES cells were examined for the expression of the neural stem cell markers Sox2, Nestin and Pax6, as well as the neuroepithelial markers Dach1, PLZF and ZO1, which were expressed and consistent with the pattern in lt-NES cells. Sox9 was occasionally expressed in this culture (Fig. 4.8B, top row). Subsequently, by applying the established protocol to primary hbNES cells, a radial glia-like population could be derived and propagated. This primary hbNES cell-derived RG-like NS cell population showed the previously described continued expression of neural stem cell markers (SOX2, Nestin and Pax6) as well as the down-regulation or changed localization of neuroepithelial markers (Dach1, PLZF and ZO1). Sox9 expression was found to increase (Fig. 4.8B, lower row). These results are in line with the hypothesis that PSC-derived neural stem cells *in vitro*, such as NES and RG-like NS cells, in fact, correspond to primary, tissue-derived neural stem cells *in vivo*.

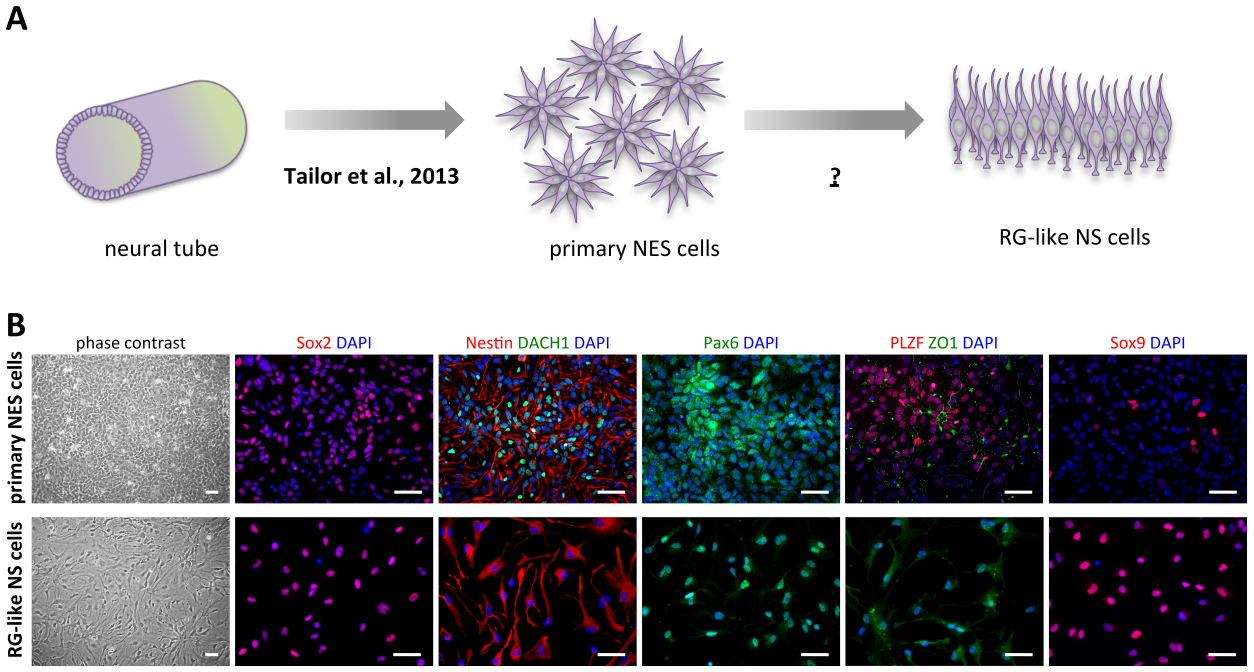


Figure 4.8: RG-like NS cells generated from primary NES cells derived from the human hindbrain

(A) Experimental outline: Primary neuroepithelial stem cells derived from the human hindbrain (hbNES) strongly resemble PSC derived lt-NES cells and should therefore be able to give rise to RG-like NS cells *in vitro* (Tailor et al. 2013). **(B)** Primary hbNES cells (upper row) show the same rosette marker expression as lt-NES cells whereas RG-like NS cells derived thereof (lower row) show typical radial glia-like marker expression. Scale bars: 50 µm

4.3 Fixation of different regional phenotypes through NES cell to RG-like NS cell transition

In early neural development, neuroepithelial stem cells that are responsive to morphogens, acquire a specific regional identity along the anterior-posterior and dorsal-ventral axis of the nervous system. These pre-patterned NES cells later give rise to radial glia cells, which no longer respond to morphogens and are fixed in their regional identity (Merkle et al. 2006).

Lt-NES cells cultured *in vitro* have, through default patterning at the time of their generation, an anterior phenotype (Falk et al. 2012; Koch et al. 2009b). However, due to growth factors required for their propagation, which also act as morphogens, they continuously posteriorize and progress to a hindbrain phenotype over time. Since lt-NES

retain some competence to be patterned, it is possible to shift their phenotype then from a hindbrain position further posterior to a spinal cord identity using retinoic acid (Koch et al. 2009b). Consequentially, the RG-like cells generated in the previous section should have a hindbrain phenotype, corresponding to their cells of origin (hindbrain-like NS cells). The following section explores whether it is possible to generate distinct radial glia-like NS cell populations that maintain their regional identity and do not shift through the influence of growth factors, by using differently patterned NES cells as a starting population (Fig. 4.9A).

4.3.1 NES cells with different regional identities along the anterior-posterior axis give rise to RG-like NS cells

For the generation of anterior forebrain RG-like NS cells, forebrain identity patterned NES cells were used as a starting population. In order to generate forebrain progenitors, human ES and iPS cell lines were directed to a neuronal fate with dual SMAD inhibition using a modified version of the protocol established by Shi et al. (Shi et al. 2012a). Following the initial differentiation for 10-12 days the cells showed neural rosette morphology and were analyzed further for the neural stem cell markers Sox2, Nestin and Pax6 as well as for the rosette markers Dach1 and PLZF, which were expressed as well as ZO1, which showed the typical accentuation at the apical center of the rosettes. The transcription factors FoxG1 (Forkhead Box G1) and Otx2 (Orthodenticle Homeobox 2) are characteristic for the telencephalic region (Cargnin et al. 2018; Acampora et al. 1999). Generated rosettes ubiquitously expressed both transcripts, therefore confirming their telencephalic forebrain identity (Fig. 4.9B). After this evaluation, the cells were further differentiated by growth factor withdrawal for up to 6 weeks. Importantly, during this process anterior rosettes were never cultured in the presence of posteriorizing factors in order to preserve their anterior phenotype. Following 6 weeks of differentiation, the cells were plated on laminin-coated plates and FGF2/EGF were added for the first time to propagate the potentially remaining radial glia-like stem cells.

Posterior spinal cord RG-like NS cells were generated using lt-NES cells as a starting population. Using retinoic acid, lt-NES cells were first posteriorized for 2 weeks to a spinal cord identity. Different Hox genes can be used to identify domains of the hindbrain and

spinal cord (Philippidou et al. 2013; Lippmann et al. 2015). Following this treatment, the cells were thus stained for the spinal cord marker HoxB4, which was now homogeneously expressed (Fig. 4.9C). After this validation retinoic acid was removed from the culture and the posteriorized lt-NES cells were cultured under differentiating conditions for 4-6 weeks. Subsequently the cells were again dissociated, plated on laminin-coated dishes and growth factors were reintroduced.

Both approaches resulted in proliferating, homogeneous cell populations that were subsequently analyzed in more detail.

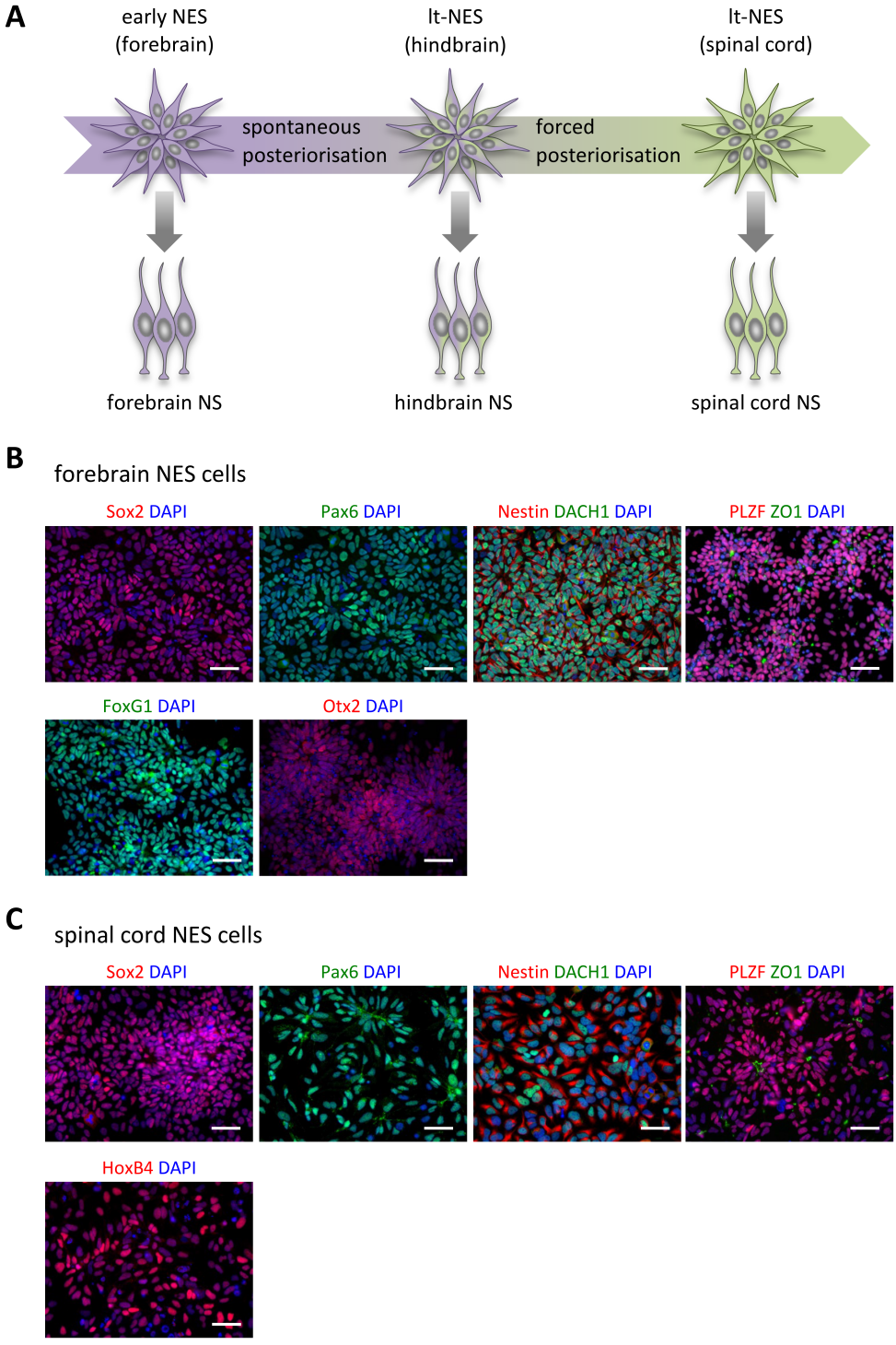


Figure 4.9: RG-like NS cells generated from NES cells with forebrain and spinal cord regional identities

(A) Experimental outline: To investigate whether RG-like NS cells can be generated from NES cells of different regional identities, the temporary growth factor withdrawal protocol was applied to early NES cells with default dorsal forebrain identity, to non-patterned lt-NES cells, which exhibit a growth factor induced hindbrain identity, and to lt-NES cells shifted with retinoic acid into a spinal cord-like identity. **(B)** Early passage (p2) forebrain-

like NES cells show typical rosette marker expression and homogeneously express the dorsal forebrain markers FoxG1 and Otx2. **(C)** Spinal cord-patterned lt-NES cells are strongly enriched for the spinal cord-fate associated marker HoxB4 and continue to express rosette markers. Scale bars: 50 µm

4.3.2 Regionally defined forebrain- and spinal cord-like NS cells retain the positional identity of their founder population

After the cell populations obtained from pre-patterned forebrain and spinal cord NES cells were expanded for 4-6 passages, both lines displayed the typical bipolar morphology of radial glia cells (Fig. 4.10A). Independent of their regional identity, the cells equally continued to express neural stem cell markers (Sox2, Nestin and Pax6) and additionally became strongly positive for the radial glia marker Sox9. The neural rosette markers DACH1 and PLZF were not detected and ZO1 no longer showed the rosette-typical apical localization (Fig. 4.10A).

Following growth factor withdrawal, these newly generated RG-like NS cells were able to give rise to βIII-tubulin- and MAP2ab-positive neurons as well as GFAP-positive astrocytes. In line with earlier results some cells remained positive for Nestin, consistent with the presence of progenitor cells or immature astrocytes. Following the afore described differentiation protocol for oligodendrocytes, some O4-positive cells could be observed in forebrain-like but not spinal cord-like NS cultures (Fig. 4.10B).

Next, comparative semi-quantitative RT-PCR analysis of both approaches was performed, comparing newly generated regionalized RG-like NS cells with the pre-patterned NES cells they were derived from. All examined cell lines shared the expression of important neural stem cell markers, including SOX1, PAX6, Notch1 and its downstream targets HES5 and HEY1 as well as RFX4, GPM6A and ASCL1. The neural rosette markers PLZF, MMRN1 and PLAGL1 were expressed by both, forebrain- and spinal cord-patterned NES cell lines, but down-regulated in the RG-like NS cell lines derived thereof. Conversely, these showed a notable up-regulation of hallmark radial glia markers, such as AQP4, HOP, CD44, SOX9, S100beta and SPACL1 (Elkabetz et al. 2008; Koch et al. 2009b) (Fig. 4.10C).

Importantly, RG-like NS cells derived from forebrain and spinal cord NES cells where subsequently analyzed for their regional identity. As hypothesized, forebrain-like NS cells

were able to retain a strong expression of the forebrain associated transcription factor FoxG1, whereas spinal cord-like NS cells remained positive for the spinal cord marker HoxB4. Forebrain-like NS showed no immunoreactivity for HoxB4 and spinal cord-like NS were found negative for FoxG1 respectively (data not shown). In addition, the regional identity along the dorsal-ventral axes of the brain was assessed in both populations.

Forebrain-like NS cells, as described before, express the dorsal neural stem cell marker Pax6, whereas the ventral forebrain-associated transcription factor Nkx2.1 was not expressed. This observation is consistent with the default dorsal phenotype of neural stem cells generated from pluripotent stem cells (Shi et al. 2012a). Spinal cord-like NS cells also expressed the dorsal neural stem cell marker Pax6 (Fig 4.10A). Conversely the transcription factors Nkx2.2 and Olig2, characterizing the ventral spinal cord, were only occasionally expressed, confirming a predominantly dorsal identity (Fig. 4.10D).

These procedures were repeated and validated using ES cell lines with different genetic backgrounds (H9 and I3) as well as induced pluripotent cell lines yielding corresponding results (data not shown).

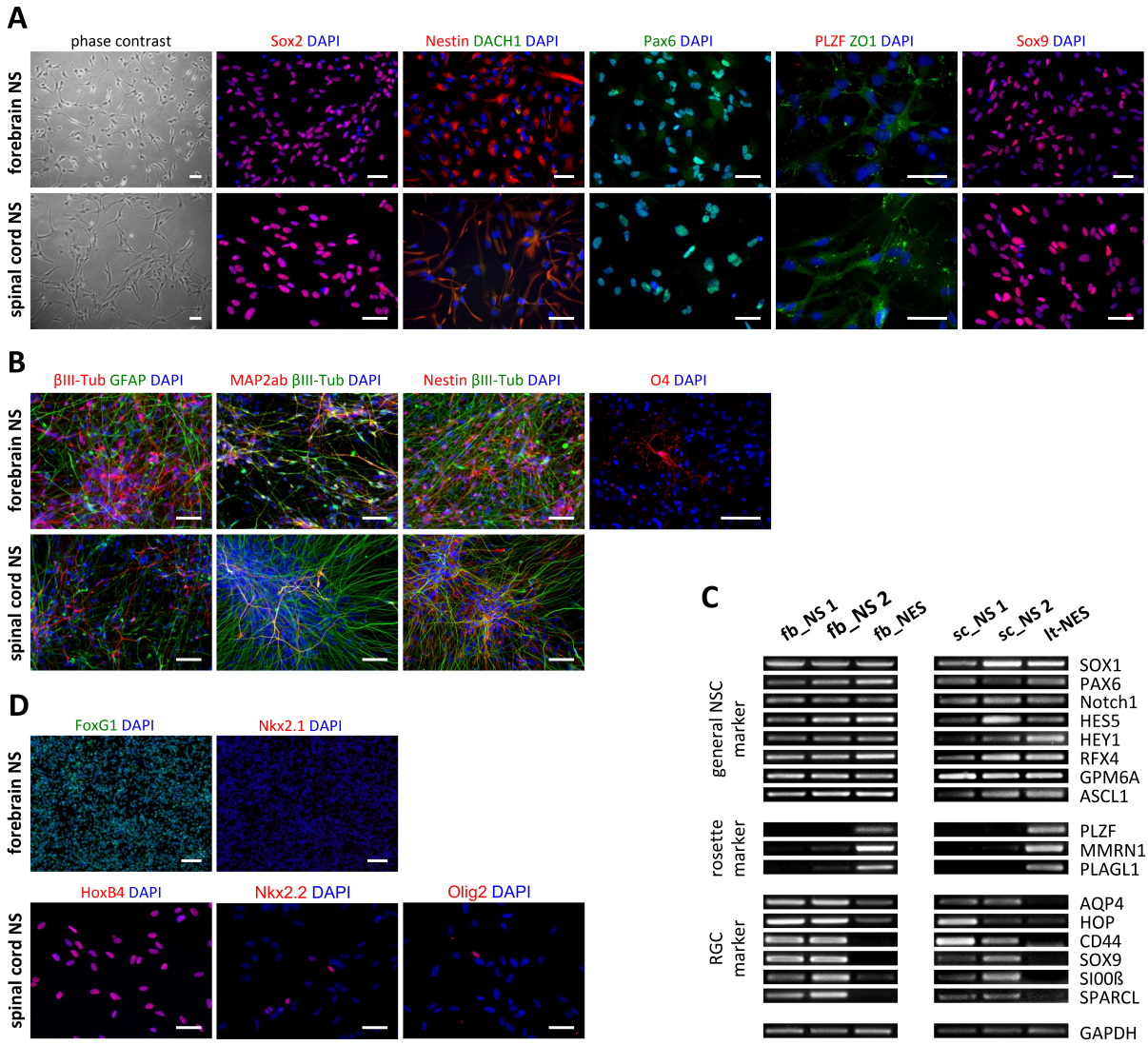


Figure 4.10: RG-like NS cells generated from NES cells with different regional identities

(A) RG-like NS cell generated from forebrain-like and spinal cord-like NS cells exhibit typical radial glia cell morphology, homogeneous expression of Sox2, Nestin and Sox9 and down-regulation of Dach1 and PLZF and loss of pronounced polarized expression of ZO1. Both cell populations also express the dorsal neural stem cell marker Pax6. **(B)** Regionalized RG-like NS cells differentiate into βIII-tubulin- and Map2ab-positive neurons as well as GFAP-positive astrocytes following growth factor withdrawal. Following appropriate inductive cues some O4-positive oligodendrocytes could be observed in forebrain-like but not spinal cord-like NS cultures. **(C)** Neural stem cell marker expression characterizes forebrain-like NES cells (fb_NES) and lt-NES cells as well as the forebrain-like (fb_NS) and spinal cord-like (sc_NS) NS cells derived thereof. Rosette-typical markers are exclusively expressed by NES cells and absent in RG-like NS cell lines. Regionalized RG-like NS cell populations share the expression of typical radial glia-associated genes, which are not expressed in NES cells. **(D)** Forebrain-like NS cells also stain positive for the forebrain marker FoxG1 and the dorsal marker Pax6 (see 4.10A) while lacking expression of the ventral progenitor marker Nkx2.1. Hindbrain-like NS cells

exhibit homogeneous expression of the hindbrain-marker HoxB4 and the dorsal marker Pax6 (see 4.10A). Cells stained positive for markers associated with progenitors of the ventral hindbrain (Olig2 and Nkx2.2) could only occasionally be detected in this cell population. Scale bars: A, B and D 50 µm.

4.3.3 Committed RG-like NS cells are regionally restricted and preserve positional identity over time

Following the initial evaluation, it was now of great interest if these RG-like NS cells, generated from forebrain- and spinal cord-patterned NES cells respectively, would indeed be able to consistently maintain their regional identity even under the influence of posteriorizing growth factors. To this end, the cell lines were propagated using FGF2/EGF and analyzed over several passages for the expression of regional transcription factors.
Even after extensive passaging, forebrain-like NS cells continued to express the markers FoxG1 and Otx2 (P25) whereas the spinal cord-like NS cells remained positive for the spinal cord transcription factor HoxB4 (P20) (Fig. 4.11A, B).
To analyze the expression of additional regional transcription factors, semi-quantitative RT-PCR was performed of forebrain- and spinal cord-like NS cells at passage >10 as well as of the previously established RG-like NS cells derived from lt-NES cells with assumed hindbrain identity (hindbrain-like NS cells). Analysis of forebrain-like NS cells confirmed a strong expression of FOXG1 and OTX2 while spinal cord- and hindbrain-like NS cells did not express these markers. In addition, forebrain-like NS cells expressed the anterior marker DLX2. Hindbrain-like NS cells expressed the HOX transcription factor HOXB2, in line with the original lt-NES cells, while spinal cord-like NS cells in addition showed the expression of the further posterior spinal cord Hox genes HOXB4 and HOXB6 (Fig. 4.11C).

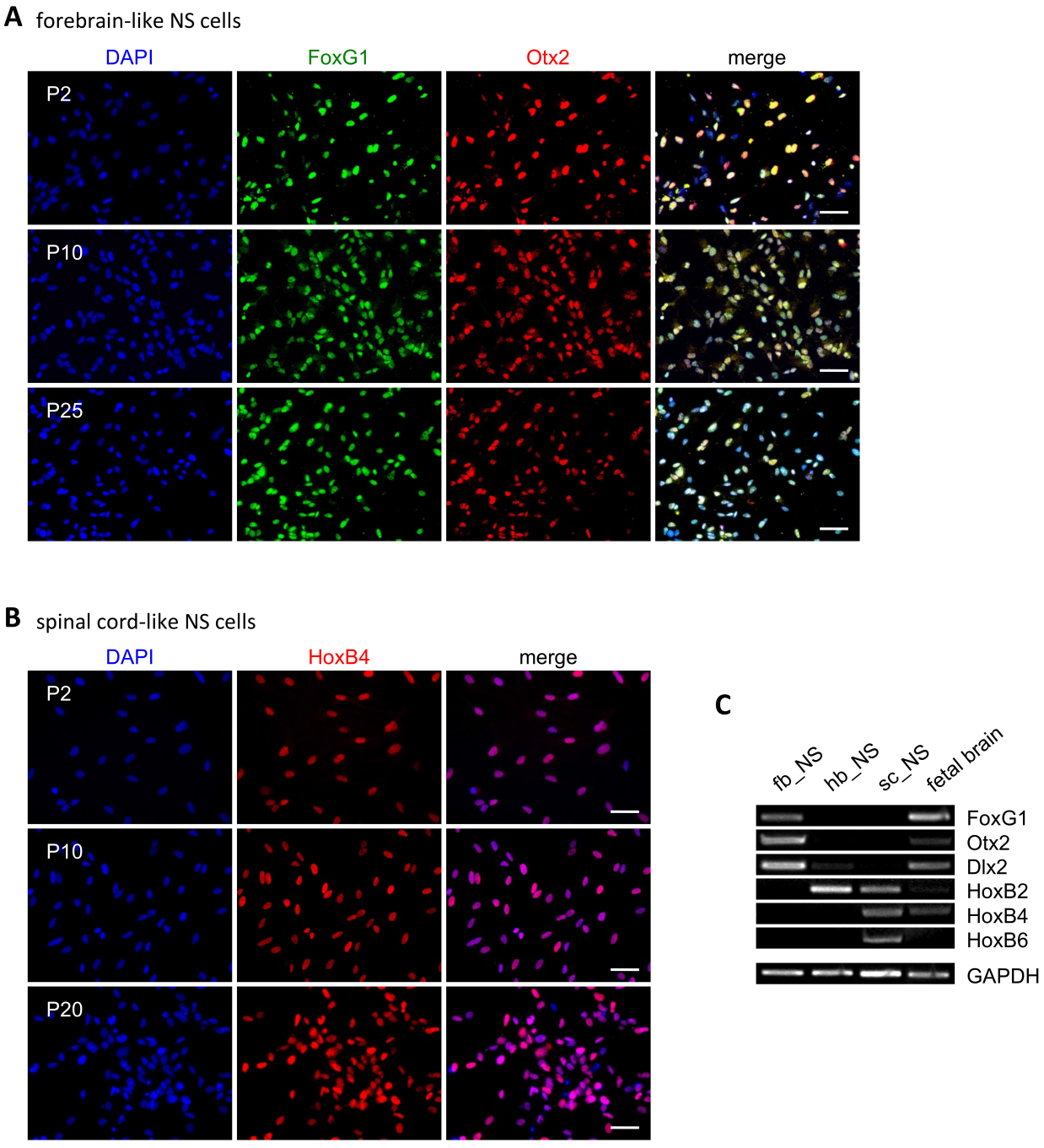


Figure 4.11: Forebrain-like and spinal cord-like NS cells stably express respective regional markers over passages

(A) Immunofluorescence analysis of forebrain-like NS cells shows stable expression of the telencephalic markers FoxG1 and Otx2 (depicted are passages 2, 10 and 25). **(B)** RG-like NS cells derived from spinal cord-patterned lt-NES cells show stable expression of HoxB4 (depicted are passages 2, 10 and 20). **(C)** Comparative RT-PCR analysis of differentially regionalized RG-like NS cells. Forebrain-like NS cells (fb_NS) express the telencephalic genes FOXG1, OTX2 and DLX2, default hindbrain-patterned NS cells (hb_NS) express HOXB2 and spinal cord-like NS cells (sc_NS) the more posterior HOX genes HOXB4 and HOXB6. Scale bars: 50 µm

In order to challenge the stability of the regional phenotype more rigorously, forebrain-like NS cells were exposed to high concentrations (10μM) of retinoic acid for 14 days. Interestingly, the cells continued to homogeneously express the telencephalic markers Otx2 and FoxG1, even after continued exposure to this potent posteriorizing factor, demonstrating the enduring resistance of their positional identity to regionalizing conditions (Fig. 4.12).

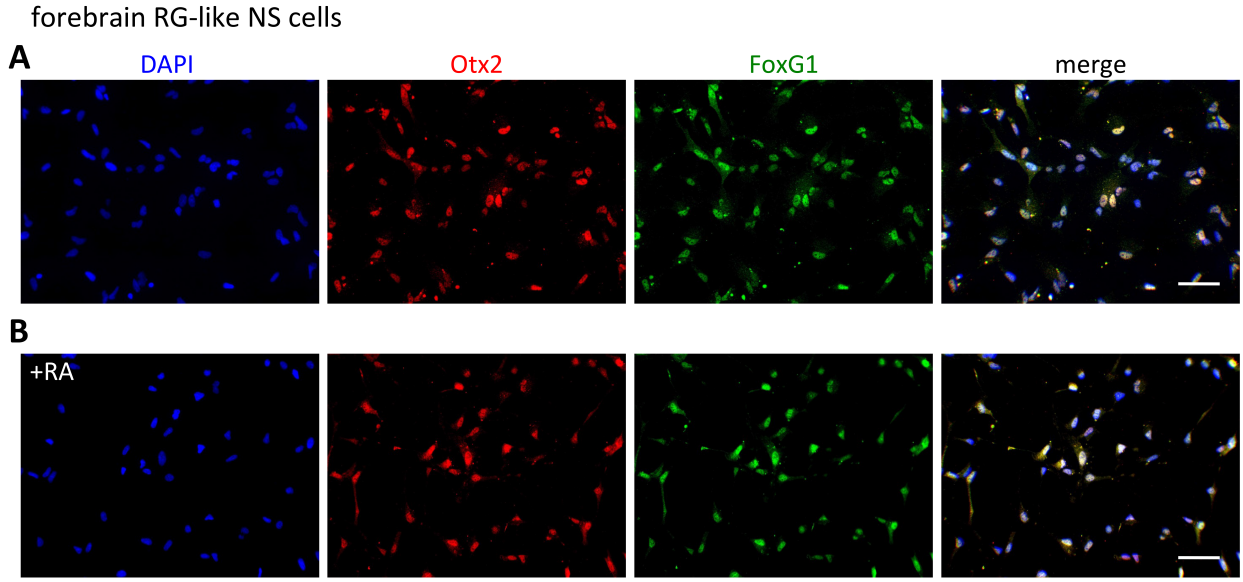


Figure 4.12: Resistance of forebrain-like NS cells to forced posteriorization

(A) RG-like NS cells with forebrain identity express the anterior (telencephalic) transcription factors Otx2 and FoxG1. **(B)** Despite prolonged exposure to high concentrations of the posteriorizing factor retinoic acid for 2 weeks, forebrain-like NS cells maintain expression of these transcription factors and thus their anterior regional identity. Scale bars: 50 μm.

Next, a genome wide microarray gene expression analysis of forebrain-, hindbrain- and spinal cord-patterned RG-like NS cell lines together with non-patterned hindbrain lt-NES cells, was conducted. Unsupervised hierarchical clustering (Fig. 4.13A) together with principal component analysis (Fig. 4.13B) separates lt-NES and the three differently patterned RG-like NS cells into distinct groups, clearly distinguishing lt-NES cells from the RG-like NS cell populations based on global transcriptional profiles.

The analysis of microarray gene expression data was focused on selected, regionally specific transcription factors. This analysis confirmed the expected positional identity of the forebrain-like NS cells, which have a high expression of the anterior transcription factor FOXG1, the dorsal forebrain marker LHX2 and the more ventral forebrain marker DLX1. Hindbrain lt-NES cells as well as the hindbrain-like NS cells derived from them express the hindbrain-associated transcription factors IRX3 and HOXB2 (Koch et al. 2009b). Spinal cord-like NS cells derived from posteriorized lt-NES cells are positive for the spinal cord marker HOXB4 as well as the more caudal markers HOXA5, HOXB8 and HOXB5 (Fig. 4.13C).

Together this data confirms that RG-like NS cells robustly preserve the regional identity of the pre-patterned NES cell population they are derived from in contrast to NES cells, which are unable to maintain an anterior phenotype *in vitro* (Elkabetz et al. 2008; Koch et al. 2009b).

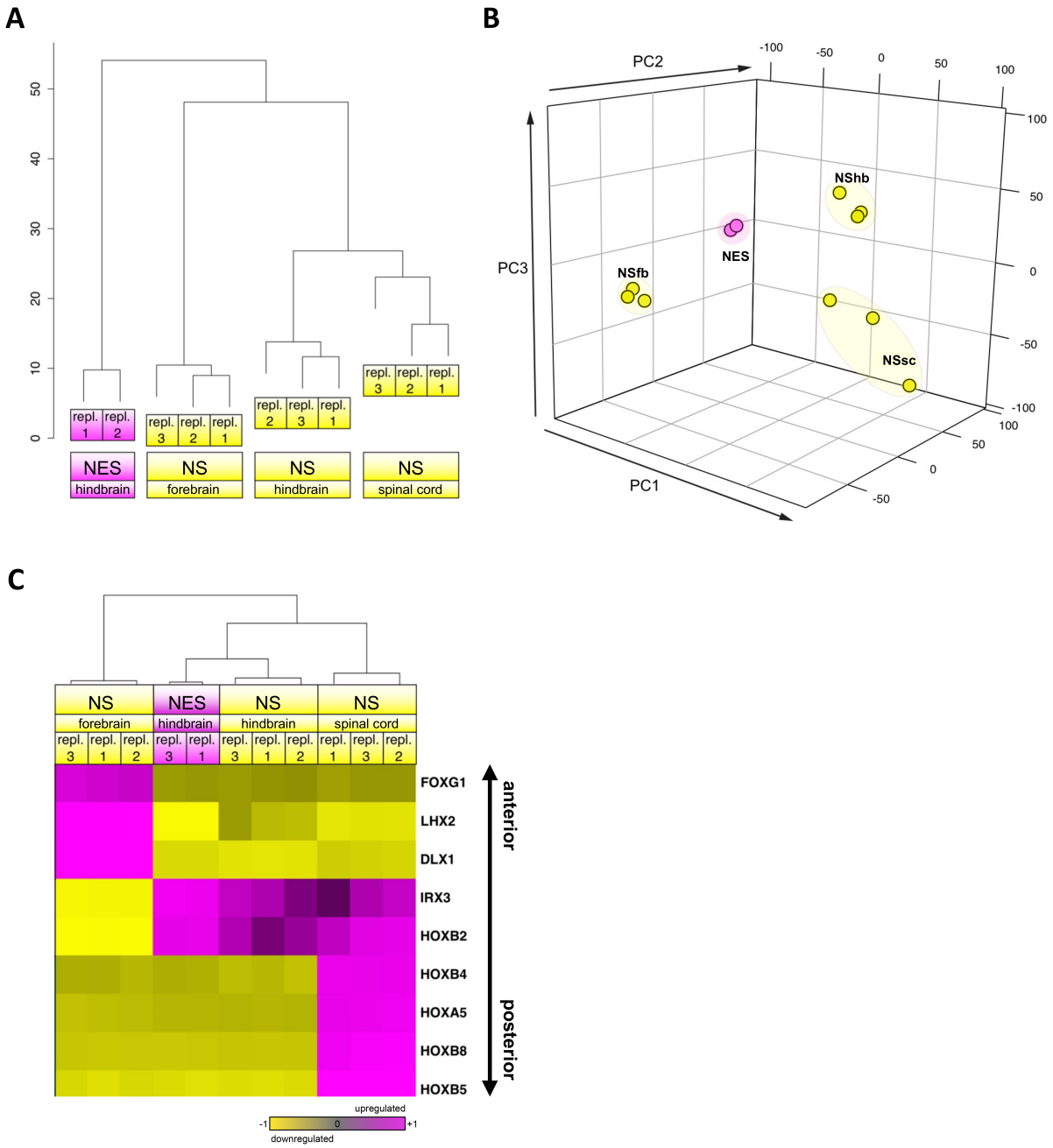


Figure 4.13: Transcriptome analysis of regionally defined RG-like NS cells

(A) Unsupervised hierarchical clustering **(B)** and Principal Component Analysis separate lt-NES and regionally defined RG-like NS cell populations into distinct groups based on global transcriptional profiles. **(C)** A heatmap of unsupervised, hierarchically clustered CNS regional markers (FOXG1, LHX2, DLX1, IRX3, HOXB2, HOXB4, HOXA5, HOXB8 and HOXB5) selected from the microarray gene expression data presented in (A) and (B) confirms the distinct *in vitro* regionalization of NES cells and RG-like NS cells and the sample clustering in (A) and (B). Transcriptome data analysis was performed in cooperation with Franz-Josef Müller (University of Kiel).

4.3.4 Regionally fixed RG-like NS cells generate neuronal subtypes appropriate for their positional identity

Following the confirmation that RG-like NS cells can be generated with a the regionally distinct and stable forebrain, hindbrain or spinal cord phenotype, it is subsequently important to determine whether these neural progenitors are indeed able to generate region-specific neurons that correspond to their respective regional identity.

To this end, growth factors were withdrawn from forebrain- and spinal cord-like NS cell cultures and the cells were differentiated for 6-8 weeks. The resulting neurons were then analyzed using immunohistochemistry to stain for region-specific neuronal subtypes. *In vivo* the cortex is generated in an inside-out manner with deep layer neurons, marked by Ctip2 and Fezf2, first and then upper layer neurons, marked by Satb2 and Brn2 (Greig et al. 2013; Dominguez et al. 2013; Chen et al. 2008). Neurons derived from forebrain RG-like NS cells in fact expressed the deep cortical layer marker Ctip2 and the upper cortical layer markers Satb2 and Brn2, following a longer period 8-10 weeks of differentiation. Ctip2 together with the marker Darpp32 is also associated with medium spiny neurons of the striatum (Onorati et al. 2014; Arlotta et al. 2008). However, Darpp32 was only identified in some few neurons (Fig. 4.14A). The cells were additionally examined for the expression of vGlut1, a glutamate transporter indicating excitatory neurons in the cortex, and GAD67, marking a glutamate-decarboxylase expressed in interneurons, both in combination with Ctip2. GAD67 could only be detected in a small subgroup of neurons and not in combination with Ctip2, whereas the majority of neurons expressed vGlut1. This staining pattern suggests, that the majority of Ctip2+ cells represent glutamatergic cortical projection neurons and few if any correspond to Ctip2+/Darpp32+ GABAergic striatal medium spiny neurons. Together this indicates that forebrain-like NS cells predominantly generate dorsal glutamatergic neurons but are also able to give rise to neurons with ventral forebrain phenotype (Fig. 4.14B).

Spinal cord neuronal subtypes comprise various subpopulations of inhibitory interneurons, marked i.a. by the transcription factors Lim1/2, Lim3, Sim1 and Isl1, as well as motor neurons generated ventrally, which selectively express the homeobox gene HB9 (Lu et al. 2015; Arber et al. 1999). Spinal cord RG-like NS cells differentiated into neurons that

were positive for Lim1/2, Lim3 as well as Isl1. The neurons generated from spinal cord RG-like NS also remained positive for HoxB4 (Fig. 4.14C). In line with the lack of expression of ventral markers by the spinal cord progenitors, HB9-positive motoneurons could not be detected.

To investigate the phenotype of the forebrain and spinal cord RG-like NS cell derived neurons in more detail, transcriptional analysis using semi-quantitative PCR was performed. Neurons derived from forebrain RG-like NS cell exhibited strong expression of the deep cortical layer markers Ctip2 and Fezf2 while neurons derived from spinal cord RG-like NS cell showed no expression of these marker. In accordance with their regional identity these neurons instead expressed the spinal cord interneuron markers Lim3 and Sim1. *In vivo*, the vesicular glutamate transporter 1 (vGlut1) marks glutamatergic, excitatory neurons, whereas GAD65 a GABA-producing enzyme shows the presence of GABAergic, inhibitory neurons (Erlander et al. 1991; Bellocchio et al. 2000). Analysis for the neurotransmitter phenotype showed that forebrain RG-like NS cell derived neurons, in agreement with the previous identification of excitatory and inhibitory neurons in these cultures, strongly expressed vGlut1, in addition to GAD65. Spinal cord RG-like NS derived neurons, which prevailingly differentiate into inhibitory spinal cord interneurons, mainly expressed GAD65, displaying a GABAergic phenotype (Fig. 4.14D).

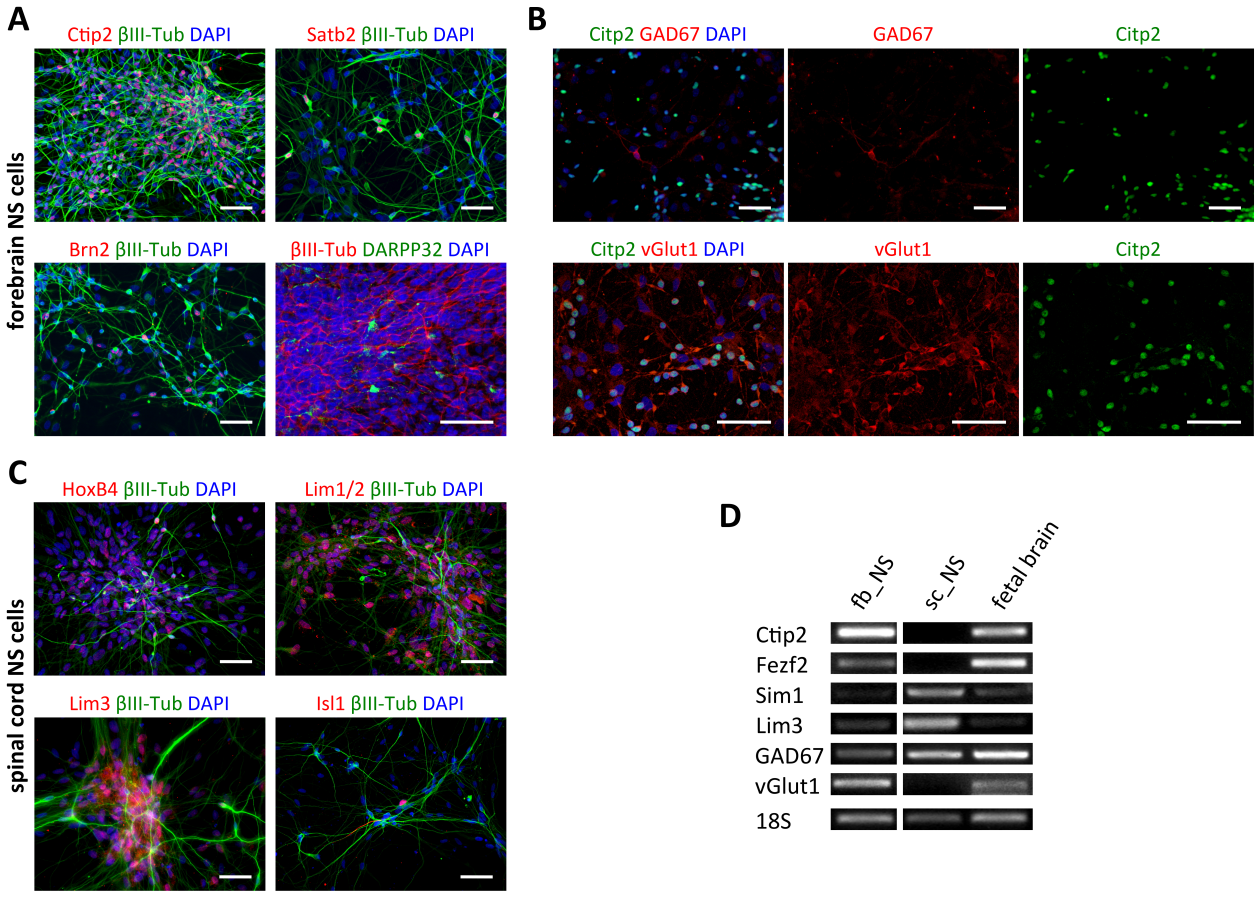


Figure 4.14: Region-specific NS cells give rise to region-specific neuronal subtypes

(A) Following growth factor withdrawal for 4 weeks, dorsal forebrain-like NS cells generated neurons with predominantly cortical phenotypes. Differentiated neurons stained positive for transcription factors typically expressed in the cortex such as Ctip2, Satb2 and Brn2. Few striatal neurons expressing DARPP32 were also present. **(B)** Following growth factor withdrawal for 6 weeks, forebrain-like NS cells generated Ctip2 and vesicular glutamate transporter 1 (vGlut1)-positive neurons. Occasionally GAD67-positive neurons could be observed. **(C)** RG-like NS cells with spinal cord identity stained positive for the posterior marker Hoxb4 as well as Lim1/2, Lim3 and occasionally Isl1, markers typical for hindbrain and spinal cord interneurons. **(D)** Using RT-PCR, cortical transcription factors such as Ctip2 or Fezf2 could be detected in differentiated forebrain-like NS cells (fb NS). In contrast, neurons from spinal cord-like NS cells (sc NS) preferentially expressed transcription factors associated with a hindbrain/spinal cord fate such as Sim1 or Lim3. The vesicular glutamate transporter 1 is expressed predominantly in differentiated cultures derived from forebrain-like NS cells whereas spinal cord-like NS cell-derived differentiated cultures primarily express GAD67 (glutamic acid decaboxylase), a GABA-producing enzyme. Scale bars: 50 µm

4.3.5 Forebrain-like NS cells generate neurons of different cortical layers in a time-dependent manner

One of the significant differences in human cortical development to other species is the exceptionally increased neocortical seize, resulting from a greater proliferative potential of human neural stem cells and the amplification of the progenitor pool through intermediate progenitor cells (Sessa et al. 2008). Whereas NES cells mostly divide symmetrically until the onset of neurogenesis, one of the characteristics of radial glia cells is their propensity for asymmetric division, generating nascent neurons directly or through intermediate progenitors (Paridaen and Huttner 2014).

In cultures of forebrain-like NS cells under proliferating conditions intermediate progenitors, identified by Tbr2, could only be detected occasionally, likely owing to the fact that the propagation through growth factors successfully overrides the natural tendency to differentiate. Following the omission of FGF2 for only 4 days, the number of Tbr2-positive intermediate progenitors significantly increased and Sox2 expression markedly decreased, suggesting that *in vitro* generated forebrain-like NS cells start to divide asymmetrically upon the initiation of differentiation (Fig. 4.15A) (Huttner and Kosodo 2005; Bultje et al. 2009).

Cortical neurons are generated in a stereotypic inside-out temporal order in which deep cortical layers appear before upper layers (Leone et al. 2008; Gaspard et al. 2008). To assess whether forebrain-like NS cells follow this sequential development of neurons, cultures were differentiated for an extended period of 60 days and the expression of deep- (Ctip2+) and upper- (Satb2+) layer markers was monitored and quantified over time. Interestingly, the results indicate that forebrain-like NS cells indeed follow the sequential order of corticogenesis with layer 5 Ctip2-positive neurons generated before layer 4 Satb2-positive neurons appear in the cultures (Fig. 4.15B, C). Importantly, the capacity of forebrain-like NS cells to give rise to cortical layer specific neurons was not influenced by the passage numbers, as shown for cells differentiated from passage 9 and 21 (Fig. 4.15D). These findings indicate that forebrain-like NS cells recapitulate major features of human cortical development, such as the generation of IP and the subsequent formation of deep-layer neuron first, followed by upper-layer neurons.

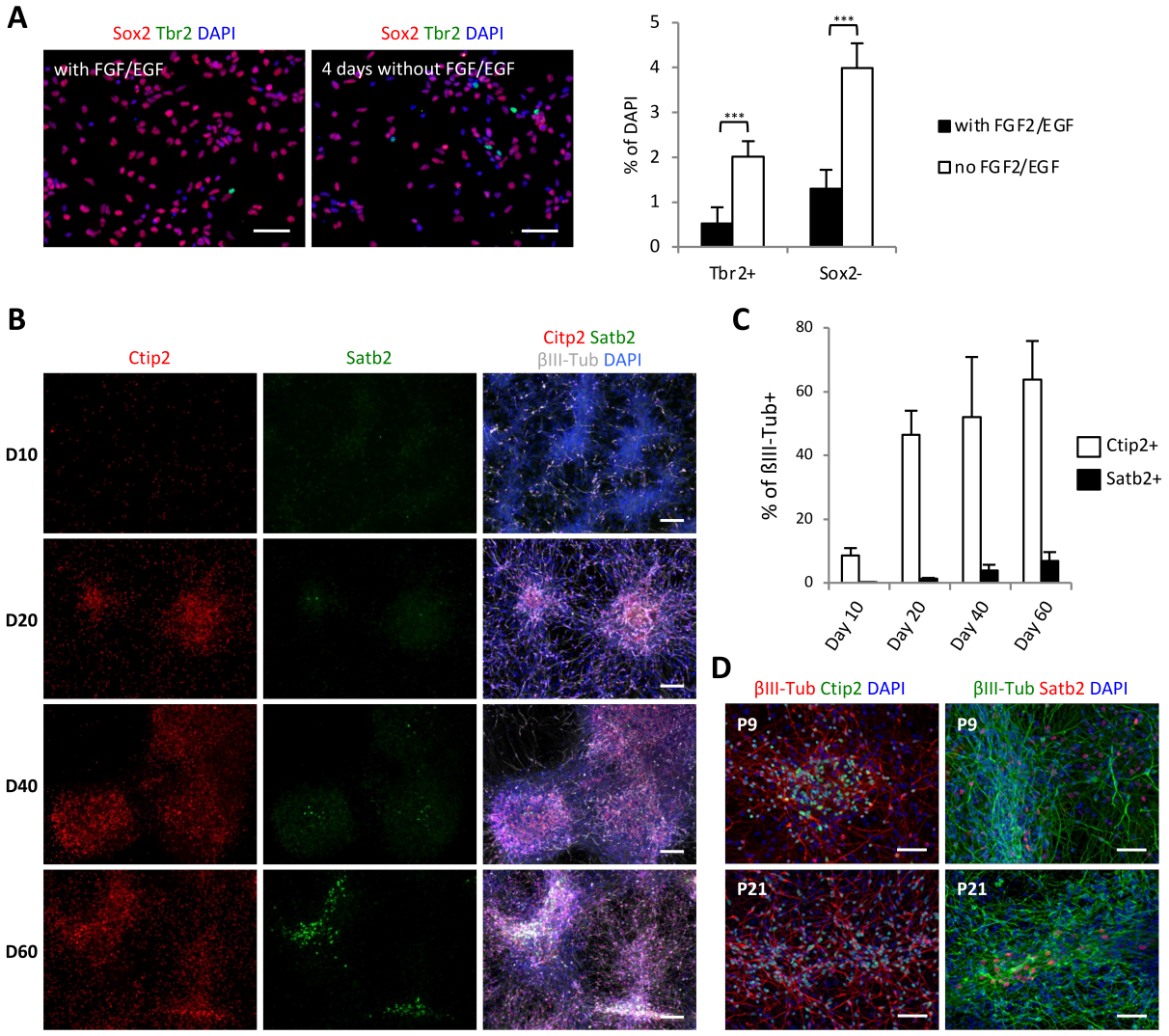


Figure 4.15: Forebrain-like NS cells follow a stereotypic temporal order of cortical neurogenesis.

(**A**) Undifferentiated forebrain-like NS cells (cultured with FGF/EGF) are largely negative for the intermediate progenitor marker Tbr2. 4 days following growth factor withdrawal a significant increase in Tbr2-positive / Sox2-negative cells are detectable. (**B**) Forebrain-like NS cells co-stained for the neuronal marker βIII-tubulin, as well as a deep (Ctip2+) and upper (Satb2+) cortical layer specific transcription factor 10-, 20-, 40- and 60-days following growth factor withdrawal with deep layer Ctip2-positve neurons generated before upper-layer Satb2-positive neurons. **(C)** Quantification of deep (Citp2+) and upper-(Satb2+) layer neurons across 60 days of *in vitro* differentiation. **(D)** Passage 9 and passage 21 forebrain-like NS cells differentiated for 7 weeks give rise to comparable amounts of Ctip2- and Satb2-positive βIII-tubulin expressing neurons. Scale bars: 100 µm; Error bars: ± SD; ***p< 0.001

4.3.6 Forebrain-like NS cells can be specified along the dorsal-ventral axis

As the previous findings could demonstrate that RG-like NS cells can be specified in their regional identity along the anterior-posterior axis, it was consequentially important to investigate if the same holds true for the dorsal-ventral axis of the developing brain. To address this idea, early forebrain rosettes which are dorsally patterned by default (Shi et al. 2012a), were ventralized through the exposure to the sonic hedgehog (Shh) pathway activators purmorphamine and SAG for 10 days (Maroof et al. 2013) (Fig. 4.16A). Subsequent to this treatment, the cells were analyzed for the expression of the forebrain marker Foxg1 as well as for the ventral forebrain-associated transcription factor Nkx2.1. Immunohistochemical staining confirmed, that ventralized forebrain NES cells continue to express Foxg1, however, in contrast to the default patterned forebrain NES they gained the marker Nkx2.1, which was now homogeneously expressed. Additionally, they express the neural stem cell marker Nestin as well as the rosette markers PLZF, and ZO1 (Fig. 4.16B).

Ventral forebrain-like NES cells were then subjected to the established protocol and the resulting RG-like NS cell population analyzed. As a first step, the cells were analyzed for the expression of the transcription factors Sox2 and Sox9, confirming their radial glia-like identity. Next, to characterize their regional phenotype, the RG-like NS cells were stained for Foxg1 and Nkx2.1, which they continued to express confirming their ventral forebrain identity. Their phenotype was further supported by the fact that the dorsal marker Pax6, which is expressed in default patterned forebrain-like NES, could no longer be detected (Fig. 4.16.C).

Upon growth factor withdrawal, the cells were able to differentiate into βIII-tubulin-positive neurons and GFAP-positive astrocytes. Interestingly, ventral forebrain-like NS cells were also able to generate region-specific neurons. In line with their regional phenotype, the majority of generated neurons appeared GABAergic, with some cells also staining positive for calbindin and parvalbumin (Fig. 4.16D). These two calcium-binding proteins are expressed *in vivo* in mostly nonoverlapping cortical interneuron populations originating in the medial and lateral ganglionic eminences (DeFelipe 1997; Lim et al. 2018; Liu et al. 2004). Taken together, these results confirm the additional ability of RG-like NS cells to conserve their identity along the dorsal-ventral axis of the forebrain.

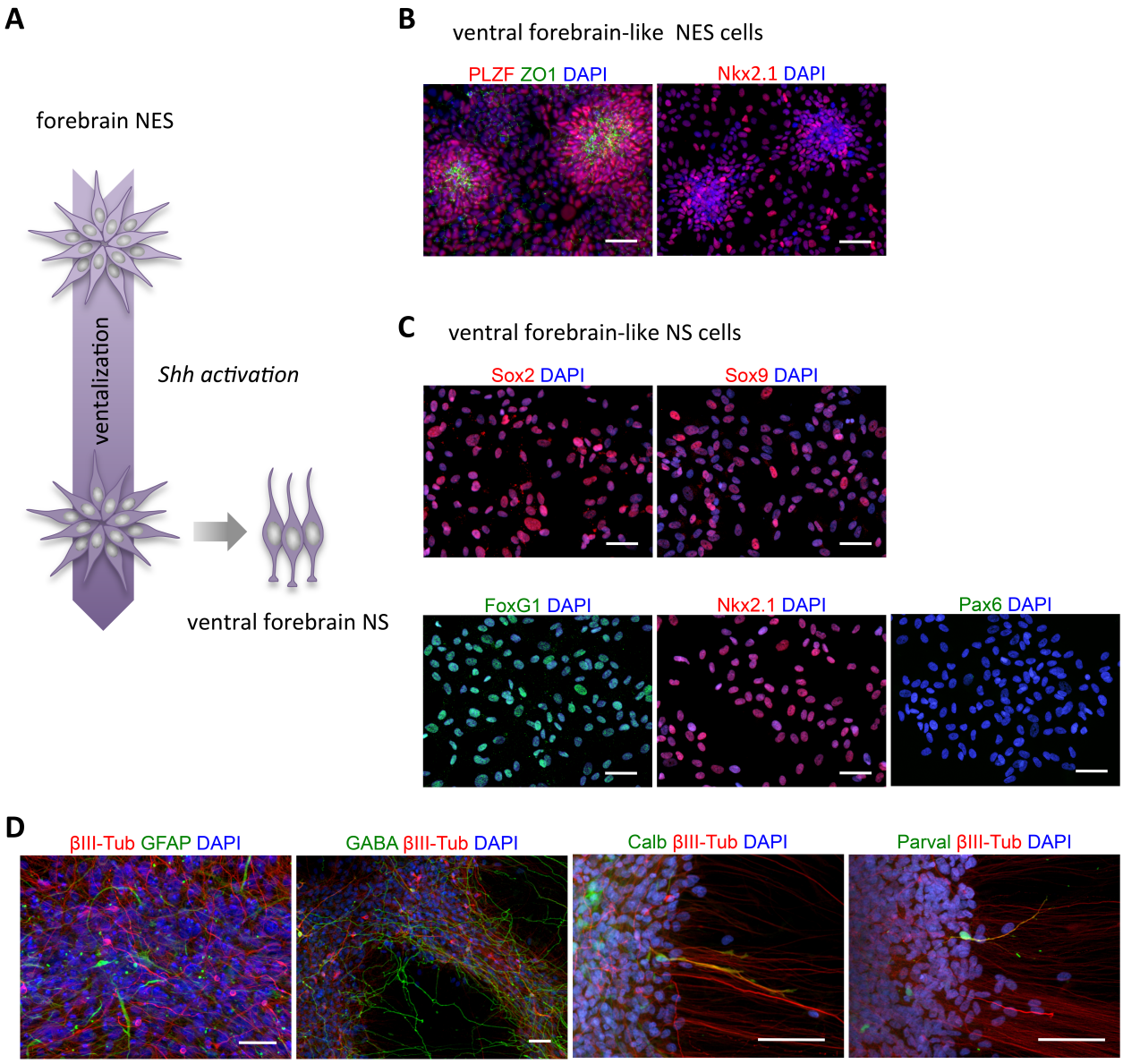


Figure 4.16: RG-like NS cells generated from ventralized forebrain NES cells.

(A) Experimental outline: To explore whether RG-like NS cells can be generated from ventralized NES cells, temporary growth factor withdrawal was applied to early NES cells shifted through Shh activation into a ventral forebrain-like identity. **(B)** Ventralized forebrain-like NES cells form PLZF-positive rosettes with central ZO1 localization. They homogeneously expressed the ventral progenitor marker Nkx2.1. **(C)** RG-like NS cell generated from ventralized forebrain-like NES cells exhibited homogeneous expression of Sox2 and Sox9. Ventral forebrain-like NS cells also stained positive for the forebrain marker FoxG1 and the ventral progenitor marker Nkx2.1 while lacking expression of the dorsal progenitor marker Pax6. **(D)** Ventral forebrain-like NS cells differentiated into βIII-tubulin-positive neurons as well as GFAP-positive astrocytes following growth factor withdrawal. βIII-tubulin-positive neurons largely co-expressed GABA. Inhibitory neurons, which stained positive for calbindin (Calb) or parvalbumin (Parval), could also be detected. Scale bars: 50 µm

4.3.7 Transcriptome analysis of PSC- and fetal tissue-derived neural stem cell populations at different developmental stages

In order to investigate the relationships between the neural stem cell populations used or generated in this study in more detail, microarray gene expression profiling of the different NSC classes was performed. This analysis included human PSC-derived NES cells and primary fetal CNS-derived NES cells (hbNES), as well as RG-like NS cells generated from NES with different regional identities, primary NS cells with different regional identities and primary NES cell-derived NS cells (Sun et al. 2008; Tailor et al. 2013). A complete linkage hierarchical clustering based on the 3541 transcripts previously identified to be differentially expressed in NES cells vs. NS cells was carried out and represented in a heatmap. This analysis showed that radial glia-like neural stem cells, independent of their origin (derived from different ES cell lines or brain tissue) and regional identity (forebrain, hindbrain or spinal cord) cluster together and are clearly separated from NES cells of different origins (Fig. 4.17).

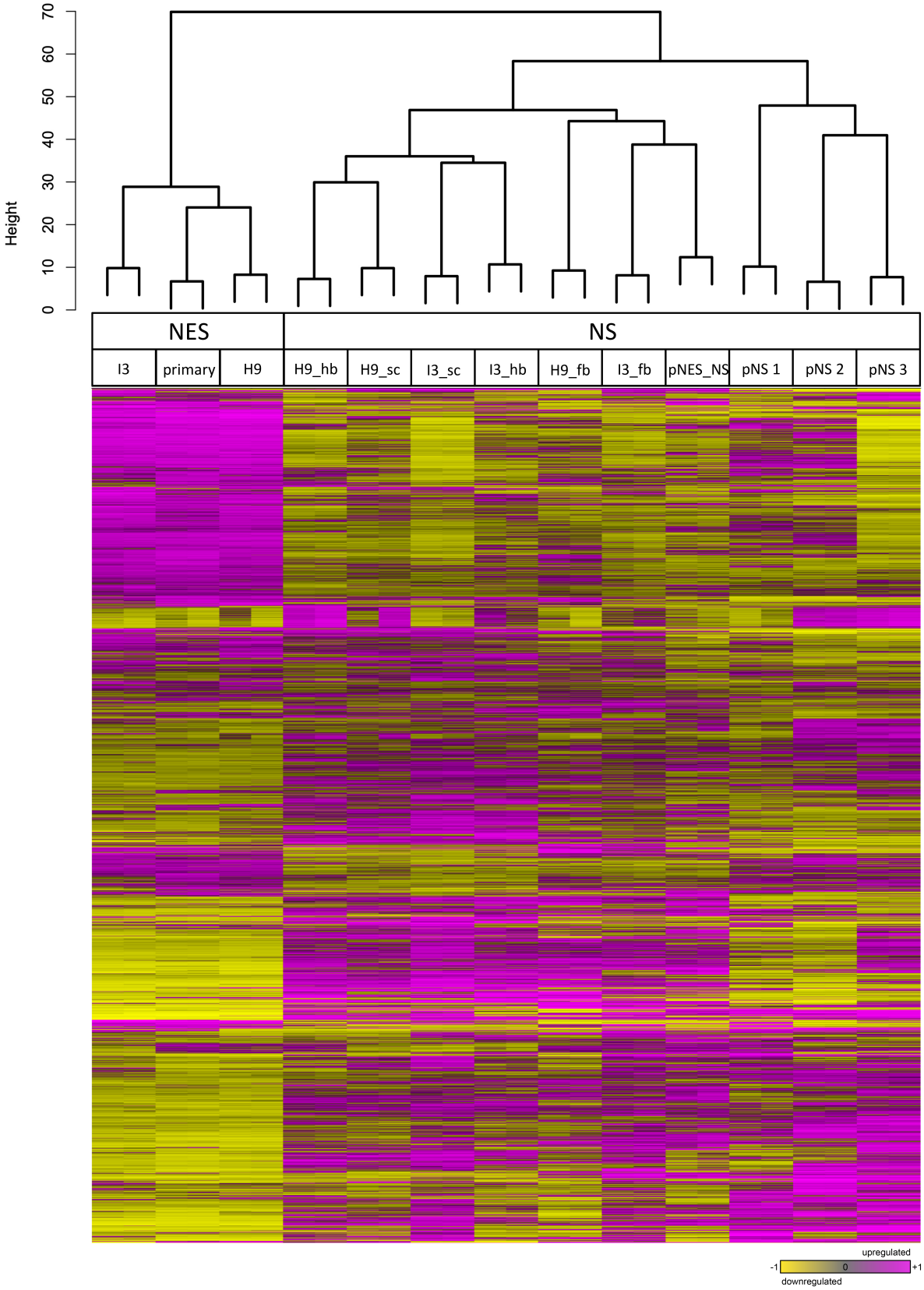


Figure 4.17: MicroArray analysis of NES cells and RG-like NS cells derived from hESC and primary tissue.

Hierarchical clustering and heat map performed with MicroArray data from NES cells (primary or hESC-derived (I3 and H9.2 ESCs) and regionally distinct RG-like NS cells derived either from NES cells or human fetal tissue (3 independent lines: pNS1, 2 and 3). The heat map is generated with the approximately 3600 genes identified initially as being differentially regulated between NES and RG-like NS cells. RG-like NS-like cells cluster together independently of their origin and clearly segregate from NES-type cells. Transcriptome data analysis was performed in cooperation with Franz-Josef Müller (University of Kiel). Fb: forebrain; hb: hindbrain; sc: spinal cord; primary NES: pNES; primary NS: pNS

5 Discussion

5.1 Recapitulation of the NES to RG cell transition *in vitro*

In the first part of this study, NES-like cell populations generated from PSCs and believed to correspond to NES cells found *in vivo* (lt-NES), were used to generate a later and more defined neural stem cell population. This radial glia-like neural stem cell can be propagated for multiple passages while retaining the ability to differentiate into neurons and glia. Importantly, detailed characterization of this new population revealed a strong resemblance to radial glia cells isolated directly form fetal tissue while a similar RG-like NS cell population can be generated from primary hindbrain derived NES cells (hbNES) (Tailor et al. 2013).

In light of the scarce availability of viable human brain tissue, neural stem cells generated from pluripotent stem cells represent an exceptional tool for the study of human neural development as well as disease modeling and possible cell replacement therapies (Scheffler et al. 2006; Suzuki et al. 2015; Brennand et al. 2015; Koch et al. 2009a). Many different systems for the generation of neural stem cells exist to date, making the comparison of results between different studies challenging. In addition, the relationship of these long-term *in vitro* cultured populations to *in vivo* existing transient neural stem cell populations is not fully understood and the influence of an artificial *in vitro* environment not always taken into consideration (Conti et al. 2010).

Therefore, it needs to be carefully considered to which extend developmental steps *in vivo* can be recapitulated *in vitro* using the protocol established in this work.

5.1.1 Evidence for the successful transformation of NES cells into RG-like NS cells *in vitro*

During neurogenesis *in vivo* the generation of different neuronal and glial subtypes occurs via transient neural stem cell populations, which over development become more and more fate restricted. The ability to culture hPSCs and direct them to a neuronal fate opens the exciting possibility to follow this developmental process *in vitro*.

Following this advance, different protocols have been developed over the years to capture transiently occurring neural stem cells during the neural induction of PSCs (Reinhardt et al. 2013; Conti et al. 2005; Li et al. 2011a; Koch et al. 2009b).

However, the fidelity and physiological relevance of neural stem cells cultured outside of their *in vivo* niche in an artificial *in vitro* environment is still under debate and requires further investigation. An important step in this process is to show that *in vitro* cultured neural stem cells retain the ability to follow the natural course of neurogenesis in reaction to appropriate inductive cues.

In this project, lt-NES cells generated from PSCs and believed to correspond to the neural tube stage of development, were applied to investigate the capacity of *in vitro* cultured neural stem cells to follow the progression of neural stem cells *in vivo*. This part specifically explored the ability of lt-NES cells to transition from an earlier neuroepithelial stem cell stage to a more defined radial glia stem cell stage.

Indeed, a cell population with properties of radial glia cells could be retrieved and proliferated from differentiating lt-NES cell cultures through the subsequent reintroduction of growth factors. Consistent with radial glia cells *in vivo*, RG-like NS cells are characterized by the expression of neural stem cell markers (Sox2, Nestin Pax6 and CD133), which are shared with lt-NES cells. Ablation of Sox2 specifically in NS cells (RG-like NS cells generated from mice) is reported to cause loss of proliferative capacity as well as progenitor cell identity through the downregulation of radial glia markers (BLBP and Nestin) (Conti et al. 2005; Gómez-López et al. 2011). Similarly, loss of Pax6 in NS cells led to major proliferative defects, changes in cell morphology from bipolar to multipolar and deficits in gliogenesis, showing that both transcription factors are essential not only stem cell but also for radial glia cell identity.

In addition to newly expressing radial glia markers, important rosette stage associated markers (PLZF and DACH1) are downregulate in RG-like NS cells, indicating that the transition to a later radial glia stage cell type was successfully induced (Elkabetz et al. 2008; Castiglioni et al. 2018).

Radial glia associated markers up-regulated in RG-like NS cells include BLBP and GLAST (Hartfuss et al. 2001; Malatesta et al. 2000; Pérez-Álvarez et al. 2008; Shibata et al. 1997). These proteins together with several other glial markers are expressed from radial glia through to astrocytes during development and speak to the glial characteristics of radial

glia cells (Kriegstein et al. 2009). BLBP (also known as FABP7), a member of the intracellular lipid-binding protein family, is found in the cytoplasm and nucleus of radial glia *in vivo* during development (Feng et al., 1994). It is required for the establishment of the radial glial fiber system along which new born neurons migrate to their accurate cortical layers. FABP7-deficient mice show traits associated with schizophrenia while FABP7 polymorphisms in humans are associated with schizophrenia and autism spectrum disorders (Watanabe et al. 2007a; Iwayama et al. 2010; Maekawa et al. 2010). Radial glia cells also specifically upregulate proteins associated with the extracellular matrix. This includes CD44, a trans-membrane adhesion molecule, as well as vimentin, an intermediate filament. The monoclonal antibody 3CB2 used in this study recognizes three vimentin isoforms and has first been described to mark radial glia in the chick embryo (Pérez-Álvarez et al. 2008; Prada et al. 1995).

One important aspect of the progression of neuroepithelial to radial glia cells is the concomitant down-regulation of some epithelial features such as tight junction formation (Aaku-Saraste et al. 1996; Aaku-Saraste et al. 1997). Lt-NES cells show strong polarity with the adhesion molecules ZO1 together with N-cadherin and β-catenin localized at the apical pole at the center of the rosette (Koch et al. 2009b). In the absence of tight junctions *in vivo* ZO1 is re-localized to adhesion junctions at the apical pole, which is connected to the ventricle (Aaku-Saraste et al. 1996). It is important to note that RG-like NS cells in this study, similar to previous *in vitro* cultured NS cells, even though they continue to express adhesion proteins and tight junction-associated markers, only displayed a clear polar distribution as described *in vivo* in a subset of cells and grow at low density in reticulate not in rosette structures (Fietz and Huttner 2011; Higginbotham et al. 2013; Rasin et al. 2007; Pollard and Conti 2007; Pinto et al. 2007). This could be owed to the fact that important structures like the basal lamina or amniotic cavity are missing in *in vitro* culture. To investigate this feature further, RG-like NS cells could be analyzed after transplantation *in vivo* e.g. into the developing mouse brain or given a biomaterial structure *in vitro* to support radial alignment.

In this study the sequential differentiation protocol was applied to lt-NES cell from different sources including ESCs (I3 and H9) and iPS cells resulting in the generation of corresponding RG-like NS cell lines. This reproducibility supports the robustness of the protocol and opens the possibility for the generation of disease-specific iPSC derived RG-

like NS cells. Intriguingly, RG-like NS cells could also be isolated from NES cell cultures that had been differentiated for extended times of up to 6 months, which possibly reflects the prolonged development and gestational period in humans compared to other mammals (Van den Ameele et al. 2014).

When comparing these results to previous studies in which radial glia-like cell types have been derived from mouse and human pluripotent stem cells important similarities but also marked differences come to light. 'NS cells' were derived by Conti et al. in 2005 from mouse ESCs through neural induction in serum-free adherend monoculture and subsequently replated after 7 days in basal neuronal medium with FGF2 and EGF. They express Pax6, Glast, and BLBP transcripts and are homogenously positive for nestin, Sox2, vimentin, 3CB2, and CD133 (Prominin). In addition, their bipolar morphology and lattice-like growth strongly resembles RG-like NS cells. Interestingly, NS cells generated from mouse ESCs do not express a conclusive regional code corresponding to a specific brain area (Conti et al. 2005). This could be owed to the fact that the induction protocol does not include a developmentally relevant set of regionalizing cues, resulting in an un-physiological combination of regional transcription factors. Any potential for regional patterning was not addressed in this study (Conti et al. 2005). Elkabetz et al. described in 2008 an early rosette NES cell population (R-NSCs), which has a broad differentiation potential towards PNS and anterior CNS fates but, in contrast to lt-NES cells is not stably expandable in the presence of FGF2 and EGF. Under these conditions, R-NSCs undergo a similar NES to RG-like cell transition to so called $NSC^{FGF/EGF}$. This later cell population shares many glia-specific transcripts such as S100B, PMP2, HOP, AQP4 and SPARCL1 with RG-like NS cells. Likewise, the rosette specific transcripts PLZF, DACH1 and PLAGL are down-regulated and a polar expression of ZO1 is not discernable. NSC markers shared between R-NSCs and $NSC^{FGF/EGF}$ are also present in lt-NES and RG-like NS cells. Upon exposure of R-NSCs to FGF2 and EGF and subsequent conversion to $NSC^{FGF/EGF}$ the expression of FOXG1 (BF1) is markedly reduced and forebrain identity is lost. The resulting regional identity is not further taken into consideration (Elkabetz et al. 2008).

Taken together, the possibility to derive RG-like cell lines, which show a remarkably similar morphology and radial glia marker expression, through different methods *in vitro* supports the notion that this population could actually recapitulate aspects of an *in vivo* existing developmental program. What other models, however, fail to recapitulate is a coherent

regional identity corresponding to distinct brain regions and the subsequent generation of region-specific neuronal subtypes.

In vivo the transition of NES cells to RG cells signifies the switch from early progenitor expansion through symmetric proliferative divisions to the beginning of neurogenesis through asymmetric divisions or symmetric differentiating divisions. Indeed, it would be beneficial to have a model of this decisive step in development. The correct timing and sequence of this transition has a significant influence on the size of the progenitor pool and the number of neurons generated. Deregulation of this process has therefore severe consequences and may lead to developmental defects such as micro- or macrocephaly (Homem et al. 2015).

The molecular mechanisms controlling this transition are currently under investigation and are vital in understanding how the timing of development is regulated and cell numbers determined in different brain regions. The morphogen FGF10 for example has been shown to be transiently expressed in rostral cortical NSCs during the transition from NES to RG cells. In mice deletion of FGF10 delays RG cell generation through enhancement of symmetric divisions in NES cells and increases the stem cell pool. Later on, this consequently leads to the overproduction of neurons in the rostral cortex whereas overexpression of FGF10 has the opposite effect causing premature differentiation and a reduction in neuron production (Sahara and O'Leary 2009). Similarly Retinoic acid (RA) seems to play a role in the shift of division mode in neural progenitor cells. In FOXC1 mutant mice the formation of forebrain meninges, a source of RA, is disrupted leading to prolonged symmetric divisions and reduction in neuron production. This cortical phenotype could be rescued by in utero RA treatment (Siegenthaler et al. 2009). Another important cell intrinsic factor that influences the switch from symmetric to asymmetric divisions and ultimately brain seize is thought to be cell cycle length (Borrell et al. 2014). The underlying idea of the ‘cell-cycle length hypothesis’ is that a longer G1 phase allows for the accumulation and distribution of fate-determining factors (Calegari and Huttner 2003). Two studies could show that in the brain increasing the cell cycle length by overexpressing G1 regulators such as Cdk4/cyclinD1 or cyclins alone triggers premature neurogenesis (Lange et al. 2009; Pilaz et al. 2016). An increase in cell cycle length, particularly the G1 phase, coincides with the transition from symmetrically dividing NES

to asymmetrically dividing RG cells. Interestingly, in this study an increase in doubling time from approximately 38 to 96 hours can likewise be observed following the transition from lt-NES to RG-like NS cells without, however, resulting in accompanying increase in differentiation, most likely due to the continued growth factor exposure.
Taken together, extrinsic factors as well as cell autonomous mechanisms and epigenetic programs are considered to influence the NES to RG cell transition. In order to further elucidate the mechanisms involved, RG-like NS cells could serve as a human specific model to recapitulate this process.

5.1.2 Self-renewal and differentiation of RG-like NS cells

NSCs *in vitro* are characterized through the ability to self-renew and generate the main cell types of the human brain, neurons, astrocytes and oligodendrocytes.
In vivo NES cells divide symmetrically to expand the stem cell pool while RG cells during neurogenesis mainly show asymmetric divisions and divide more slowly. Corresponding to the phases of neurodevelopment *in vivo*, lt-NES cells have a much higher proliferation rate compared to RG-like NS cells (Gotz et al. 2005; Matsuzaki et al. 2015). However, *in vivo* RG cells give rise to differentiated cells mainly through asymmetric divisions, which should also diminish the progenitor population *in vitro*. FGF2 is well known to support self-renewal of neural stem cells *in vitro* and *in vivo* (Svendsen et al. 1996; Vaccarino et al. 1999). Recently, a study on cultured mouse RG cells could show that *in vitro* FGF2 supports self-renewal of RG cells by three different mechanisms: increasing the growth fraction through inducing cell-cycle reentry of quiescent cells, promoting symmetric proliferative divisions and increasing the proliferation rate by shortening the cell-cycle (Ledesma-Terron et al. 2020). These three effects enable the propagation and expansion of radial glia *in vitro*. The interplay of FGF2 and EGF in promoting self-renewal and inhibiting differentiation in RG cell cultures could be elucidated in future studies.
Under FGF2 and EGF treatment, RG-like NS cells are able to homogeneously proliferate for more than 30 passages without accompanying differentiation. This could, however, not be sustained by FGF2 alone as previously described for some NSC populations (Pollard et al. 2006; Tropepe et al. 1999). In addition, the cells could be reliably recovered following several months of cryopreservation.

Upon sequential withdrawal of EGF followed by FGF2, RG-like NS cells were able to consistently generate neurons positive for βIII-tubulin, MAP2ab and NeuN with no significant decline after 25 passages. Neurons were produced at a rate of about 30% compared to 40% GFAP-positive astrocytes consistent with the glial bias of later stage NSCs. Generation of oligodendrocytes could not be observed under neurogenic culture conditions. However, RG-like NS populations were able to generate oligodendrocytes following appropriate inductive clues. The higher number of astrocytes early on in *in vitro* culture could be beneficial for the overall survival and long-term culture of neurons and allows for easily attainable co-culture conditions (Johnson et al. 2007; Roy et al. 2006). Encouragingly, neurons generated by RG-like NS cells displayed electrophysiological properties in line with functional maturation including the generation of action potentials and spontaneous postsynaptic currents consistent with functional synapses. Under exposure to serum, RG-like NS cells rapidly adopted astrocyte morphology and became strongly positive for GFAP in line with the response of NS cells generated by Conti et al. (Conti et al. 2005). In order to demonstrate that RG-like NS cells indeed exhibit tripotent differentiation potential at single cell, rather than population level, clonal analysis would be required. However, studies of radial glia isolated from primary brain tissue could show that *in vivo* only a subset of cells are tripotent with some clones generating only astrocytes or neurons (Malatesta et al. 2000; Malatesta et al. 2003; McCarthy et al. 2001). Importantly, during development the potency of radial glia follows a timeline dependent progression from neuro- to gliogenesis.

Based on the combination of radial glia morphology and marker expression together with the ability to generate functional neurons and glia, the PSC-derived population generated in this study was defined as ‘radial glia-like’ neural stem cells. It is important, however, to appreciate the lack of some other hallmarks of physiological radial glia such as polar morphology with apical adherens junctions and asymmetric division. It is conceivable that these discrepancies are caused by the continuous exposure to growth factors as well as the lack of anatomical structures *in vitro*. Future experiments *in vivo* are needed to investigate the characteristics of RG-like NS cells in a physiological environment.

5.1.3 Reflection of distinct neurodevelopmental stages in the expression profile of *in vitro* cultured NES and RG-like NS cells

In a global gene expression analysis a detailed comparison of lt-NES versus RG-like NS cells confirmed the transition from an earlier neuroepithelial-like to a later radial glia-like expression profile. Encouragingly, a first analysis of their Pluripotency score already places lt-NES cells closer to an early, rather undifferentiated state, while RG-like NS cells are characterized as more differentiated (Müller et al. 2011).

In total, 1700 transcripts could be identified as significantly up-regulated in lt-NES cells compared to RG-like NS cells. Among them, some of the rosette-associated genes already detected in immunocytochemistry and/or PCR analysis, such as MMRN1, PLZF and NR2F1, could be confirmed. Interestingly, a subsequent overall interpretation of the 1700 transcripts specific for lt-NES cells by gene ontology annotation revealed several processes associated with cell cycle regulation and embryogenesis. When looking specifically at the top 20 genes significantly up-regulated in lt-NES cells, a combination of well-documented cell cycle-associated genes including ID3, LIN28 and NMYC could be identified (Ruzinova and Benezra 2003; Cimadamore et al. 2013; Knoepfler 2002). These results are in line with the rapid expansion of the stem cell pool by NES cells *in vivo* and the higher proliferation rate of lt-NES cells. In addition, important transcriptional regulators of early developmental programs in mammalian embryogenesis such as ZIC2 or ZFHX3 (also known as ATBF1) are up-regulated in lt-NES cells (Nagai et al. 2000; Jung 2005). In line with the developmental stage of lt-NES cells, ZIC2 plays an important role regulating the kinetics of neurulation while mutations in the human ZIC2 gene can cause severe neural tube defects such as spina bifida and holoprosencephaly (Brown et al. 1998; Ishiguro et al. 2018).

Genes shared between both populations include neural stem cell markers SOX2, NESTIN and ASCL1 as well as CITED2, a transcriptional co-regulator broadly expressed by progenitors in the SVZ and implicated in the generation of callosal projection neurons as well as the self-renewal and cell fate decisions of ESCs (Fame et al. 2016; Kranc 2015; Pacheco-Leyva et al. 2016).

In RG-like NS cells 1900 genes were found to be differentially up-regulated compared to lt-NES cells, which include the characteristic radial glia markers SOX9, BLBP, SPARCL1,

CD44, GLAST, HOPX and GFAP. Theses transcripts were previously identified in studies involving *in vivo* as well as *in vitro* cultured human radial glia cells (Hartfuss et al. 2001; Elkabetz et al. 2008; Sun et al. 2008; Araque and Navarrete 2010; Shibata et al. 1997; Anthony et al. 2004). Especially the astrocyte marker GFAP is selectively up-regulated in humans and is not found in rodent radial glia cells (Malatesta et al. 2000; Campbell and Götz 2002; Sun et al. 2008; Kriegstein et al. 2009). In accordance with the role of radial glia cells in the developing brain, gene ontology analysis of the 1900 transcripts specific for RG-like NS cells highlighted several terms associated with brain, glial or neuronal morphology and synaptic transmission. Among the top 20 up-regulated genes identified in RG-like NS cells were characteristic radial glia markers including tenascin C (TNC), CD44, NFIB and NFIX. Several studies have identified the nuclear factor one (NFI) gene family as important regulators of CNS development involved in processes such as axon-guidance and outgrowth as well as neuron and glia differentiation (Betancourt et al. 2014; Matuzelski et al. 2017; Fraser et al. 2019; Deneen et al. 2006). Interestingly, overexpression of NFIA as well as NFIB in combination with SOX9 in human PSCs has recently been shown to generate a homogeneous population of mature astrocytes (Tchieu et al. 2019; Canals et al. 2018).

An important theme highlighted in the expression profile of RG-like NS cells is the upregulation of several extracellular matrix-associated (ECM) transcripts. Tenascin C (TNC), an extracellular matrix protein characteristic for radial glia cells, is playing an important regulatory role in NSC self-renewal and differentiation and is implicated in the guidance of migrating neurons. In addition, it has the ability to bind morphogens like FGF2 and can stimulate EGF receptor expression (Garcion et al. 2004; De Laporte et al. 2013). TNC persists in the adult brain and is also an essential component of the adult neurogenic niche. CD44, a transmembrane receptor for the glycosaminoglycan hyaluronan (HA), is highly expressed in radial glia (Su et al. 2017; Goodison et al. 1999) . Other transcripts among the top 20 genes connected to cell-matrix interactions include LGALS1, a soluble carbohydrate-binding protein which is implicated in cell-cell and cell-matrix interactions and NSC proliferation (Sakaguchi et al. 2007), IGFBP7, an insulin-like growth factor receptor (Hwa et al. 1999; Oh et al. 1996; Pen et al. 2008), transgelin (TAGLN), an actin cross-linking protein (Kronenberg et al. 2010; Boldog et al. 2018), CAV1, a scaffolding protein associated with neural progenitor cell fate decision (Li et al. 2011b; Grande-García

and del Pozo 2008), Annexin 2 (ANAX2), a membrane-associated calcium-dependent phospholipid-binding protein which complexes to actin (Reeves et al. 1992; Tatenhorst et al. 2006), or GAP43, a protein highly expressed in neuronal growth cones (Kawasaki et al. 2018; Holahan 2017).

This prominent expression of ECM-related transcripts in RG-like NS cells is especially interesting as interactions between the ECM and radial glia cells are thought to be essential for their sustained proliferation and self-renewal *in vivo*. ECM constitutes and their receptors are vital compounds of the neural stem cell niche, creating a microenvironment supportive of self-renewal. Major functions of the EMC include controlling the local concentration of growth factors, morphogens and cytokines and supporting cell morphology and migration (Faissner and Reinhard 2015). Recent studies examining differences in gene expression underlying the increased proliferation capacity of human compared to mouse neural stem cells have indicated a cell-autonomous production of ECM components by NSCs and especially basal progenitors such as outer radial glia cells (oRG cells). Fietz et al. isolated specific germinal zones of the mouse and human cortex and by comparing their expression profile showed an upregulation of EMC production in the inner and outer SVZ of humans in contrast to the mouse SVZ (Fietz et al. 2012). Transcriptome analysis of single cells from the human fetal neocortex found a prominent upregulation of ECM compounds in oRG cells (Thomsen et al. 2016; Pollen et al. 2015). Since oRG delaminate from the ventricular surface, they lack contact to the apical lamina and access to stimulating factors from the cerebral spinal fluid. The evolutionary increase in proliferative capacity of basal progenitor such as oRG might therefore in part be due to their ability to create their own EMC supported niche. These transcriptome studies were also able to identify genes preferentially expressed in oRG compared to vRG, including TNC, PTPRZ1, FAM107A, LIRF as well as MOXD1 and HOPX, some of which relate to the ECM. Interestingly, TNC, HOPX and MOXD1 are also among the transcripts specifically up-regulated in RG-like NS cells. In order to examine the significance of this finding, a direct comparison with defined human oRG and vRG cells would be ideal to determine how RG-like NS cell relate to these diverse radial glia populations present *in vivo*.

Taken together, this specific gene expression profile of RG-like NS cells correlates to the developmental role of native radial glia cells during neurogenesis, which are central to the

establishment of the complex histoarchitecture of the developing brain through cell-cell interactions such as radial neuronal migration and close cell-matrix interactions within their supportive niche.

5.1.4 Connecting *in vitro* cultured neural stem cells to their *in vivo* counterpart

Stable *in vitro* cultured neural stem cell populations represent an attractive opportunity to model basic cellular and molecular processes in development. Various strategies have been applied to derive neural progenitors from different sources and to propagate them *in vitro*. However, the *in vitro* environment is distinctly different from their natural *in vivo* niche and therefor the authenticity and physiological relevance of *in vitro* propagated NSCs need to be closely investigated (Conti et al. 2010).

Neural stem cells isolated from human fetal tissue at different time points reflect the progression of neural progenitors *in vivo* from neuroepithelial to radial glia to adult neural stem cells. Cells isolated at Carnegie stage 15–17 show NES cell properties while those at Carnegie stage 18–23 have radial glia characteristics (Sun et al. 2008; Tailor et al. 2013).

In order to examine the relationship of PSC-derived radial glia-like NS cells to endogenous neural stem cells, a detailed comparison with primary human fetal derived NS cells was carried out. Primary NS cells, kindly provided by the Austin Smith Laboratory, are established from human neural tissue at Carnegie stages 18 to 23 (day 44-56) after the transition of NES to radial glia cells has occurred *in vivo*. Intriguingly, RG-like NS cells closely resemble primary NS cells in morphology with bipolar cells growing in lattice-like patterns. A direct comparison of their marker expression profiles confirmed the shared homogeneous expression of neural stem cell and radial glia markers. Corresponding to RG-like NS cells, primary derived NS cells showed downregulation of rosette specific transcripts and do not exhibit polarized expression of ZO1.

The similarities between NES-derived RG-like NS cells and primary NS cells suggest that pluripotent stem cell-derived NS cells reflect the phenotype of native human radial glia-like NS cells rather than representing a cell culture artifact (Fig 5.1).

Important to consider, however, is the influence of long-term growth factor expansion, which might result in the general acquisition of new properties of *in vitro* cultured cell

populations. In particular exposure to FGF2 and EGF, growth factors commonly applied in neural stem cell cultures, reportedly alters the expression of several genes in neural progenitor cells (Pollard et al. 2008; Doetsch et al. 2002; Bithell et al. 2008). This also includes the radial glia surface marker CD44, which is solely present in primary and RG-like NS cells but does not appear to get induced at the lt-NES cell stage. Interestingly, under the same culture conditions, lt-NES cells and RG-like NS cells together with primary NS cells show a distinct and homogeneous marker profile characteristic of the NES cell and radial glia cell stage *in vivo,* respectively.

Tailor et al. in 2013 were able to derive primary neuroepithelial stem cells from the anterior hindbrain of Carnegie stage 15–17 (week 5–7) human embryos (hbNES cells). This population forms rosettes and can be propagated for over 50 passages in identical medium used to culture PSC-derived lt-NES cells. HbNES and lt-NES cells showed a surprisingly similar marker profile including neural stem cell and rosette marker expression and clustered together in global transcriptome analysis and apart from primary hindbrain-derived NS cells (Tailor et al. 2013). Interestingly, hbNES cells retained an anterior hindbrain regional marker profile under long-term growth factor expansion, which is comparable to primary anterior hindbrain tissue. This regional marker profile is also shared with lt-NES cells, even though lt-NES cells initially pass an anterior phenotype and are regionalized through *in vitro* culture conditions. This data suggests that PSC-derived lt-NES cells correlate to primary hbNES cells and represent a physiologically relevant NSC population.

Consequently, hbNES cells are able give rise to RG-like NS cells following sequential differentiation and proliferation. Primary hbNES-derived RG-like NS cells are indistinguishable from lt-NES-derived populations in morphology and marker expression and give rise to neurons and glia. Therefore, RG-like NS cells derived from different sources (primary tissue and PSC-derived RG-like NS cells) show similar RG-like characteristics when cultured under the same conditions. Taken together, these findings suggest that neural stem cell populations can be generated and propagated *in vitro*, which follow the developmental progression of transient neural stem cells *in vivo* (Fig 5.1). However, this does not necessarily allow the conclusion that *in vitro* cultured cells faithful recapitulate all aspects of bona fide neural progenitor cells as important aspects of the *in vivo* tissue context are missing in this model.

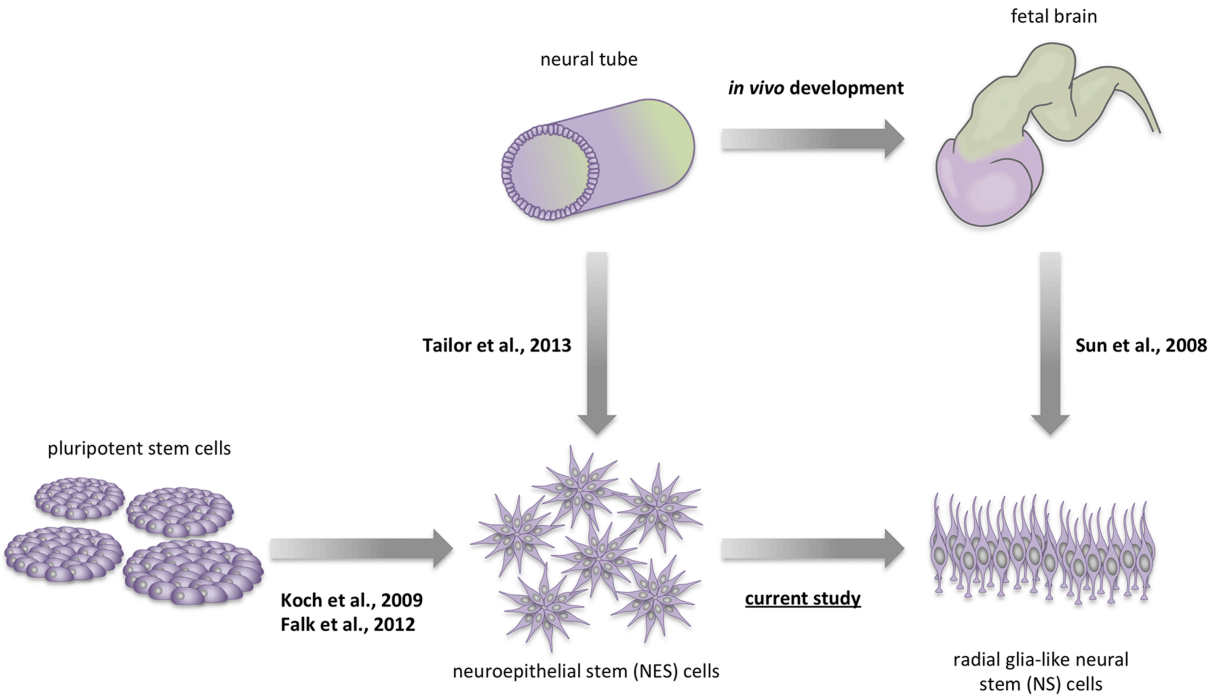


Figure 5.1: Neural stem cell systems *in vitro* and their *in vivo* counterparts

Cartoon showing the presumptive relationship of *in vitro* cultured neural stem cell populations used in this study to their native counterparts. *In vivo*, neuroepithelial stem cells of the neural tube give rise to radial glia cells of the fetal brain. Stably expandable NES cells can be derived from pluripotent stem cells and directly from early-stage human embryos. Later stage human embryos give rise to RG-like NS cells. The current study demonstrates that RG-like NS cells can be directly generated from *in vitro* expanded NES cells.

5.2 Preservation of regional identity in a stable RG-like NS cell population

Cultures of human neural stem cells and neurons generated from pluripotent stem cells represent an attractive tool to study cell types that are directly affected by neurological diseases. In order to generate various different neuronal subtypes without batch-to-batch variation a substantial challenge in neural stem cell research has been to culture stable intermediate NSC populations, which are patternable, yet can be propagated with a stable regional identity *in vitro*. Rosette-like NES cells generated from PSCs have been demonstrated to be amenable to patterning cues and initially show a broad differentiation potential towards anterior and posterior fates (Elkabetz et al. 2008; Koch et al. 2009b).

Under standard *in vitro* culture conditions this plasticity, however, also causes progressive restriction of the patterning potential to more posterior identities such as hindbrain and spinal cord. Radial glia cells isolated from different regions of the CNS in contrast appear to be stable in their regional identity when exposed to different environmental cues *in vitro* as well as *in vivo* following heterotopic transplantation (Onorati et al. 2011; Hook et al. 2011). In this study these two neural stem cell systems were combined to enable the generation of RG-like NS cell populations with different regional phenotypes, including forebrain identity. Regionally specified PSC-derived RG-like NS cells are able to maintain regional stability during proliferation *in vitro* and differentiate into neuronal subtypes corresponding to their regional identity.

5.2.1 Assessing and challenging the preservation of regional identity in RG-like NS cells

The first part of this study demonstrated that lt-NES cells with hindbrain identity could give rise to RG-like NS cells that share important properties with radial glia cells *in vivo*. Consequentially this led the question whether PSC-derived RG-like NS cells with different regional phenotypes, in accordance with native radial glia cells, have the ability to retain positional identity under *in vitro* expansion.

Regional stability has traditionally posed a challenge to the long-term culture of neural stem cells *in vitro.* Much like their *in vivo* counterpart, cultured NES cell populations are particularly susceptible to the influence of regionalizing morphogens. Characteristically, early NES cells when generated from PSCs exhibit a default anterior forebrain phenotype that cannot be maintained in culture and is shifted through growth factor exposure to an anterior hindbrain positional identity (Pankratz et al. 2007; Elkabetz et al. 2008; Falk et al. 2012; Koch et al. 2009b). However, as was alluded to previously, regionally stable NSC populations would have significant advantages.

To investigate the regional stability of RG-like NS cells along the anterior-posterior as well as the dorsal-ventral axis the sequential differentiation protocol was modified to include an additional patterning step before the withdrawal of growth factors (Fig 5.2).

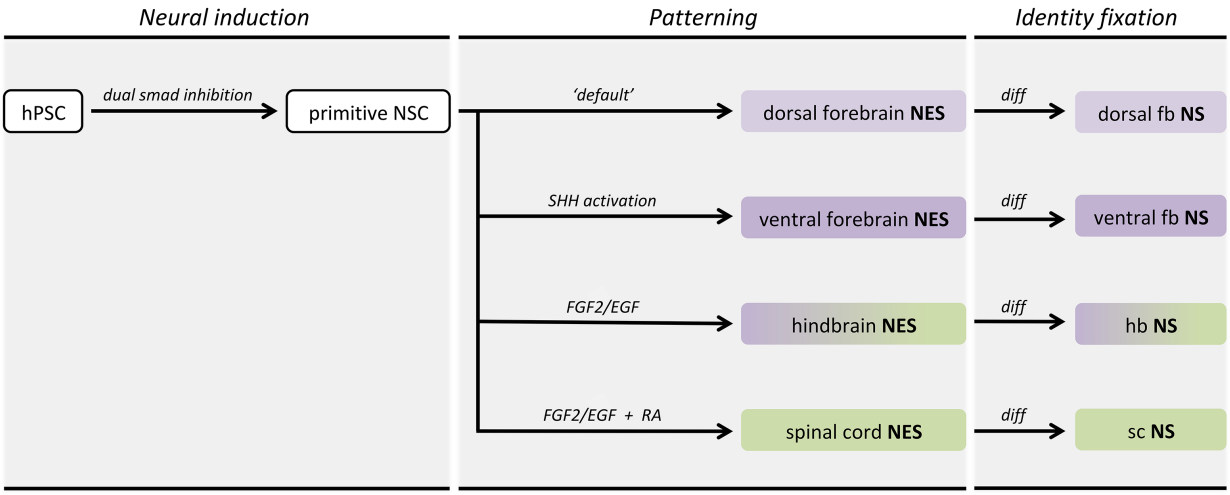


Figure 5.2: From hPSC to RG-like NS cells with fixed regional identities

Overview of RG-like NS cell regional identities and the patterning approach applied to hPSCs in this study. Neural induction of hPSC is achieved by dual SMAD inhibition (Chambers et al. 2009). Primitive NSCs are patterned through different morphogens to NES cells with a specific regional identity. Consecutively respective identities are consolidated through the transition of pre-patterned NES cells to RG-like NS cells taking place over 4 weeks of differentiation.

Forebrain identity RG-like NS cells were generated from early FoxG1- and Pax6-positive anterior neural rosettes directly, omitting any GF mediated propagation steps to avoid posteriorization. For ventral specification early forebrain rosettes were ventralized by activation of the SHH pathway to express the ventral marker Nkx2.1 and downregulate Pax6 before differentiation into RG-like NS cells (Maroof et al. 2013; Nicholas et al. 2013; Liu et al. 2013).

RG-like NS cells derived from NES cells previously propagated in FGF2/EGF (lt-NES cells) adopted a hindbrain identity whereas spinal cord identity RG-like NS cells were generated from lt-NES cells which were first posteriorized through exposure to RA.

Previous studies have disputed whether positional identity in NSCs is maintained through cell-intrinsic mechanisms or requires continued exposure to appropriate morphogens (Merkle et al. 2007; Ihrie et al. 2011; Fuccillo et al. 2006). To avoid ambiguities, morphogens used to induce the desired regional identity, such as RA or SHH, were therefore withdrawn from NES cultures together with GF following confirmation of the positional phenotype. Importantly, RG-like NS cell lines generated from dorsal and ventral forebrain-, hindbrain- and spinal cord-patterned NES cells share the characteristic radial

glia morphology and homogeneous marker expression observed previously. This conformity again supports the reproducibility of the sequential differentiation protocol developed in this study.

Importantly, dorsal forebrain-like NS cells were able to maintain their positional identity and continued to express the anterior markers FoxG1 and Otx2 following extensive passaging of up to 30 passages in neural stem cell medium containing FGF2 and EGF. In contrast, anterior marker expression could reportedly only be detected in very early NES cells up to passage 3 and was lost in higher passages under propagation with GF (Falk et al. 2012). Likewise, ventral forebrain-like NS cells retained the expression of FoxG1 as well as the ventral marker Nkx2.1 showing that RG-like NS cells can also be specified along the dorsal-ventral axis of the forebrain.

In line with forebrain-like NS cells, spinal cord-like NS cells also appear to be fixed in their posterior positional identity and express the spinal cord marker HoxB4 at higher passages without the continued influence of retinoic acid supporting the notion that the maintenance of regional identity is based on cell-intrinsic mechanisms.

These results are consistent with previous studies observing regional marker retention in late stage primary NSC populations cultured *in vitro*. In 2008 Sun et al. could show that human fetal-derived radial glia cells, isolated from the forebrain, maintained expression of Otx2 and Dlx1, while cell lines derived from the spinal cord continued to express HoxB9 under long-term expansion *in vitro* (Sun et al. 2008). Ventral SVZ neural progenitors isolated form mice maintain the expression of Nkx2.1 *in vitro* even after serial passaging (Delgado et al. 2016). Other studies in rodents also support regional fate restriction in later-stage neural stem cells from the forebrain (Hitoshi et al. 2002; Na et al. 1998), midbrain (Hitoshi et al. 2002; Studer et al. 1998), hypothalamus (Markakis et al. 2004), and spinal cord (Mayer-Proschel et al. 1997; Hook et al. 2011).

Indeed, heterotopic transplantation studies could show that native radial glia, similar to RG-like NS cells, are not only independent of morphogen instruction but also resistant to external regionalizing cues and retain the identity of their region of origin (Frantz and McConnell 1996; Merkle et al. 2007).

Similarly, forebrain-like NS cells preserved their anterior regional identity even when cultured under the influence of the potent posteriorizing morphogen, retinoic acid. This observation could be further validated in future experiments by transplanting RG-like NS

cells heterotopically into murine brains and exploring to what extend RG-like NS cells remain regionally stable *in vivo* and how they adapt to local environmental cues.

Taken together, these results support the notion that, while morphogen gradients establish positional identity in NSCs and induce the expression of specific transcription factors, retaining this regional patterning is dependent on cell-intrinsic mechanisms. The mechanisms responsible for this cellular identity memory are likely based on epigenetic changes and involve chromatin regulators. Interestingly, Polycomb group (PcG) and trithorax group (trxG) genes, an evolutionary conserved class of regulators initially described in Drosophila, appear to play an impotent role in maintaining appropriate regional patterning in mice (Schuettengruber et al. 2017). Through modification of the chromatin structures PcG and trxG proteins are able to maintain silencing or activation of Hox genes as well as many other fate-determining transcription factors (Beck et al. 2010; Delgado and Lim 2017). In a recent study Delgado et al. could show that mixed-lineage leukemia 1 (Mll1), a mammalian trxG homolog, is required in the murine brain for the maintenance, but not establishment, of ventral identity and Nkx2.1 expression in forebrain NSCs. Even transient inhibition of Mll1 resulted in permanent loss of ventral transcription factors and partial adoption of dorsal forebrain fate in ventral forebrain NSCs (Delgado et al. 2020). Further important factors in cell memory are likely non-coding RNAs, including lncRNA and miRNA, which both have been shown to interact with PcG/trxG proteins and regulate their expression (Davidovich and Cech 2015; Engreitz et al. 2016; Neo et al. 2014).

The current study shows that it is possible to recapitulate the phenomenon of regional patterning in NES cells and consolidation and maintenance of regional identity in the absence of morphogens through the transition to an RG-like phenotype *in vitro*. This sequential protocol could therefore serve as an attractive model to investigate the underlying mechanism of maintenance of NSC positional identity and the potential roles of chromatin regulators in humans. Illuminating these processes could have important implications for our understanding of human developmental disorders as well as the culture of stable human NSCs.

Further investigation of the specific regional phenotype of RG-like NS cells generated in this study using RT-PCR and microarray gene expression analysis at a medium high passage (p16) showed additional HOX genes such as HOXB6, a marker for thoracic

spinal cord progenitors, in spinal cord-like NS cells (Lippmann et al. 2015). In addition, they expressed the dorsal marker Pax6 but only few cells stained positive for more ventral spinal cord markers such as Nkx2.2 or Olig2 expressed in the most ventral progenitor domains responsible for motoneuron and interneuron generation. Forebrain-like NS cells expressed the hem marker Lhx2 and the dorsal marker Pax6 but were negative for the ventral forebrain marker Nkx2.1 expressed *in vivo* in the medial ganglionic eminence (MGE). They, however, show some expression of the pan-GE markers DLX1 and 2. This expression pattern is in line with the applied neural induction protocol, which generates by default an anterior dorsal identity, but does not enforce a more specified dorsal phenotype through factors blocking ventralization (eg. cyclopamin) (Floruta et al. 2017). It is conceivable that RG-like NS cells could be generated with even more defined regional identities using newly developed precise patterning protocols. Additional experiments are required to explore the full potential of such region-specific RG-like NS cell populations and their differentiation capacity as well as their proficiency to model region-specific progenitor disorders.

5.2.2 PSC- and fetal tissue-derived stable neural stem cells lines show similarities in gene expression profiles depending on their developmental stage

Radial glia cells *in vivo* represent a very heterogeneous cell population with diverse regional identities as well as temporal specification. In addition, the propagation of RG-like NS cells under artificial conditions could alter the identity of *in vitro* cultured neural stem cells. These factors might complicate biomedical applications, such as cell replacement or disease modelling which require neural progenitor populations to be standardizable as well as comparable to their *in vivo* counterparts.

Remarkably, when differently patterned (forebrain, hindbrain or spinal cord) RG-like NS cells are compared to lt-NES cells from the same genetic background (hESC line I3) in unsupervised hierarchical clustering and principal component analysis, RG-like NS cells cluster together and away from lt-NES cells independent of their regional identity. This speaks to the fact that RG-like NS cells are indeed a defined *in vitro* stem cell population entity separate from NES-like stem cells.

In a next step *in vitro* generated, hPSC derived NES and RG-like NS cells were compared to primary fetal tissue-derived NES and NS cells. The microarray gene expression profiling was carried out based on the previously identified 3541 transcripts that were differentially expressed between lt-NES and RG-like NS cells. RG-like cell lines included in this analysis comprised the hPSC-derived RG-like NS cells with forebrain, hindbrain and spinal cord identity generated in this study as well as primary tissue derived RG-like NS cells with forebrain, hindbrain and spinal cord identity. In addition, primary hbNES derived RG-like NS cells with hindbrain regional identity were included. The NES cell lines included in the analysis were hPSC-derived lt-NES cells and primary derived NES cell (hbNES cells) both solely with hindbrain identity.

Intriguingly, despite their differing regional identity and independent of their origin (hPSC or primary tissue) RG-like NS cells and NES cells again cluster in two distinct groups. Differences in the expression profile are, however, more pronounced among different RG-like NS cell lines compared to the three NES cell lines, which are rather homogeneous. This could be owed to the fact that radial glia represent a neural stem cell with more diverse features including regional specification but also temporal differences with regards to the progeny they generate during the course of neuro-and gliogenesis (Edri et al. 2015). In addition, the RG-like NS cell lines analyzed included different regional identities, while primary and hPSC-derived NES cell lines shared a hindbrain identity, which could also account for their greater conformity. The overall comparable expression pattern of different regional identity RG-like NS and primary NS cells with regard to a radial glia-like profile, however, offers the possibility to identify a defined and standardized *in vitro* population with resemblance to native radial glia cells.

5.2.3 Regionally defined RG-like NS cells allow the reliable generation of region-specific neurons

Strategies for the generation of specific neuronal subtypes from PSCs often involve 'run-through' induction protocols mimicking developmental patterning events. These protocols guide terminal differentiation of pluripotent stem cells into neuronal cell types without a defined intermediate progenitor state, which can result in batch-to-batch variation. Regionally fixed, expandable RG-like NS cells as starting populations have the potential

to reduce variability and improve reproducibility by facilitating stable generation of specific neuronal subtypes. To that end, the regional transcription code present in RG-like NS cells needs to yield corresponding region-specific neurons and glia.

Spinal cord-like NS cells when differentiated for 4 weeks give rise to neuronal cultures positive for HoxB4 as well as Lim1/2 and Lim3, transcription factors typical of various inhibitory spinal interneuron populations. Isl1 could only be detected infrequently while the motor neuron marker HB9 was absent from the cultures. In accordance with this phenotype PCR analysis showed expression of GAD67, a GABA producing enzyme. This neuronal subtype specification is also in line with the ventral-dorsal regional identity of spinal cord-like NS cells, which lack more ventral markers necessary for motor neuron generation. Spinal cord neurons include more than 30 distinct subgroups and have important roles in the generation of locomotion and the processing of sensory information (Lu et al. 2015; Zhang et al. 2024; Yadav et al. 2023). Applying targeted neural patterning regimes to the NES cell starting population, including SHH activation for more ventral phenotypes, could in the future allow the generation of RG-like NS cell lines able to give rise to specific interneuron or motor neuron populations. These cell types are essential in the pathological processes of disorders such as ALS and neuropathic pain as well as spinal cord injury and therefor are attractive neuronal populations for possible therapeutic approaches or disease modelling (Hossaini et al. 2011; Salamatina et al. 2020; Zavvarian et al. 2020).

Dorsal forebrain-like NS cells predominately give rise to glutamatergic neuronal cultures and several deep and upper cortical layer markers such as Ctip2, Satb2 and Brn2 could be detected. In accordance with the regional code of forebrain-like NS cells, which is prevailingly dorsal but includes some MGE markers (Dlx1/2), only few neurons expressed GAD67 and DARPP32, a marker expressed in striatal medium spiny neurons, which degenerate in Huntington’s disease. However, no overlay of GAD67 and Citp2 could be observed, suggesting that Ctip2 expressing cells are indeed glutamatergic deep-layer neurons and not of striatal origin. On a transcriptional level, in addition to Citp2, FEZF2, a gene specifically expressed by subcortical projection neurons, could be detected (Cheng 2008). Neurons derived from the human cortex are particularly interesting for research purposes since this brain region has greatly evolved in humans, making the study of

disorders such as Alzheimer's or psychiatric diseases challenging to reproduce in animal models (Dawson et al. 2018).

Neuronal cultures derived from ventral forebrain-like NS cells are enriched for GABAergic neurons, some of which express calbindin or parvalbumin, markers for cortical interneurons originating in the medial and lateral ganglionic eminences, which are not present in dorsal forebrain-like NS cell neuronal cultures. Cortical interneurons are particularly affected in psychiatric diseases such as schizophrenia or in epilepsy and therefore represent an attractive population for disease-modifying drugs or cell replacement approaches (Cunningham et al. 2014; Southwell et al. 2014).

Taken together, these results show that RG-like NS cells with specific regional identities give rise to specific neuronal subtypes corresponding to their phenotype. Regionally stable RG-like NS cells generated from pre-patterned NES cell populations might therefore serve as a platform to generate and expand region-specific neural precursors and neurons of diverse positional identities. In the future, RG-like NS cells could be generated form several more defined brain regions including specific cortical or subcortical, diencephalic and midbrain regions or the cerebellum. Additional experiments are, however, required to explore the full differentiation potential of region-specific RG-like NS cells.

RG-like NS cells with different regional identity have also been shown to generate astrocytes even more efficiently than earlier NSCs. In addition, RG-like NS cells could therefore be an attractive and expandable source for macroglia, which are possibly also regionally defined. However, the maturity of these astrocytes as well as their possible regional phenotype needs to be further investigated. Interestingly, glia cells have recently been shown to play significant roles in common neurological diseases. Genes associated with sporadic Alzheimer's disease, such as APOE, APOJ and SORL, are mainly expressed by glial cells (Arranz and De Strooper 2019). Astrocyte dysfunction has also been implicated in other diseases including Parkinson's, frontotemporal dementia, ALS and MS (di Domenico et al. 2019; Hallmann et al. 2017; Haidet-Phillips et al. 2011; Perriot et al. 2018). Animal models are frequently used in researching glial aspects of neurological diseases. However, much like neurons, glia cells show significant differences between humans and commonly used rodent models. Human astrocytes appear to be larger, more numerous and exhibit increased complexity compared to rodent astrocytes (Oberheim et

al. 2006; Oberheim et al. 2009). In 2016 Zhang et al. isolated and analyzed both human and mouse glial cells and compared their transcriptome using RNA sequencing. Surprisingly, of the most highly expressed genes in human astrocytes they found only 30% to be also highly expressed in mice (Zhang et al. 2016). Interestingly, astrocytes are no longer believed to be a homogeneous population (Zhu et al. 2010; Rowitch et al. 2010). Evidence suggests, that regional patterning of the neural tube also leads to the generation of diverse astrocyte populations. The resulting differential gene expression has recently been shown to cause important differences in physiological properties of astrocytes in different brain regions (Hu et al. 2019). These include membrane properties, calcium homeostasis and effects of astrocyte subtypes on neurite outgrowth and the blood-brain barrier (Bradley et al. 2019). It would therefore be deeply interesting to see if RG-like NS cell-derived astrocytes are regionally specified, if they differ in their function between brain regions and share human specific expression profiles.

Going forward it would also be interesting to further analyze the propensity of RG-like NS cells to generate oligodendrocytes. Similar to astrocytes human oligodendrocytes show considerable differences to their mouse counterpart. When grafted into hypomyelinated shiverer mice human glial progenitors display such a competitive advantage over endogens oligodendrocytes that virtually all endogenous glial cells are replaced leading to a chimeric mouse brain (Windrem et al. 2014; Goldman et al. 2015). In contrast to astrocytes, it is less clear if regional differences influence oligodendrocyte function. However, there is some evidence of variations in myelination phenotypes, in particular between the brain and spinal cord as well as grey and white matter (Bechler et al. 2015; Viganò et al. 2013). A recent study could show region-specific vulnerabilities of gray matter oligodendrocytes in response to stress-induced behavior (Long et al. 2021). In addition, ventrally and dorsally derived forebrain oligodendrocytes appear to have some distinctive properties and functions (Foerster et al. 2024). It would therefore be interesting to see if RG-cells of various dorsal and ventral regional identity could be coaxed into generating region-specific oligodendrocytes respectively.

In future experiments RG-like NS cell-derived patient-specific glial cells could serve as human disease models and provide a platform for the generation of region-specific macroglia.

5.2.4 Forebrain-like NS cells emulate the inside-out development of the human cortex

The development of the 6-layer structure of the human cortex requires intricate control of the timing and numbers of neuronal subtypes being generated. It is therefore an important question to what extend neural stem cells propagated *in vitro* under artificial conditions retain the ability to recapitulate the complex mechanisms involved in human corticogenesis.

In vivo the 6-layer cortex is generated in an inside-out fashion with deep-layer generated before upper-layer neurons (Hevner et al. 2003). In humans, neurogenesis occurs mostly in an indirect manner whereby radial glia stem cells give rise to committed transient amplifying precursors through asymmetric divisions, which in turn migrate outward and divide several times before generating postmitotic neurons (Cárdenas et al. 2018; Florio et al. 2014; Sun et al. 2014). Especially precise control of the balance between symmetrical self-renewing divisions and the generation of committed neurogenic precursors in asymmetric divisions of radial glia cells is crucial for the formation of a functional cortex (Lancaster et al. 2013; Iefremova et al. 2017). Cortex specific intermediate progenitor cells (IP) are identified by the transcription factor Tbr2 (Gene: EOMES) (Englund et al. 2005). Tbr2+ IPs exclusively generate cortical projection neurons and Tbr2 itself plays an important role in laminar fate regulation. Knockout experiments in rodents could show that laminar differentiation is disrupted and in particular the generation of later upper cortical layer neurons severely negatively affected by loss of Tbr2 due to the depletion of radial glia cells (Mihalas et al. 2016).

During *in vitro* culture, neural stem cells are kept in an artificial state of constant self-renewal under the influence of mitogens, which might have the capacity to change their potency and obscure their natural state (Doetsch et al. 2002; Pollard et al. 2008). *In vivo* NES cells divide symmetrically to increase the progenitor population before transitioning to radial glia, which at the onset of neurogenesis, divide increasingly asymmetrically to generate IPs and neurons. FGF signaling specifically inhibits the generation of Tbr2+ IPs from radial glia in asymmetric divisions (Kang et al. 2009). This can be appreciated in dorsal forebrain-like NS cell cultures proliferating in the presence of FGF2, which results in very low numbers of Tbr2-positive or Sox2-negative differentiated cells. However, when

FGF2 is removed from the culture, the number of Tbr2+ IPs and differentiated cells rapidly increases, indicating that asymmetric divisions typical for radial glia are reinstated and indirect neurogenesis is occurring in the culture.

Following the continuation of developmental progression in forebrain-like NS cells after the removal of growth factors, the question arises whether forebrain-like NS cells also faithfully recapitulate the sequential generation of neurons as observed during physiological corticogenesis. Recent studies using single cell transcriptome analysis indicate that cell-autonomous mechanisms together with extrinsic cues regulate the timing of laminar fate transition from deep- to upper-layer neurons (Kohwi et al. 2013; Nowakowski et al. 2017; Okamoto et al. 2016).

Interestingly, cortical progenitor cells generated *in vitro* from PSCs and directly differentiated are indeed able to recapitulate the inside-out layer formation by generating deep-layer before upper-layer neurons, supporting the notion that this timing is partially a cell-intrinsic mechanism (Eiraku et al. 2008; Espuny-Camacho et al. 2013; Shi et al. 2012b). However, these progenitor cells were neither subjected to growth factors nor propagated prior to differentiation into cortical neurons. When forebrain-like NS cells, which were previously proliferated using FGF2 and EGF, are differentiated for up to 60 days, neurons expressing the deep-layer marker Ctip2 are generated first, followed later by Satb2-positive upper-layer neurons. Encouragingly, this indicates that the timing of layer generation is maintained in RG-like NS cells despite prolonged *in vitro* propagation. In addition, the ability to generate deep- and upper-layer neurons is maintained from early to late passages.

5.3 RG-like NS cells in the context of established NSC protocols and recently developed *in vitro* models of human neurodevelopment

There exist various approaches to date for the *in vitro* generation of neural stem cells, each with its own advantages and drawbacks. One approach is the isolation of neural stem cells *in vivo* and consecutive propagation *in vitro*. This method promises bona fide NSCs but is of course limited through the scarce availability of human fetal tissue and ethical concerns. Therefore, the most suitable source for NSCs, as previously alluded to, are pluripotent stem cells and especially iPS cells. Starting from iPSCs different model

systems have been developed to study human neurodevelopment and disease mechanisms. These include ‘run-through’ protocols for the derivation of specific neuronal subtypes, neural stem cell models such as described in this study and, most recently, brain organoids (Lancaster et al. 2013; Kadoshima et al. 2013).

The generation of NSC from PSC has made great progress form protocols with initially low efficiency and high heterogeneity to the now prevailing neural induction protocol through dual SMAD inhibition, with a yield of 96% NSC marker expressing cells. However, protocols allowing stable NSC self-renewal following neural induction remain rare. Neural rosettes (R-NSC) generated form ESCs by Elkabetz et al., cultured in serum free medium supplemented with FGF2, EGF, FGF8, SHH, AA, BDNF and N2 can only be expanded for 4 passages before losing their rosette architecture and broad differentiation potential (Elkabetz et al. 2008). The long-term self-renewing NES (lt-NES cells) applied in this study can be extensively propagated using FGF2, EGF, B27 and N2 for more than 150 passages without losing NSC properties and show a stable differentiation potential toward neurons and glia. Lt-NES cells have been used in the study of disorders such as Alzheimer’s and Machado-Joseph disease (Falk et al. 2012; Mertens et al. 2013a; Doerr et al. 2017; Koch et al. 2011; Koch et al. 2012; Poppe et al. 2018). Similarly, small molecule neural progenitor cells (smNPCs) developed by Reinhard et al. in 2013, which are cultured using CHIR, PMA, AA, N2 and B27, can be extensively passaged. They appear to correspond to a pre-rosette stage and form typical neural rosettes upon exposure to FGF2 (Reinhardt et al. 2013). One important caveat of both these self-renewing intermediates, however, is the spontaneous posteriorization they undergo due to growth factor-expansion. Lt-NES cells as well as smNPCs after propagation can therefore only be directed towards hindbrain and spinal cord fates, which might lead to false results when studying disorders normally affecting the forebrain. Therefore, the regionally stable RG-like NS cells developed in this study could fill an important gap by providing disease specific neurons and glia from the appropriate brain region.

Further, the benefits of homogeneous 2D cell culture should be weighed against the higher complexity of 3D models. IPS cell derived 3D brain organoids exploit the ability of neural stem cells to self-organize to model aspects of early neurodevelopment. In recent years, a number of different protocols have been developed which give rise to either whole brain organoids or 3D models of various specific brain regions. Brain organoids represent

a highly dynamic model of brain development over time. Forebrain organoids in part recapitulate the inside-out generation of the cortical plate and the orientation of specific germinal regions such as the VZ and SVZ. Interestingly, features important for human cortical expansion, including the formation of outer radial glia have been observed in forebrain organoids (Qian et al. 2016; Bershteyn et al. 2017). The improved spatial organization and complex architecture compared to conventional 2D models offer an opportunity to study intricate cell-cell and cell-matrix interactions in developmental processes as well as neurological and psychiatric diseases in a human background. Brain organoids have therefore been widely used in recent years and are considered a more clinically relevant model system. However, the increased complexity of these 3D structures, which include a great diversity of brain cell types, also brings some limitations. In addition, the generation of brain organoids requires more technically challenging and expensive culture conditions and can result in increased batch-to-batch variability. Therefore, depending on the application, 2D NSC and neuronal culture, such as RG-like NS cells and their progeny, might be more suitable for certain application in drug screening or electrophysiological test and readouts where simplicity and easy access are a benefit. In the end, the combination of the simplicity and controllability of 2D cultures combined with the greater complexity and fidelity 3D models will likely be the best path for future studies.

5.4 Outlook - Application of RG-like stable neural stem cell populations

In this study a protocol was established which allows the generation of regionally stable human radial glia-like neural stem cells form pre-patterned neuroepithelial cells. In the past, the study of human specific features of the brain was aggravated by the difficulty to access fetal or diseased brain tissue. Even though animal models have greatly increased our understanding of neurodevelopment and neurological disorders fundamental differences in human brain biology have limited the translation of knowledge gained in these studies to human disorders and led to the failure of clinical trials (Zhao et al. 2018). In recent years human ES and iPS cells have therefore been instrumental in studying human specific characteristic of development and disease. Different *in vitro* model systems have been developed to be applied in disease modelling, compound screening

and modelling of developmental processes as well as in developing possible cell-based therapies.

The RG-like NS cells developed in this study are a homogeneous monolayer of specified neural progenitors, which remain stable over extended passaging and can be expanded and stored. The homogeneity together with stable expansion and easy handling of these cultures potentially reduces batch-to-batch variation. In addition, the 2D system allows easy access for more complex manipulations and the option to constantly monitor individual cells in culture. These qualities are especially advantages for high-throughput screening approaches and facilitate efficient and reproducible downstream analysis.

RG-like NS cells have the additional advantage of stably maintaining their regional identity under growth-factor expansion. They also show stable neuronal and glial differentiation independent of passage number and a consistent transcription profile across cell lines characteristic for radial glia cell *in vivo*. Such regionally stable human neural stem cell populations could have interesting applications in future research and for therapeutic approaches by combining the advantages of stable intermediates with regional specificity, potentially for any brain region. Along this line, various new additional patterning protocols used for the run-through production of region-specific neurons could be applied to the sequential differentiation paradigm developed in this study. In this way generated regionally stable RG-like NS cells lines could serve as a platform for the derivation of region-specific neurons with reduced batch-to-batch variation compared to commonly applied run-through protocols. Additionally, the expansion of intermediate cell populations reduces the effort and expenses necessary for pluripotent stem cell cultivation and the variation prone patterning process. With this in mind, the capacity of RG-like NS cells to generate a variety of relevant neuronal subtypes needs to be further evaluated and the resulting neurons analyzed for their maturity and function.

Interestingly, not only human neurons but also astrocytes and oligodendrocytes show significant evolutionary differences compared to rodent models (Li et al. 2021; Zhang et al. 2016). In addition, regional differences in glial cells might play an important role in disease mechanisms and could be examined in co-culture settings of neurons and glia cells generated from patient- and healthy control-derived regionally defined RG-like NS cells (Molofsk et al. 2012). To this end, the potential of RG-like NS cells to generate astrocytes with a higher efficiency and state of maturation could be further explored. Using

recently published improved differentiation protocols, the ability of RG-like NS to generate oligodendrocytes should also be investigated (TWC 2017; Rodrigues 2017).

Stable and well-defined iPSC-derived neural stem cell populations could also be a potential source for cell-based therapies. However, in particular for any future therapeutic applications it will be paramount to validate *in vitro* generated region-specific RG-like NS cells through *in vivo* transplantation studies in rodents. Following transplantation RG-like NS cells could be analyzed for their ability to survive *in vivo* and to generate neurons and astrocytes. By transplanting regionalized RG-like NS cells heterotopically we could investigate whether their regional identity remains stable *in vivo* and how the cells react to local environmental cues. In addition, RG-like NS cell-derived neurons could be analyzed for their integration capacity into existing neuronal networks as previously shown for lt-NES cells (Doerr et al. 2017). IPSC-derived cells are currently already being tested as a source for cell-based therapies in disorders like Parkinson's disease or macular degeneration (Maeda et al. 2021; Parmar et al. 2020; Kobold et al. 2020). In this context radial glia-like cell types could be an attractive starting population as they can be regionally defined while retaining the ability to proliferate potentially without showing extensive overgrowth (Kajikawa et al. 2020).

Aside from their potential for biomedical use the authenticity of RG-like NS cells, as with any *in vitro* generated cell type, remains imperfect. Lacking the tissue context of the developing brain many aspects of cell physiology such as cell polarity or interactions with other cell types in a 3-dimensional structure cannot be represented in this model. In this context it is important to recognize the limitations of RG-like NS cells developed in this study as they only reproduce certain aspects of bona fide RG cells including the expression of RG specific markers, differentiation into neuros and glia as well as the ability to conserve regional identity. Accordingly, this system might be used to interrogate the cell intrinsic mechanisms controlling the consolidation of regional identity occurring during the transition from NES to RG-like NS cells.

RG cells *in vivo* have a pivotal role in human specific brain development. ORG cells in particular, through their increased proliferation capacity, play a key role in the expansion of the human neocortex (Kalebic et al. 2019; Florio et al. 2017). A simplified and stable model of this important cell type could therefore be an attractive tool to investigate certain aspects of human specific features in developmental disorders. Interestingly, RG-like NS

cells display a transcriptional identity, which includes genes thought to be crucial to maintain the proliferative capacity of oRG cells in the OSVZ. Recently, single cell RNA sequencing of the developing human brain has revealed the temporal and spatial heterogeneity of NES and RG cells and has allowed the identification of previously unknown subtypes of neural progenitor cells and their molecular signatures (Fan et al. 2020; Eze et al. 2021). Therefore, it would be interesting to compare the transcriptome of differently patterned RG-like NS cells to *in vivo* single cell data sets to determine which stage of RG cell development is represent the closest by this model and in what way cells generated in 2D *in vitro* differ from bona fide neural progenitor cells. Several oRG and vRG cell specific characteristics, such as anatomical position or division mode, cannot be modelled in a 2D environment. To investigate if RG-like NS cell show behavior representative for specific cell types in a 3D setting, they could be integrated into cortical brain organoids or transplanted into developing mice brains.

Taken together the data presented in this study allude to the potential applications of regionally stable RG-like NS cells in disease modelling, drug testing and, with some caveats, in modelling human development. In the end, it is important to appreciate, that every model is flawed when it comes to emulating all aspects of the human brain and its development. Therefore, depending on the application, the advantages and limitations of each approach, 2D and 3D human cell culture as well as animal models, need to be considered to ensure that gained insights can be translated to the human brain.

6 Abbreviations

Abbreviation	Full Name
3CB2	Anti-Vimentin Antibody Cone 3CB2
AA	Ascorbic acid
ALK receptor	Anaplastic lymphoma kinase
ALPL	Alkaline Phosphatase
ALS	Amyotrophic lateral sclerosis
ANAX1	Annexin 1
ANXA2	Annexin 2
APOE	Apolipoprotein E
APOJ	Apolipoprotein J
AQP4	Aquaporin 4
ARHGAP11B	Rho GTPase Activating Protein 11B
ARHGEF6	Rac/Cdc42 Guanine Nucleotide Exchange Factor 6
ASCL1 (MASH1)	Achaete-Scute Family BHLH Transcription Factor 1
BDNF	Brain derived neurotrophic factor
bFGF	Fibroblast growth factor
bFGF	Basic fibroblast growth factor
BLBP	brain lipid binding protein
BMI1	B Lymphoma Mo-MLV Insertion Region 1
BMP	Brain morphogenic protein
BrdU	Bromdesoxyuridin
BRN2 (POU3F2)	Brain 2
BTBD17	BTB Domain Containing 17
C1orf61	Chromosome 1 open reading frame 61
C1QL1	Complement C1q Like 1
Calb	calbindin
CAMKV	CaM Kinase Like Vesicle Associated
CAV1	Calveolin 1
CD133 (prominin1)	Cluster of differentiation 133
CD44	Cluster of differentiation 44
Cdk4	Cyclin Dependent Kinase 4
cDNA	Complementary DNA
cGMP	Good manufacturing guidelines for medical products
CHST15	Carbohydrate Sulfotransferase 15
CITED2	Cbp/P300 Interacting Transactivator with Glu/Asp rich Carboxy-Terminal Domain 2
CNS	Central nervous system
CNTNAP2	Contactin Associated Protein 2

CRABP1	Cellular Retinoic Acid Binding Protein 1
CRYAB	Crystallin Alpha B
CTIP2 (BCL11B)	Coup-TFI interacting protein 2
Cyp26	Cytochrome P450 Family 26
Cyp26	Cytochrom P26
DACH1	Dachshund Family Transcription Factor 1
DARPP32 (PPP1R1B)	Dopamine- and cAMP-regulated neuronal phosphoprotein
DCX	Doublecortin
DLK1	Delta Like Non-Canonical Notch Ligand 1
DLX1	Distal-Less Homeobox 1
DLX2	Distal-Less Homeobox 2
DNA	Deoxyribonucleic acid
EBiSC	European Bank for induced Pluripotent Stem Cells
EBiSC	European Bank for induced Pluripotent Stem Cells
EBs	Embryoid bodies
ECM	Extracellular matrix
EGF	Epithelial growth factor
ESC	Embryonic stem cells
FABP5L2	Fatty Acid Binding Protein 5 Pseudogene 2
FABP7 (BLBP)	Fatty Acid Binding Protein 7
FAM107A	Family With Sequence Similarity 107 Member A
FAM70A (TMEM255A)	Family With Sequence Similarity 70, Member A
fb	Forebrain
FBS	Fetal bovine serum
FEZF2	FEZ Family Zinc Finger 2
FGF10	Fibroblast Growth Factor 10
FGF2	Fibroblast growth factor 2
FGF8	Fibroblas growth factor 8
FGF8	Fibroblast Growth Factor 8
Forebrain-like NS cells	Forebrain-liken neural stem cells
Forse-1 (FOXG1)	Forebrain-surface-embryonic monoclonal *antibody 1*
FOXG1	Forkhead Box G1
FP	Floore plate
FRZB1	Frizzled Related Protein
FRZB1	Frizzled Related Protein
FRZB1	Frizzled Related Protein
FZD9	Frizzled Class Receptor 9
GABA	Gamma-Aminobutyric Acid
GABBR2	Gamma-Aminobutyric Acid Type B Receptor Subunit 2
GAD65	Glutamic acid decarboxylase 65

GAD67	Glutamic acid decarboxylase 67
GAP43	Growth Associated Protein 43
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GBX2	Gastrulation Brain Homeobox 2
GE	Ganglionic eminence
GF	Growth factor
GFAP	Glial Fibrillary Acidic Protein
GLAST	Glutamate aspartate transporter
GNPDA1	Glucosamine-6-Phosphate Deaminase 1
GPM6A	Glycoprotein M6A
hb	Hindbrain
HB9 (MNX1)	Homeobox *HB9*
hbNES cells	Hindbrain neuroepithelial stem cells
HES5	Hairy and Enhancer of Split 5
hESC	Human embryonic stem cells
HEY1	Hairy/Enhancer of Split related with YRPW Motif 1
HipSci	Human iPSC initiative
HipSci	Human iPSC initiative
HLA-DRA	Major Histocompatibility Complex, Class II, DR Alpha
HOP	Homeodomain only Protein
HOPX	HOP Homeobox
HOXA5	Homeobox A5
HOXB2	Homeobox B2
HoxB4	Homeobox B4
HOXB5	Homeobox B5
HOXB6	Homeobox B6
HOXB8	Homeobox B8
hPSC	Human pluripotent stem cells
ID3	Inhibitor Of DNA Binding 3
IGDCC3	Immunoglobulin Superfamily DCC Subclass Member 3
IGFBP7	Insulin Like Growth Factor Binding Protein 7
INM	Interkinetic nuclear migration’
IP	Intermediate progenitor
iPSC	Induced pluriponten stem cells
IRX3	Iroquois Homeobox 3
ISCI	International Stem Cell Initiative
KCNMB1	Potassium Calcium-Activated Channel Subfamily M Regulatory Beta Subunit 1
KLF4	KLF Transcription Factor 4
KLF4	KLF Transcription Factor 4

LGALS1	Galectin 1
LGE	Lateral ganglionic eminence
LHX1 (Lim1)	LIM Homeobox 1
LHX2 (Lim2)	LIM Homeobox 2
LHX3 (Lim3)	LIM Homeobox 3
LHX6	LIM Homeobox 6
DLX1	Distal-Less Homeo Box 1
DLX2	Distal-Less Homeo Box 2
LIF	Leukemia inhibiting factor
LIFR	LIF Receptor Subunit Alpha
LIFR	LIF Receptor Subunit Alpha
LIM1/2	LIM Homeobox 1/2
LIM3	LIM Homeobox 3
LIN28B	Lin-28 Homolog B
LIRF	Ring Finger Protein 39
lncRNA	Long non-coding RNA
LOC648390	S-Phase Kinase Associated Protein 1 Pseudogene
Lt-NES cells	Long-term self-renewing neuroepithelial stem cells
MAGEC2	MAGE Family Member C2
Map2ab	Mikrotubule-associated Protein 2ab
MASH1 (ASCL1)	mammalian achaete scute homolog-1
MGE	Medial ganglionic eminence
MGI	Mouse Genome Informatics
MHB	Midbrain-hindbrain boundary
miRNA	Micro RNA
Mll1	Mixed-lineage leukemia 1
MMRN1	Multimerin 1
MOXD1	Monooxygenase DBH Like 1
mRNA	Messenger-RNA
MS	Multiple sclerosis
MST	Mitotic somal translocation
MYC	MYC Proto-Oncogene
NEPs	Neural progenitor cells
NES cells	Neuroepithelial stem cells
NESTIN	neuroepithelial stem cell protein
NeuN (RBFOX3)	neuronal nuclei antigen
NFIA	nuclear factor one A
NFIB	nuclear factor one B
NFIX	nuclear factor one X
NKX2.1	NK2 Homeobox 1

NKX2.2	NK2 Homeobox 2
NMYC (MYCN)	N-Myc Proto-Oncogene
Notch1	Neurogenic locus notch homolog 1
NPC	Neural progenitor cells
NR2F1 (COUP-TF1)	Nuclear Receptor subfamily 2 group F member 1
NS cells/ NSC	Neural stem cell
Oct4	Octamer-binding transcription factor 4
Olig2	Oligodendrocyte Transcription Factor 2
oRG	Outer radial glia
Otx2	Orthodenticle Homeobox 2
Paval	Pavalbumin
PAX6	Paired Box 6
PcG	Polycomb group genes
PCR	Polymerase chain reaction
PLAGL1	Pleiomorphic Adenoma Gene-Like 1
PLZF	promyelocytic leukemia zinc finger
PMA	Phorbol myristate acetate
PMP2	Peripheral Myelin Protein 2
pNES	Primary neuroepithelial stem cells
pNS	Primary radial glia neural stem cells
PNS	Peripheral nervous system
PSC	Pluripotent stem cells
PTPRZ1	Protein Tyrosine Phosphatase Receptor Type Z1
R-NSCs	Rosette neuroepithelial stem cells
RA	Retinoic acid
RC2	NES antibody
RFX4	Regulatory Factor X4
RG cells	Radial glia cells
RG-like NS cells	Radial glia-like neural stem cells
RNA	Ribonucleic acid
ROCK inhibitor	Rho-associated kinase inhibitor
RPRML	Reprimo Like
RT-PCR	Reverse transcriptase PCR
S100beta	S100 Calcium Binding Protein B
SATB2	SATB Homeobox 2
sc	Spinal cord
SCG2	Secretogranin II
SFRP2	Secreted Frizzled Related Protein 2
SHH	Sonic Hedgehog Signaling Molecule
SIM1	SIM BHLH Transcription Factor 1

SIX3	SIX Homeobox 3
SLC1A3 (GLAST)	Soulute Carrier Family 1 Member 3
smNPCs	Small molecule neural stem s´cells
SORL	Sortilin Related Receptor
SOX2	SRY-Box Transcription Factor 2
SOX9	SRY-Box Transcription Factor 9
SPARCL1	SPARC Like 1
StZG	Stammzellgesetz
SVZ	Subventricular zone
TAGLN	transgelin
TAGLN2	Sortilin Related Receptor 1
TBR1	T-box brain protein 1
TBR2 (EOMES)	T-box brain protein 2
TF	Trancription factor
TGFß	Transforming growth factor β
TMEM14B	Transmembrane Protein 14B
TNC	tenascin C
TRNP1	TMF1 Regulated Nuclear Protein 1
trxG	Trithorax group genes
TTX	Tetrodotoxin
TUJ1	Anti-β-Tubulin III antibody
UBB	Ubiquitin B
vGlut1	Vesicular glutamate transporter 1
vRG	Ventricular radial glia
VZ	Ventricular zone
WNT	Wingless Type MMTV Integration Site
ZBTB16 (PLZF)	Zinc finger and BTB domain-containing protein 16
ZFHX3 (ATBF1)	Zinc Finger Homeobox 3
ZIC2	Zic Family Member 2
ZO1	zona occludens 1
βIII-tub	βIII-tubulin

7 Abstract

Progress made in pluripotent stem cell (PSC) technology potentially enables the production of almost any cell type in unlimited quantities for human specific models. This is particularly interesting for the generation of neuronal cells as the human brain differs considerably from commonly used animal models and human brain tissue samples are scarce, difficult to obtain and to propagate *in vitro*.

In recent years it has become possible to isolate and expand neural stem cells (NSCs) from different sources using growth factor-based protocols. However, a still open question is to what extent these diverse stem cell systems reflect physiological stem cell states observed *in vivo* (Conti et al. 2010).

During nervous system development, early neuroepithelial stem (NES) cells with a highly polarized morphology and responsiveness to regionalizing morphogens give rise to radial glia (RG) cells, which generate region-specific neurons. Stable neural cell populations reminiscent of NES cells have been obtained from pluripotent stem cells (lt-NES cells) and the fetal human hindbrain (hbNES cells) (Tailor et al. 2013; Koch et al. 2009b).

In this study a sequential differentiation protocol was developed enabling the *in vitro* generation of RG-like neural stem cells from NES-like cells (lt-NES and hbNES cells). By employing differentiating conditions NES-like cells were coaxed into following the *in vivo* developmental transition of neural stem cells into a RG-like phenotype. Indeed, this newly established NSC population exhibits important features of multipotent neuro- and gliogenic RG cells. These RG-like NS cells could be expanded for at least 25 passages and express classical NSC markers such as Nestin and SOX2 as well as markers typically associated with RG cells including SOX9, CD44, AQP4 and HOP, while the NES cell markers PLZF, DACH1, ZO1 and MMRN1 were down-regulated.

Importantly, RG-like cells generated from PSC-derived lt-NES cells and from primary hbNES cells showed similar properties as primary RG cells obtained from fetal tissue, indicating that conversion of NES-like cells into RG-like cells recapitulates the developmental progression of early NES cells into radial glia cells occurring *in vivo*.

Key challenges associated with the biomedical application of PSC-derived neural stem cells are their controlled patterning towards distinct regional subtypes and the

maintenance of an acquired regional phenotype across multiple passages of *in vitro* expansion.

Previous studies have shown that lt-NES cells, shortly after their derivation from PSC, undergo gradual posteriorization into an anterior hindbrain phenotype, which might be due to regionalizing effects of the growth factors employed for *in vitro* proliferation (Bithell et al. 2008; Koch et al. 2009b). This behavior is in line with their *in vivo* role in which they respond to morphogens in order to establish different regions of the brain. In a second step this regional identity is consolidated in RG cells which can no longer be influenced by regionalizing factors.

In order to harness this property of RG cells, RG-like NS cells were generated from dorsal forebrain-, ventral forebrain-, hindbrain- and spinal cord-patterned NES-like cells. The resulting regionalized RG-like NS cell populations express transcription factors appropriate for their positional identity across multiple passages of *in vitro* expansion while maintaining their differentiation potential into neurons and glia. Reminiscent of heterotopic transplantation studies, even under the influence of strong rationalizing factors *in vitro* the positional identity of dorsal forebrain-like NS cells remained stable (Onorati et al. 2011). Following differentiation RG-like cells generated region-specific neurons appropriate for their positional identity. These results indicate that conversion into RG-like cells may provide a route for conserving the regional identity of pre-patterned early NES cells and establishing regionally stable neural stem cell lines for biomedical application.

8 Zusammenfassung

In den letzten Jahren erzielte Fortschritte in der pluripotenten Stammzelltechnologie ermöglichen es potentiell, alle Zelltypen des Körpers in nahezu unbegrenzter Menge für die Verwendung in humanspezifischen Modellen zu generieren. Insbesondere das menschliche Gehirn unterscheidet sich wesentlich von dem der üblicherweise eingesetzten Tiermodelle und zudem sind Gewebeproben des menschlichen Gehirns schwer zugänglich und die daraus gewonnenen Zellen nur unzureichend *in vitro* vermehrbar. Daher ist besonders die Aussicht, humane neuronale Zellen aus pluripotenten Stammzellen (PSZ) zu gewinnen, sehr attraktiv.

Neuste Entwicklungen in den letzten Jahren haben es ermöglicht neurale Stammzellen aus unterschiedlichen Quellen zu isolieren oder zu generieren und unter Einsatz von Wachstumsfaktor-basierten Protokollen zu vermehren. Eine noch offene Frage ist jedoch, inwieweit diese verschiedenen Stammzellsysteme *in vitro* physiologische, *in vivo* existierende Stammzellstadien nachbilden.

Während der physiologischen Entwicklung des Nervensystems entstehen aus frühen, neuroepithelialen Stammzellen (NES), welche eine hochgradig polarisierte Morphologie aufweisen und empfindlich auf sogenannte regionalisierende Morphogenen reagieren, späte radiale Gliazellen (RG), die dann wiederum regionalspezifische Neurone generieren.

In vitro konnten bislang stabil proliferierenden neurale Stammzellpopulationen, welche neuroepithelialen Stammzellen gleichen, sowohl von humanen pluripotenten Stammzellen (lt-NES Zellen) als auch direkt aus dem fetalen humanen Hinterhirn (hbNES Zellen) gewonnen werden.

Darauf aufbauend wurde in dieser Arbeit ein sequentielles Differenzierungsprotokoll entwickelt, das die Generierung von RG-ähnlichen neuralen Stammzellen (RG-ähnliche NS Zellen) aus NES-ähnlichen Zellen (lt-NES und hbNES Zellen) *in vitro* ermöglicht. Durch die zeitlich begrenzte Anwendung differenzierender Zellkulturbedingungen werden NES-ähnliche Zellen dazu gebracht, die Entwicklung neuraler Stammzellen *in vivo* nachzuvollziehen und RG-ähnliche Zellen zu generieren.

Tatsächlich weist diese neu etablierte neurale Stammzellpopulation wichtige Merkmale von neuro- und gliogenen RG Zellen auf. RG-ähnliche NS Zellen können für mindestens

25 Passagen expandiert werden und exprimieren klassische neurale Stammzellmarker wie NESTIN und SOX2 sowie typischerweise RG Zell-assoziierte Marker wie SOX9, CD44, AQP4 und HOP während die NES Zell Marker PLZF, DACH1, ZO-1 und MMRN1 herunterreguliert werden.

Eine wichtige Erkenntnis dieser Studie ist, dass RG-ähnliche NS Zellen - generiert von PSZ-abgeleiteten lt-NES Zellen oder primär isolierten hbNES Zellen - ähnliche Eigenschaften zeigen wie primär aus fetalem Gewebe gewonnene RG Zellen. Dies deutet darauf hin, dass der Übergang von NES-ähnlichen Zellen in RG-ähnliche Zellen *in vitro* in der Tat die Entwicklung von frühen NES Zellen in RG Zellen während der Neurogenese *in vivo* nachvollzieht.

Eine große Herausforderung, im Hinblick auf die biomedizinische Anwendung von aus PSZ-abgeleiteten neuralen Stammzellen, ist ihre kontrollierte Entwicklung in unterschiedliche regionale Subtypen und die Aufrechterhaltung eines erworbenen regionalen Phänotyps während ihrer Kultivierung und Expansion *in vitro*. Vorangegangenen Arbeiten haben gezeigt, dass lt-NES Zellen kurz nach ihrer Generierung aus PSZ einen anterioren Vorderhirn Phänotyp aufweisen und in der Folge eine sukzessive regionale Posteriorisierung in einen Phänotyp durchlaufen, welcher dem ventralen Hinterhirn entspricht. Dieses Phänomen ist vermutlich auf die regionalisierenden Effekte der für die Proliferation *in vitro* notwendigen Wachstumsfaktoren (FGF2 und EGF) zurückzuführen.

Dieses Verhalten entspricht auch der Rolle von NES Zellen *in vivo*, wo sie zunächst auf Morphogene ansprechen und auf diese Weise die verschiedenen Regionen des Gehirnes generieren. In einem zweiten Schritt wird diese regionale Identität durch den Übergang in RG Zellen, welche nicht mehr durch regionalisierend Faktoren beeinflussbar sind, konsolidiert.

Um die Eigenschaft von RG Zellen, eine stabile regionale Identität zu besitzen, nutzbar zu machen, wurden zunächst RG-ähnliche NS Zellen von NES-ähnlichen Zellen mit unterschiedlicher regionalen Phänotypen abgeleitet (ventrales Vorderhirn, dorsales Vorderhirn, Hinterhirn, Rückenmark). Die hieraus gewonnenen regionalisierten RG-ähnlichen NS Zellpopulationen exprimieren trotz Exposition zu Wachstumsfaktoren über zahlreiche Passagen stabil Transkriptionsfaktoren, welche ihrer regionalen Identität entsprechen ohne zu posteriorisieren und behalten über die Zeit, die Fähigkeit sowohl in

Neurone, als auch in Gliazellen zu differenzieren. In Anlehnung an Studien zur heterotopen Transplantation von RG Zellen in Mäusen *in vivo* wird die regionale Identität von RG-ähnlichen Zellen selbst unter dem Einfluss starker regionalisierender Faktoren wie beispielweise Retinsäure aufrechterhalten. Werden diese regionalisierten RG-ähnliche NS Zellen differenziert, generieren sie zudem neuronale Subtypen, welche ihrer regionalen Identität entsprechen.

Die Ergebnisse dieser Studie deuten zusammenfassend darauf hin, dass die Überführung in RG-ähnliche NS Zellen eine Methode darstellt, die regionale Identität von frühen regionalisierten NES Zellen zu konservieren und so regional stabile neurale Stammzelllinien für biomedizinische Anwendungen zu etablieren.

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