Stage- and area-specific control of stem cells in the developing nervous system

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Abstract

The spatiotemporal control of proliferation and differentiation in neural stem cells (NSCs) is essential to produce a functional nervous system (NS). Stem cells in different areas and at different time points during development have to produce different types of cells in a precise manner. Recent studies uncovered a plethora of cell extrinsic as well as intrinsic factors that play crucial roles in the area-specific and stage-specific control of NSCs. Moreover, recapitulation of the spatiotemporal specification of NSCs in vitro opens new avenues for future applications. In this review, we have selected some key molecules to discuss the mechanisms underlying the spatiotemporal regulation of NSC development.
Stage- and Area-Specific Control of Stem Cells in the Developing Nervous System

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Summary of recent advances

The spatiotemporal control of proliferation and differentiation in neural stem cells (NSCs) is essential to produce a functional nervous system (NS). Stem cells in different areas and at different time points during development have to produce different types of cells in a precise manner. Recent studies uncovered a plethora of cell-extrinsic as well as intrinsic factors that play crucial roles in the area- and stage-specific control of NSCs. Moreover, recapitulation of the spatiotemporal specification of NSCs \textit{in vitro} opens new avenues for future applications. In this review, we have selected some key molecules to discuss the mechanisms underlying the spatiotemporal regulation of NSC development.

Introduction

During neural development a fairly small and seemingly homogenous starting population of neural stem cells (NSCs) build up what is often thought to be the most advanced and complex organ in the body, the nervous system. These NSCs are operationally defined as multipotent, self-renewing progenitors that acquire attributes to produce multiple types of neurons and glia in a time- and region- specific manner. In this review we focus on the question of how stem cells with a similar set of characteristics can generate all the different kinds of cell types of the nervous system in the right spatiotemporal succession. The relevance of this question becomes apparent when looking at the difference in size between distinct areas of the nervous system and the differences of the same areas between different species. In a given species the forebrain is bigger than the midbrain, while the forebrain in mice – also in relative terms – is much smaller than in humans. In addition, stem cells in different areas and at different time.
**Development and patterning of the early nervous system**

The vertebrate nervous system is derived from the neural plate, an epithelial sheet in the dorsal ectoderm induced during gastrulation. During neurulation the neural plate invaginates to form the neural tube. Around the time of neural tube closure, cells in the dorsal part of the neural tube become specified as neural crest cells that undergo an epithelial to mesenchymal transition and emigrate from the dorsal neural tube to give rise to most of the peripheral nervous system and various non-neural tissues [1]. The cells remaining within the neural tube give rise to the brain and the spinal cord, which together comprises the central nervous system (CNS). Neuronal subtype specification within the CNS begins with the molecular regionalization of the neuroepithelium. This involves a three dimensional patterning process imposed by morphogens gradients with distinct spatial orientation and signaling ranges [2] (Figure 1). Patterning along the anterior posterior axis depends, among others, on rostro-caudal gradients of FGF’s and Retinoic acid (RA) that induce different Hox expression profiles. These, in turn, determine neuronal subtypes along the neuraxis, such as specific motor neuron subpopulations present at distinct axial levels [3,4]. Regionalization of the neural tube along the dorso-ventral axis depends on signaling centers localized at the midline, namely the notochord and floor plate ventrally and the roof plate dorsally. The notochord and the floor plate secret the morphogen Sonic hedgehog (Shh) that induces ventral identities [5,6], while the roof plate produces a counteracting gradient of Wnt and BMP signals promoting dorsal identities [2,7-9]. Given the regional specification of the developing nervous system the question arises of how NSCs in different areas and at different time points are regulated with respect to proliferation, fate decision, and differentiation. It is evident that all NSCs share basic properties of self-renewal and multipotency. However, NSCs exhibit specific spatiotemporal features imposed by locally initiated cell-intrinsic programs that modulate responsiveness to the microenvironment [10].

**Stage-specific control of stem cells in the CNS**

Signaling via Receptor tyrosine kinases (RTKs) has crucial function not only during early patterning of the neural tube but also as mitogenic input signals in NSCs. During development NSCs of the CNS undergo a remarkable switch from early FGF responsiveness to later EGF responsiveness [11-13]. At least in vitro the signaling network controlling this temporally regulated switch likely involves BMP signaling and FGF signaling itself [13], FGF10, a specific member of the family of FGF ligands, controls the transition from neuroepithelial (NEP) cells to radial glia (RG), the progenitors of cortical neurons and basal progenitors. FGF10 is transiently expressed at the apical side of the neuroepithelium around the differentiation step.
from NEP cells to RG. Mutation of Fgf10 leads to delayed RG generation while \textit{in vivo} overexpression has the opposite effect [14].

An evident but very fascinating feature of the developing brain is to produce different neurons at different time points during development, or more pronounced: the right neurons at the right time. In studies with clonal cultures, using stem cells derived from the developing forebrain, Shen et al. demonstrated that NSCs are able to sequentially generate specific types of neurons and glia in the precise order found \textit{in vivo} [15]. These experiments showed that the timing mechanism is harbored within the stem cells and their clonal lineage, but does not depend on factors produced outside of their clonal lineage. Knocking down FoxG1, a forebrain specific winged helix/forkhead transcription factor, in middle-aged progenitors (E12) resets the timing mechanism, allowing these cells to produce neurons normally produced by earlier progenitors (E10). In contrast, the timing mechanism in older progenitors (E15) is not susceptible to the downregulation of FoxG1. Thus FoxG1 is essential for the appropriate stage-specific production of neurons, but has this ability only in a narrow time window.

In the developing CNS, different subtypes of neurons are not only generated in a time-specific manner, but neural stem cells also undergo a remarkable switch from producing neurons before E18 to glial cells after this stage [16]. Molecules shown to be involved in the temporal specification of this switch in forebrain progenitor cells are chicken ovalbumin upstream promoter-transcription factor I and II (Coup-tfI/II). The timely acquisition of gliogenic competence is inhibited in Coup-tfI/II knockdown NSCs, directing them to differentiate predominately into early-born neurons [17]. Coup-tfI/II knockdown cells do not respond to gliogenic cytokines such as LIF and BMP2. In addition, due to epigenetic regulation, the promoter for the glial marker GFAP is silenced in knockdown cells. Taken together, Coup-tfI/II appear to be essential for the acquisition of gliogenic competence in a temporally regulated manner. In addition, these molecules are also important for the temporal specification of NSCs to produce neurons in the right sequence [17].

\textbf{Stage-specific control of stem cells in the PNS}

During neural crest development, Wnt and BMP act synergistically on early, emigrating neural crest stem cells (eNCSC) to suppress differentiation and keep the cells in a multipotent stem cell state [18]. Wnt signaling on its own instructively promotes sensory neurogenesis in eNCSCs [19,20]. BMP signaling alone, on the other hand, instructively drives eNCSCs into the autonomic lineage \textit{in vitro} [21], while \textit{in vivo} BMP signaling regulates proliferation, differentiation and survival of NC-derived cells in a lineage specific fashion [22-24]. Cells with NCSC-like features can also be found in various postmigratory target structures of the neural crest, like in the embryonic sciatic nerve [25], adult skin [26,27], gut [28], adult dorsal root ganglia (DRG), whisker pad, and bone marrow [29]. However, such late stage NCSCs (e.g.
those found in the sciatic nerve or the DRG) do not undergo sensory neurogenesis upon treatment with Wnt1. Similarly, NCSCs maintained in culture with Wnt1 and BMP2 cease to respond to Wnt1 promoting sensory neurogenesis [18]. Taken together, in vitro and in vivo data demonstrate that NCSCs acquire cell intrinsic differences over time, leading to changes in the interpretation of the Wnt signal.

Since during development NCSCs loose responsiveness to cues controlling their fate and growth, the question arises of what molecules regulate self-renewal, proliferation, and fate decisions in late stage NCSCs. The small Rho GTPases Cdc42 and Rac1 are molecular switches that play a crucial role in transmitting extracellular stimuli to downstream effectors triggering specific cellular responses. In NC development, however, the requirement for these two factors is fully dependent on the specific stage of the cells. Deletion of either Cdc42 or Rac1 in the dorsal neural tube before emigration of the neural crest cells does not affect induction, migration, proliferation, and fate acquisition of eNCSCs. However, both Cdc42 and Rac1 are required for cell cycle control of virtually the entire pool of later NC stem and progenitor cells after they have reached their target structures [30] (Figure 2). During this developmental process neural crest cells undergo a remarkable transition in their growth factor responsiveness. eNCSCs emigrating from the neural tube first are only FGF, but not EGF, responsive. At later stages, NC cells start to express the EGF receptor and acquire EGF responsiveness, a process much resembling a transition also observed in the CNS [11-13]. Rac1 acting upstream of Cdc42 is required specifically to transduce EGF, but not FGF, signaling to control proliferation of later NC stem and progenitor cells. Thus, even though both early and late NCSCs display similar stem cell features, the mechanisms regulating their proliferation are distinct.

**Area-specific control of stem cells in the CNS**

Besides its role in the temporal specification of stem cells FoxG1 has also been reported to be responsible for region-specific differences in responsiveness of NSCs to regulatory cues. While deletion of Bmi-1 results in a progressive growth constraint due to reduced self-renewal of NSCs [31], overexpression of Bmi-1 enhances self-renewal of NSCs in culture. Interestingly, the effect of Bmi-1 overexpression is much more striking in FoxG1-expressing forebrain-derived NSCs than in FoxG1-negative cells derived from the developing spinal cord, and this increase in self-renewal of forebrain NSCs upon overexpression of Bmi-1 is FoxG1 dependent [32] (Figure 2). The Bmi-1-induced increase in self-renewal is potentiated with increasing age and passage number, showing a gradual change in the mechanism controlling proliferation of NSCs. However, when using a transgenic mouse line expressing Bmi-1 from the Nestin promoter, the effect of Bmi-1 overexpression on proliferation was only marginal [33], in striking contrast to the significantly induced proliferation observed upon acute Bmi-1 overexpression by in utero electroporation at E14 and in the adult subventricular zone [32].
Thus, levels and/or timing of its expression might influence the biological activity of Bmi-1 in NSCs.

Wnt represents an example for an extracellular signal that elicits local differences in the cellular interpretation of the active pathway. Wnt signaling exerts multiple functions in different types of stem cells, but in many it plays a crucial role in controlling stem cell maintenance and proliferation [for review see: 34]. In the forebrain overexpression of a stabilized, constitutively active form of β-catenin, an essential intracellular signaling component of the canonical Wnt-pathway, leads to an enlargement of the brain with increased cerebral cortical surface area, which is due to an increase in the number of stem and progenitor cells [35]. In the spinal cord, constitutively active Wnt-signaling also leads to an expansion of the progenitor pool [36,37]. In this region of the CNS, however, the effect on the progenitor pool is much more prominent in ventral as compared to dorsal regions, revealing local differences in signal interpretation [38] (Figure 2). Clonal culture experiments demonstrated that all cells in the developing spinal cord (i.e. dorsal and ventral) are responsive to Wnt. This shows that differences in the local environment rather than different cell-intrinsic properties are responsible for the differential interpretation of the same signal in vivo [38]. The authors proposed dorsally expressed BMPs to be responsible for the local modulation of Wnt/β-catenin-induced proliferation, leading to a model according to which the integration of Wnt and BMP signals regulates the progenitor pool size in the dorsal neural tube (Figure 2).

The progenitor pool size is also locally regulated by TGFβ signaling. Inactivation of TGFβ signaling in the developing CNS results in the lateral expansion of a specific area only, namely the dorsal midbrain. In this region, but not in the ventral midbrain or the dorsal forebrain, TGFβ signaling regulates cell cycle exit and self-renewal of neuroepithelial stem cells [39] (Figure 2). In this case it seems that cell-intrinsic differences as well as the region-specific extracellular microenvironment are responsible for the area-specific interpretation of the same signal. The latter is suggested as deletion of the TGFβ receptor type II (Tgfbr2) affects phosphorylation of the downstream signaling mediators Smad2/3 only in the midbrain, but not in the forebrain. Therefore, it is likely that other TGFβ superfamily members, signaling via a different type II receptor, compensate for the loss of TGFβ signaling. Proof for intrinsic differences comes from in vitro neurosphere [40] experiments, in which self-renewal of midbrain, but not forebrain NSCs was reduced by the addition of TGFβ1. This differential responsiveness likely involves the transcription factor FoxG1 that is specifically expressed in the forebrain neuroepithelium. Similar to wildtype midbrain cells, FoxG1-deficient forebrain cells acquire responsiveness to TGFβ in vitro, showing an increase in cell cycle exit and upregulation of p21 [41,42].

Concomitant with the enlargement of the dorsal midbrain, ectopic expression of Wnt and FGF ligands was observed. Furthermore, the enhanced self-renewal of Tgfbr2-mutant cells in culture is dependent on Wnt signaling and the presence of FGF [43] [39]. The combined data
leads to a model where the main function of TGFβ signaling is to counteract the Wnt and FGF pathways to control the size of a specific brain area (Figure 2). Interestingly, ectopic expression of Wnt1 in the dorsal and ventral mid/hindbrain region also leads to specific expansion of the dorsal midbrain, while leaving the ventral midbrain unaffected [44]. Therefore, in contrast to the spinal cord where canonical Wnt signaling has a more drastic effect on ventrally localized cells [38], in the midbrain dorsal cells are susceptible to misregulation of the Wnt pathway.

**Spatiotemporal recapitulation of NSCs features *in vitro***

During normal development, embryonic stem cells (ESC) are the primary source for all different cell types found in the body including NSCs and their progeny. In the culture dish ESCs can be differentiated into endodermal, mesodermal, and ectodermal cell types. Different protocols have been established to generate NSCs *in vitro*. While some use adherent monolayers and specific growth factors [45,46], other protocols employed 3D cell aggregates called embryoid bodies using RA [47] to induce NSCs. Using such protocols it has been possible to generate homogenous populations of specific neuronal subtypes, such as glutamatergic [47] or mesencephalic dopaminergic neurons [48]. Interestingly the spatiotemporal specification of NSCs isolated from the developing forebrain [15] can also be recapitulated in ESC *in vitro* systems [49,50]. Using a medium containing cyclopamine to counteract the ventralizing activity of Shh it is possible to sequentially generate the different kind of neurons found in the same temporal sequence in the cortex *in vivo* [49]. Employing clonal assays it was shown that this timing mechanism is encoded intrinsically within single cell lineages, similar as in NSCs taken out of the developing brain [15]. Moreover, the neurons generated *in vitro* when transplanted into the cerebral cortex form axonal projections corresponding mainly to areas of the occipital/visual cortex. This indicates that neurons with this cortical area-identity can be specified *in vitro* without the extrinsic influence of the surrounding tissue [49].

In similar experiments, Okada et al. derived NSCs from embryonic stem cells via embryoid bodies, using a neurosphere-based culture system [50]. These NSCs were capable of generating different types of neurons and glia. Importantly, when passaged in culture the cells exhibited a temporal progression analogous to the situation during CNS development *in vivo* [11-13]. NSCs from early passages were FGF-responsive, highly neurogenic progenitors whereas over repeated passages they became EGF-responsive, gliogenic NSCs with a late temporal identity [50]. To specify the rostro-caudal identity of these ESC-derived NSCs, the BMP pathway inhibitor Noggin and different concentrations of RA were used. Likewise, to regulate the dorso-ventral character of the progenitors in culture, Shh (expressed in the ventral floor plate and notochord), Wnt3a and BMP (both expressed in the dorsal roof plate of the developing CNS) were added. By using these molecules implicated in patterning of the
NS in vivo, it was possible to recapitulate the spatial identity of stem cells in vitro. It has to be noted, however, that these culture systems do not allow the generation of a homogenous population of one particular NSC type, and, as a consequence, also not of a specific subpopulation of neurons. Rather, the outcome of these cultures is always a mixed cell population reminiscent of the cells generated from a particular spatiotemporal origin in the developing CNS.

Conclusion and perspectives

In order to form a functional organ during development, stem cells in that organ have to be controlled such that they produce all different cell types needed. In addition, the right number of the appropriate cell type has to emerge at the right place and the right time. To achieve this stem cell proliferation and differentiation have to be finely tuned in a spatiotemporal manner. Several recent publications revealed multiple microenvironmental factors and cell-intrinsic determinants to play crucial roles in processes that designate the possible and the actual progeny of a given stem cell in the nervous system. This knowledge opened the possibility to recapitulate in cell culture a specific sequence of events occurring in vivo, which should provide a deeper understanding of the molecular and cellular mechanisms underlying development and disease. In addition, such systems are marvelous tools to screen for compounds influencing the generation, expansion, and survival of specific neuronal cell types as a first step towards novel pharmacological therapies. Thus, the thorough characterization of the mechanisms regulating the spatiotemporal specification of stem cells is a prerequisite to unleash the full potential of stem cells for future applications.
Acknowledgments

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Figure Legends

Figure 1

Neural tube patterning

Signaling pathways control the three-dimensional patterning of the neural tube. Along the rostro-caudal axis, gradients of FGF and RA are involved in the formation of different regional identities. Dorso-ventral regionalization of the neural tube depends on Shh released from the floor plate (FP) and Notochord (N) and Wnt and BMP produced by the roof plate (RP), respectively. Wnt/BMP is also involved in the specification of neural crest cells (NC) emigrating from the dorsal neural tube. For reviews introducing the different signaling pathways see: Shh, [51]; RA, [52]; Wnt, [53]; TGFβ superfamily, [54]

Figure 2

Signaling pathways governing area- and stage-specific control of stem cells.

The interplay between cell extrinsic and cell intrinsic factors determines NSC behavior in a spatiotemporal manner, as illustrated by selected examples from the recent literature.

**TGFβ**: In the dorsal midbrain (3), but not in the ventral midbrain (2) or dorsal forebrain (1), TGFβ antagonizes Wnt signaling and thereby negatively regulates self-renewal of NSCs [39].

**Wnt**: In the dorsal (4), but not ventral (5), spinal cord Wnt/BMP signal integration regulates proliferation and differentiation of NSCs [38].

**Bmi-1**: Bmi-1 promotes self-renewal of FoxG1-positive forebrain NSCs (1) to a much greater extent than in FoxG1-negative spinal cord NSCs (4 + 5) [32].

**Rac1 & Cdc42**: The small Rho GTPases Cdc42 and Rac1 are essential for the proliferation control through the EGF pathway in late (7) but not early (6) NCSCs [30].
References and recommended reading


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The timely transition from neuroepithelial cells to radial glia cells depends on the morphogen Fgf10. Deletion of Fgf10 leads to delayed radial glial differentiation selectively in the rostral cortex. The result is an increase in symmetric cell division, yielding more neuroepithelial cells and initially less neurons. The bigger pool of neuroepithelial cells eventually leads to an excess in neurons and basal progenitors resulting in an increased laminar thickness.


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COUP-TFI and II are essential molecular switches in the temporal specification of ESC-derived NSCs, including the acquisition of gliogenic competence with time. Injecting shRNA-encoding lentivirus into the ventricles of E10.5 embryos *in utero*, complemented by *in vitro* studies, identified Coup-tfI/II as crucial players in the timing mechanism that intrinsically determines NSC competence.


**Isolation and characterization of NCSC-like cells from various adult tissues revealed differences in self-renewal as well as differentiation potential according to the tissue source. This study also provides a potential explanation for the origin of neural cells formed from bone marrow in culture.**

**A combination of in vivo and elaborate in vitro assays demonstrated that the small Rho GTPases Cdc42 and Rac1 are essential for the proliferation control specifically of late EGF-responsive, but not of early FGF-responsive NCSCs. In Cdc42 and Rac1 mutant animals most if not all NC target tissues are reduced.**
in size due to an increase in cell cycle exit. Thus, these small Rho GTPases appear to control growth of virtually the entire pool of postmigratory NCSCs at fetal stages.


Overexpression of Bmi-1 in cell culture and by electroporation in vivo increases self-renewal of NSCs mainly by repressing the cell cycle inhibitor p21. However, the effect on self-renewal is much more striking in forebrain than in spinal cord-derived NSCs. This regional difference in the interpretation of Bmi-1 is due to the presence of FoxG1, which is specifically expressed in the forebrain and is essential for in the increase in self-renewal upon Bmi-1 overexpression.


TGFβ signaling specifically regulates self-renewal of NSCs in the dorsal midbrain, but not of the ventral midbrain or the dorsal forebrain. Deletion of Tgfbr2, an essential receptor for the transduction of the TGFβ signal, leads to induction of canonical Wnt signaling and the specific expansion of the dorsal midbrain neuroepithelium. The area-specific interpretation of TGFβ depends on extrinsic as well as on intrinsic differences of NCSs in the forebrain and the midbrain.


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Starting from ESCs it is possible to completely recapitulate the spatiotemporal specification of NSCs in the cerebral cortex in vitro, resulting in the sequential generation of neurons found in the different layers of the cortex. In addition, this paper demonstrates that it is possible to specify neurons in vitro with a particular cortical area-identity, namely of the occipital/visual cortex. Thus, the intrinsic program of corticogenesis can be engaged even in cells that were never located in a brain.


Figure 1
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