

Emergence of neuronal diversity from patterning of telencephalic progenitors

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ABSTRACT

During central nervous system (CNS) development, hundreds of distinct neuronal subtypes are generated from a single layer of multipotent neuroepithelial progenitor cells. Within the rostral CNS, initial regionalization of the telencephalon marks the territories where the cerebral cortex and the basal ganglia originate. Subsequent refinement of the primary structures determines the formation of domains of differential gene expression, where distinct fate-restricted progenitors are located.

To understand how diversification of neural progenitors and neurons is achieved in the telencephalon, it is important to address early and late patterning events in this context. In particular, important questions include: How does the telencephalon become specified and regionalized along the major spatial axes? Within each region, are the differences in neuronal subtypes established at the progenitor level or at the postmitotic stage? If distinct progenitors exist that are committed to subtype-specific neuronal lineages, how does the diversification emerge? What is the contribution of positional and temporal cues and how is this information integrated into the intrinsic programs of cell identity?

INTRODUCTION

One of the biggest challenges in developmental neurobiology is to understand how hundreds of distinct subtypes of neurons are generated from spatially and temporally related progenitor pools.

Regional patterning of the neural tube along antero-posterior (AP) and dorso-ventral (DV) axes represents one of the earliest events in the formation of distinct central nervous system (CNS) structures. This is achieved through the concerted activity of a series of organising centres that release diffusible signalling factors and ultimately give rise to progenitor cells with positional identity.

The molecular mechanisms of cell fate specification have been predominantly studied in the spinal cord, where progenitor cells integrate cues from morphogen gradients to establish progenitor domains that are defined by unique transcription factor codes¹. Discrete subtypes of spinal motor and interneurons later arise from these different domains with exquisite spatial precision.

In comparison with the spinal cord, in the telencephalon, the scenario is complicated by the multitude of different neuronal types and by the complexity of their spatial organisation into tri-dimensional structures, as opposed to the simpler tubular arrangement of more posterior CNS regions.

Although progress has been made in understanding the formation of the primary telencephalic subdivisions, molecular mechanisms that underlie the diversification of initially multipotent progenitor cells remain unknown. Furthermore, the means by which individual progenitors can temporally generate a sequence of different cell types is still the subject of intensive research.

Here, we focus on the developing telencephalon and consider how intrinsic and extrinsic, spatial and temporal factors can interact to pattern neural progenitor cells, ultimately generating the immense neuronal diversity required for a functioning CNS.

1 SPECIFICATION OF TELECEPHALIC TERRITORY

During the development of the mammalian central nervous system (CNS), the coordination of growth and patterning of the neural tube shapes the single-layered neuroepithelium into distinct yet connected brain structures. The brain arises from a series of vesicles at the cephalic end of the neural tube (Fig. 1A) with subsequent development, folding

and differential enlargement giving rise to the subdivisions of the fore-, mid- and hind-brain regions ². The first stage in the specification of the telencephalic territory is the acquisition of anterior identity within the prosencephalic vesicle. A two-step model proposes that the neuroectoderm adopts an anterior identity during initial neuralisation, and in a subsequent step, the prospective caudal regions are posteriorised in response to a gradient of secreted factors such as members of the Wnt family. Prospective anterior forebrain regions are protected from this posteriorising effect through both the secretion of Wnt antagonists from the anterior patterning centres, and by reduced responsiveness of anterior progenitors to Wnt signalling ^{3,4}. Two early anterior neural fate determinants, *Hesx1* and *Six3* repress the expression of Wnt signalling components such as *Axin2* and *Wnt1* respectively; the importance of this mechanism being emphasised in the *Hesx1* and *Six3* mutant embryos that fail to maintain anterior forebrain identity and instead exhibit rostral expansion of posterior markers ⁵⁻¹⁰.

However, anterior identity is also under active control through Fgf signalling ^{5,11}. The anterior neural ridge at the most anterior neural border also secretes several Fgf ligands, including Fgf8. Fgf8, in turn, promotes induction and maintenance of the forkhead box transcription factor FoxG1 (also called brain factor 1, BF1) that is expressed at the early neural plate stages in the prospective telencephalic territory ^{12,13}. Correspondingly, over-expression of Fgf8 by *in utero* electroporation results in expansion and caudal shift of rostral areas ^{14,15}, and deletion of FoxG1 leads to a substantial reduction of the telencephalic structures ¹⁶.

Thus, a recurrent model emerges in which the coordinate activity of morphogen gradients initiate and maintain the expression of transcription factors that specify and characterise discrete domains within the CNS. Subsequent dorso-ventral patterning within the telencephalic domain gives rise to pallial and subpallial compartments, the origins of the cerebral cortex and the basal ganglia, respectively.

1.1 Establishment of dorsal pallial identity

Although initial specification of the telencephalic territory requires protection from high level Wnt activity, subsequent acquisition of dorsal identity within this domain requires the sequential activation of Wnt and Fgf signalling ¹⁷, together with additional factors such as BMPs and EGFs that may also play instructive roles ¹⁸.

Dorsally located forebrain progenitors become exposed to increasing Wnt signalling activity as development progresses. Firstly, the anterior neural ridge that secretes Wnt

antagonists at early stages becomes spatially restricted into a small patch of cells at the commissural plate, thus reducing the amount of Wnt antagonists in this region. Secondly, two additional patterning centres arise to establish a dorso-ventral gradient of Wnt signalling (Fig. 1B,C). The cortical hem at the dorsal midline starts to secrete Wnt molecules, whereas the anti-hem, located in the lateral cortex at the level of the pallium/subpallium border, secretes the Wnt antagonist Sfrp2¹⁹⁻²¹.

The way in which the Wnt signalling gradient regulates cortical patterning is not completely understood, but it seems to control the expression of transcription factors that promote the acquisition of dorsal identity²². Among these transcription factors, Empty spiracles homologue 2 (Emx2), Paired box 6 (Pax6) and Neurogenins (Neurog1 and Neurog2) play crucial roles²³⁻²⁶, Emx2 is a direct Wnt signalling target and its expression decreases in β -catenin loss-of-function mutants^{27, 28}. Pax6 expression is also altered in a Wnt signaling mutant that carries constitutively active β -catenin and this mutant also shows expression of Neurog2 in the ventral forebrain and concomitant decrease of ventral markers²⁸.

The expression of Emx2 and Pax6 establishes dorsal identity of cortical progenitors and Pax6; Emx2 compound mutants show absence of the cortex and expansion of the ventral domains throughout the telencephalon²⁴. Neurog1 and Neurog2 contribute to the dorsal specification of cortical progenitors, as shown by the loss of dorsal marker expression in the cortex of Neurog1; Neurog2 double mutants. Neurog1; Neurog2 mutant cortices also show an up-regulation of ventral markers, such as the Achaete-scute homologue Ascl1 and Distal less Dlx1-2 and 5²⁶. In addition, a member of the high mobility group of factor, Sox6, has been shown to cooperate with Neurog2 during the specification of dorsal fate²⁹. Sox6 regulates pallium-subpallium segregation by repressing the expression of a ventral transcriptional programme in pallial progenitors. Altogether, these studies indicate that these transcription factors predominantly act through inhibition of ventral specification while inducing dorsal fate.

The neocortex, which represents the phylogenetically most recent cortical area, unique to mammals, emerges from the dorsolateral region of the telencephalic vesicles and exhibits a molecular profile distinct from the adjacent medial (archicortex) and lateral (paleocortex) regions. Specification of neocortical identity requires the expression of the LIM homeobox transcription factor Lhx2, which selects neocortical progenitor fate from an already-specified dorsal telencephalic domain, partially by suppressing hem and anti-hem identity in the medial and lateral cortex, respectively³⁰⁻³².

1.2 Specification of the ventral telencephalon

Dorso-ventral specification is acquired as early as E8 in the mouse embryonic telencephalon. Whereas dorsal identity requires the presence of Wnt among other signals, ventral identity is similarly promoted by the combined activities of Sonic hedgehog (Shh), Fgf and possibly retinoic acid signalling^{33,34}. Shh is expressed in the prechordal plate at E8 (Fig. 1B), underlying the telencephalic primordium, and later becomes secreted from the hypothalamic anlage and the ventral telencephalon. Here Shh is required for the expression of subpallium restricted transcription factors, such as *Dlx1/2*, *Ascl1* and the homeobox genes *Nkx2.1* and *Gsx2*, whose expression is reduced in Shh mutants³⁵⁻⁴¹. Ventral telencephalic structures are completely absent in mutant mice that lack Shh gene⁴², whereas forebrain-specific deletion of Shh signalling from E9 in the mouse leads to early failure of ventral patterning and loss of neurons usually generated from this region³⁹, suggesting that Shh activity may control different aspects of subpallium development in addition to initial DV patterning.

The downstream mechanism of Shh activation has been extensively studied in the spinal cord, where differences in Shh concentration and exposure duration determine the balanced activity of Shh target Gli proteins^{43,44}. In the telencephalon, Shh is involved in a feedback mechanism with *Fgf8* that coordinates the functions of different patterning centres. In the absence of Shh, *Gli3* acts as a repressor and inhibits *Fgf8* expression; through *Gli3*-mediated de-repression, Shh maintains *Fgf8* expression in the anterior neural ridge, and in turn, *Fgf8* indirectly induces Shh expression in ventral patterning centres^{13,45,46}. This Shh-mediated inhibition of *Gli3* repressor function and possibly induction of *Gli1* and *Gli2* activators in restricted ventral subregions also regulates the pattern of expression of transcription factors like *Nkx2.1*, *Gsx2* and *Nkx6.2*^{35,37,47} that maintain ventral telencephalic identity and specify ventrally-derived neuronal subpopulations. However, concomitant deletion of Shh and repressor *Gli3* successfully rescues the DV patterning defects observed in Shh mutants, indicating that Shh-independent mechanisms may also operate to pattern the ventral telencephalon⁴⁸. Furthermore, Fgf signalling may have ventral specification functions that are independent of Shh. *FgfR1/2* double knock-out mice exhibit defects in DV patterning despite normal Shh expression⁴⁹, and *Fgf8* overexpression induces ventral markers even in the presence of Shh inhibitors⁵⁰. Moreover, both inhibition of Fgf/ERK signalling and compound mutation of either *Fgf3/8* or *FgfR1/2* results in DV patterning defects with ventral expansion of dorsal markers^{49,51,52} (for recent review on Fgf signalling in neural development, refer to⁵³). Thus, consistent with the model presented previously, ventral

telencephalic structures are specified through the integrated activity of multiple signalling paths that establish defined patterns of gene expression.

2 CORTICAL AREALIZATION

2.1 Specification of distinct cortical areas

Although the general pattern of CNS development is conserved throughout evolution, the subsequent elaboration of the dorsal telencephalon has led to a dramatic expansion of the cerebral cortex during mammalian development⁵⁴⁻⁵⁶. Cortical progenitors and their neuronal progeny exhibit highly organized spatial distribution, both radially in terms of the laminar microarchitecture (see below) and tangentially in terms of functional subdivisions, also known as arealization⁵⁷.

The four primary functional distinctions include one frontal motor area that controls our movements, and three sensory areas to process auditory, tactile and visual information. The mechanisms that regulate cortical arealization have been the subject of intense debate; the protomap model proposes that patterning is intrinsically encoded in cortical progenitors^{58,59}, whereas the protocortex model suggests that thalamic afferent connections that reach the cortex impart specific areal identity through a neuronal activity-dependent event⁶⁰. This has now been reconciled with the recognition that the establishment of cortical area identity begins early in CNS development, even before any functional diversification is apparent, and it is later refined by the formation of neural circuitry⁶¹.

In the last 15 years the influence of genetic factors on cortical arealization has gained increasing attention and several transcription factors that exhibit cortical patterning activity have been identified (Fig. 2). For example, the combinatorial graded expression of the transcription factors *Emx2* and *Pax6* determines the formation of distinct progenitor domains along the rostro-caudal axis. *Emx2* exhibits a high caudo-medial to low rostro-lateral gradient with *Pax6* reciprocally expressed^{62,63}. Analysis of knockout animals reveals that these counter gradients regulate the relative size of motor (frontal) and sensory (caudal) areas of the cortex. Moreover, *Emx2* overexpression induces *Pax6* down-regulation in rostral areas and results in a rostro-lateral shift of sensory and motor areas, thus establishing a direct patterning role for *Emx2*⁶⁴.

Similarly, patterning functions have been ascribed to the orphan nuclear receptor COUP-TFI (also known as *Nr2f1*) and the *Drosophila* buttonhead homologue *Sp8*. COUP-TFI is required for specification of caudal sensory areas⁶⁵, but may also have roles in ventral

specification during patterning of pallium ⁶⁶, whereas Sp8 plays a crucial role in the establishment of rostral cortical identity ^{67,68}. A reciprocal gradient of expression is generated by cross-repressive interaction between Sp8 and COUP-TFI ⁶⁹. Indeed, Sp8 mutants exhibit rostral expansion of the caudal marker COUP-TFI ⁶⁸, whereas cortex-specific deletion of COUP-TFI results in enlarged frontal motor areas at the expense of caudal sensory regions ⁶⁵.

Furthermore, alterations in cortical area identity observed in the mutants for Emx2, COUP-TFI and Sp8 are paralleled by altered thalamocortical projections that maintain area-specific connections ^{62, 65, 68}. Each cortical area receives specific inputs from the principal thalamic nuclei that relay information from subcortical regions. During embryonic and early postnatal development a conserved and stereotyped pattern of connectivity is established between the cortex and the thalamus and can be easily visualized via lipophilic dyes that follow axon tracts. The use of retrograde and anterograde axon tracing reveals that the cortical areas in Emx2, COUP-TFI or SP8 mutants, even if shifted or contracted, maintain area-specific connections with the thalamus, thus suggesting that a strong genetic pre-patterning is in operation.

2.2 Establishment of transcription factor domains

The transcription factor gradients described above are in turn established by the coordinate response of progenitor cells to secreted morphogen signals from various patterning centres located in different antero-posterior, dorso-ventral and medio-lateral positions. For example, Fgf signalling was introduced earlier with a role in initial specification of the telencephalic territory, and several Fgf factors such as Fgf8, Fgf15, Fgf 17 and Fgf18 are secreted from the anterior patterning centers to promote rostral cortical identity ⁷⁰⁻⁷². Fgf8 controls the expression of several patterning transcription factors, including induction of Sp8 in rostral regions ⁶⁷, and the repression of Emx2 and COUP-TFI that become restricted to more caudal regions of the cortex ^{13, 73}. However, distinct Fgf members can differentially regulate COUP-TFI expression and this, in turn, can feedback to refine the region of active Fgf signalling through inhibition of downstream pathways ⁷⁴. Fgf17 represses COUP-TFI expression and induces rostral fate, acting similarly to Fgf8 predominantly in the dorsomedial region ^{75,76}, whereas Fgf15, whose expression extend in the caudal-most region of the ventral pallium⁷⁷ induces COUP-TFI expression and promotes caudal cortical fate ⁷⁸. Furthermore, Fgf signalling can be modulated by secreted molecules from other patterning centres ¹⁸. For example, the cortical hem releases members of the BMP and Wnt families that regulate the expression of patterning factors, such as Emx2, through both direct Emx2 transcriptional

control and indirect modulation of *Fgf8* expression^{18, 27, 79}. Recently, a novel transcription factor, *Dmrt5* (double sex and mab3 related) has been shown to promote caudal fate by maintaining *Wnt* and *BMP* expression in the cortical hem, thus ultimately inducing *Emx2* expression⁸⁰.

Taken together, progenitor cells receive a cocktail of morphogen signals, the relative intensities of which will vary with the relative distance from the secreting patterning centres. Integration of these signalling paths determines a unique signature of transcription factor expression that defines the spatial identity of the progenitor cells; when considered as a progenitor population, this creates the spatial gradients of expression that are described above.

However, these spatial gradients must then be translated into sharp borders of gene expression that demarcate the margins of future cortical areas. The mechanisms underlying this process are not well described in the telencephalon, but studies performed in different contexts indicate that a threshold phenomenon may be involved^{1, 43}; a certain level of transcription factor is required to trigger the activation of a specific set of genes, consequently establishing the limits of target gene expression.

Additionally, a novel framework to explain the segregation of pallial subdivisions has been recently proposed⁸¹. Pattabiraman and colleagues identified several human enhancers whose activities are restricted to distinct progenitor domains at early developmental stages. By deploying an inducible Cre-mediated approach, the authors followed the fate of the progenitor domains that display single enhancer activity at E11.5; these domains maintain relative spatial locations at later embryonic stages, corresponding to specific regions of the prefrontal cortex. While the pattern of enhancer activity does not exactly match the domain of expression of any single transcription factor, several factors involved in cortical patterning (such as *COUPTFI* and *Pax6*), have been shown to bind some of these enhancer elements. Together, these results suggest that the graded expression of different transcription factors may be integrated at the enhancer level to generate distinct patterns of gene activation in different pallial subdivisions.

Downstream of these genomic and genetic factors, various cellular mechanisms may be in place to establish and maintain the areal pattern in the mature cortex. Although these are still poorly understood, several lines of evidence indicate that cell adhesion and cell repulsion molecules might contribute to the segregation of early patterned cortical domains, by promoting differential adhesiveness and cell repulsion at the borders of area subdivisions⁸². Indeed, members of the Cadherin family of adhesive factors and axon guidance molecules of

the Eph-Ephrin family exhibit restricted patterns of expression in specific cortical areas and they have been used so far as markers for area demarcation⁸³⁻⁸⁷. However, whether these molecules also play a role during the segregation of distinct cortical areas is still unclear. Recent work suggests that Cdh6 may actively function to establish or maintain area identity; Cdh6 expression at early stages demarcates the borders of areas that will only later segregate into functionally distinct cortical regions, such as the barrel field and the limb field⁸⁸.

In the future, it would be interesting to study whether factors like Cadherin and Eph-Ephrins are direct target genes regulated by the patterning transcription factors^{81, 89}.

3 NEURONAL DIVERSITY IN THE DORSAL TELENCEPHALON

3.1 Progenitor cells within the cerebral cortex

In addition to the tangential subdivisions, the cerebral cortex is characterised by its histological laminar architecture; six radially arranged layers that are populated by an astonishing variety of neuronal subtypes, each with distinct morphological, hodological and molecular properties in coordination with their final laminar positions^{90, 91}. For example, corticofugal neurons occupy deep cortical layers V and VI, projecting either to the thalamus or to subcerebral targets such as the spinal cord and the brainstem, and express specific transcription factors including Fezf2, Tbr1 and Sox5⁹²⁻⁹⁵. In contrast, upper layer II-III neurons project to the contralateral hemisphere via the corpus callosum and express transcription factors such as Cux1, Cux2, Satb2 and POU3F3/2⁹⁶⁻¹⁰⁰. Moreover, intra-laminar variation of subtype adds further complexity to the vast array of neurons that populate the cortex. One of the key questions in developmental neurobiology is how this neuronal diversification is achieved: are lineage-specific properties imparted at the post-mitotic stage or do fate-restricted progenitors exist in the cortex?

Pioneering transplantation experiments and birth dating studies gave rise to the 'progressive restriction model'^{58, 101, 102}. Neurons of lower and upper layers are generated in a temporal order, with final laminar fate determined by neuronal birth date, and progenitor cells becoming restricted in their differentiation potential as development progresses. Accordingly, neurons generated at early developmental stages (e.g prior to E14 in the mouse), differentiate into deep layer V-VI neurons, whereas later born neurons migrate radially and settle into the upper layers II-III-IV, creating an "inside-out" arrangement¹⁰³⁻¹⁰⁵ (for recent reviews refer to:^{91, 106}). This model has been predominantly interpreted as a proof for the existence of only one progenitor type that changes and restricts its fate potential over time.

However, extensive research in the last decade has identified different types of progenitor cells that vary in morphology, mitotic behaviour and molecular profile^{107, 108}. In rodents, the most abundant neocortical progenitors are radial glia cells (RGCs) and intermediate progenitors (IPCs). RGCs are present from very early stages in mouse development (E9-E10 onwards), dividing at the apical border both symmetrically to self-renew and maintain the pool of progenitors, and asymmetrically to additionally give rise to a more committed neuronal progeny^{109, 110}. IPCs instead divide in more basal regions of the subventricular zone (SVZ), and they do not make contact with the ventricle or the pial surfaces. The accumulation of dividing IPCs in the SVZ starts around E13 and IPCs generally cycle only once or twice before undergoing neurogenic symmetric divisions to produce two post-mitotic neurons¹¹¹. Importantly, IPCs produce neurons of all layers, whereas only 10% of neurons arise directly from RGC differentiation^{111, 112}. More recent works have described the existence of additional progenitor types that exhibit combined RGC and IPC properties¹¹³. For example, short neural progenitors (SNP) have lost contact with the basal surface but still divide in the ventricular zone (VZ)^{114, 115}, and the newly identified outer radial glia cells (oRG) retract the apical process and divide in more basal regions like IPCs, yet their ability to self-renew and lack of basal marker *Tbr2* are properties shared with RGCs^{116, 117}. Most of these progenitor types have been described both in rodents and in humans or non-human primates. However their relative abundance varies according to the species. In humans, the impact of oRG cell proliferation on neuronal output is much greater than in mice (oRGs in rodents represent only 6% of all murine cortical progenitors, whereas in humans oRG cells represent 40% of the progenitors in the outer SVZ^{117, 118}), and it contributes to the tangential expansion of the gyrencephalic neocortex⁵⁵. Although these progenitor types show differences in morphology and spatial locations, it is unclear whether they also differ in fate potential and preferentially give rise to specific neuronal subpopulations.

An alternative approach has characterised transcription factors that are expressed in progenitor cells and subsequently maintained in layer and neuron subtype-specific postmitotic neurons, making them attractive candidates to specify neuronal subtypes. Among them, the zinc finger transcription factor *Fezf2* and the orthodenticle homologue 1 (*Otx1*) are expressed in the VZ progenitors at the time of deep layer neuron generation, and these same factors are later maintained in post-mitotic layer V and VI neurons^{92, 93, 119, 120}. Similarly, *Lhx2* is expressed both in progenitors and in the upper layer neurons that originate from them^{31, 121}. Nevertheless, these factors may not be directly involved in the specification of deep or superficial-layer neurons, and indeed, recent lineage tracing experiments have shown that

Fezf2-positive RGCs are multipotent progenitors that differentiate into neurons of all cortical layers and into glial cells ¹²². This is consistent with clonal analysis, showing that clonally labelled radial glia cells give rise over time to neurons of different layers within the same columnar unit ^{102, 123-125}.

Interestingly, a lineage association has been reported for the transcription factor, Cut like 2 (Cux2) ¹²⁶. Franco and colleagues developed an inducible lineage tracing technique to show that only Cux2 positive progenitors give rise to Satb2 positive upper layer neurons, and Cux2 negative cells instead generate deep layer neurons, demonstrating that a lineage relationship exists between fate-restricted progenitors and layer-specific neurons. Moreover, although upper layer neurons are generally born at late developmental stages, Cux2 positive progenitors have been detected also at early time points, even before the onset of upper layer neuron generation. Indeed, forced cell cycle exit and differentiation of Cux2 positive cells at very early time points gives rise to correctly specified upper layer neurons, indicating that neuronal fate is primarily governed by intrinsic determinants and not by the time of birth ¹²⁶.

This view is, however, controversial and contrasts with other available data. For example a lineage tracing approach similar to the one employed by Franco et al revealed that Cux2 positive cells also populate lower layers and express Ctip2, a marker of lower layer neurons ¹²². It is important to note though that the analysis showing Cux2 contribution to lower cortical layers was performed only at an early postnatal stage (P0) when the cortical layers are still intermingled and cells are still migrating. Therefore analysis at a later stage is needed to strengthen the conclusion that Cux2 positive cells contribute to all layers of the cortex.

Additional evidence that challenges the view of Cux2 as a fate determinant of upper layer neuron identity in progenitors comes from the analysis of Cux2 null mutants, in which upper layers are not lost but expanded, and upper layer neurons are correctly specified ¹²⁷. Therefore, further work is needed to clarify the role of Cux2 in neuronal subtype specification and the existence of Cux2 positive (and/or Cux2 negative) fate-restricted progenitors in the cortex ¹²⁸.

3.2 Mechanisms of generating cortical progenitor diversification

A related field of investigation is directed towards elucidating the mechanisms of progenitor diversification. So far only a few signals and factors have been identified that can differentially influence the fate or cell cycle properties of specific cortical progenitors. From the discussion above it is clear that progenitors in distinct locations and/or at different

developmental stages may be subjected to diverse environmental cues that instruct their fate. It has been proposed that temporal restriction in progenitor fate potential may be caused not by intrinsic limitations, but by progressive changes in the extracellular environment that supports the proliferation and differentiation of distinct progenitors over time ¹⁰⁶. For example, lower layer neurons, which are the first to populate the cortical plate, release soluble factors like Nt3 and members of the Fgf family (Fgf18, Fgf9) that feed back to the VZ progenitors and switch them to the production of the next neocortical layer and glial cells ^{129, 130}.

Direct cell-cell communication via Notch signalling may also be influential in progenitor cell fate; the expression of Notch ligands in IPCs and neuroblasts activates Notch signalling in RGCs to maintain them as progenitors ^{131, 132}. Although differences in Notch signalling among RGCs during early or late neurogenesis have not been described, it has been proposed that Notch activation may primarily induce proliferation of RGCs during early neurogenesis, so that the progenitors of upper layer neurons expand during lower layer neurogenesis ¹⁰⁶. Intrinsic cell cycle programs and morphological properties, such as directional protrusions, can also contribute to the differential reception of the above signals. For example, the basal process that spans the entire cortical plate enables RGCs and oRGs to sense signals such as Notch ligands that promote self-renewing divisions ^{118, 133, 134}. Differences in Notch activation may also stem from asymmetrical inheritance of fate determinants that govern the fate of the daughter cells after cell division. Polarity complexes, such as Par3/aPKC regulate the distribution of intracellular components of the Notch signalling pathway (Mindbomb, Numb and Numb-like) and they also determine which cell will inherit the apical or the basal process ^{133, 135}. Additional pro-differentiative signals, such as retinoic acid and BMP7, are released from the meninges on the outer surface of the brain, possibly influencing the fate of cells that possess the basal process. However, how these signals can differentially affect progenitor lineage-restriction or the cell cycle properties is currently unknown.

Furthermore, in contrast to IPCs and oRGs, RGCs also contact the ventricular surface at the apical side and extend a primary cilium into the lateral ventricles that are filled with the corticospinal fluid (CSF). Proteomic analysis of the CSF has identified Igf2, a growth factor that binds to the Igf receptor localized to RGC cilia ¹³⁶. Interestingly, Igf2 is enriched at late stages of neurogenesis and specifically promotes the differentiation of late-born upper layer neurons, as demonstrated by the selective decrease in this neuronal population in Igf2 knockouts.

Therefore, the different morphological properties displayed by distinct progenitor types may underlie their capacity to sense and respond to environmental cues coming from the extracellular milieu. Further studies will be important to understand the contribution of different signals to the restriction of progenitor fate potential.

4 NEURONAL DIVERSITY IN THE VENTRAL TELENCEPHALON

4.1 Progenitor cells within the subpallium

The subpallium is the site of origin of multiple neuronal types that principally occupy the ventral telencephalic structures of the striatum, globus pallidus and other nuclei within the basal ganglia. The striatum is largely composed of the GABAergic medium-sized spiny projection neurons (MSNs) that receive cortical input and target other ventral forebrain nuclei to process motor and cognitive information ^{137, 138}. In addition to MSNs, distinct subtypes of GABAergic and cholinergic interneurons also settle in the striatum and modulate the activity of MSNs. The basal forebrain gives rise not only to neurons that remain within the ventral structures, but also to a large variety of GABAergic interneurons that migrate long distances to populate the cortex and the olfactory bulb ¹³⁹⁻¹⁴¹.

In contrast to neurogenesis in the dorsal pallium, it is well established that each neuronal subtype originates from different progenitors located in discrete domains of the subpallial VZ (Fig. 3). A primary subdivision of the subpallium occurs early in development with the formation of three evaginations, the ganglionic eminences (GEs), in the lateral (LGE), medial (MGE) and caudal (CGE) positions. Early transplantation studies and recent fate mapping experiments revealed that the LGE is the major source of striatal MSNs and of olfactory interneurons, whereas the MGE and CGE generate the majority of the cortical interneurons ^{142, 143}. Each domain expresses a precise combination of transcription factors that play instructive roles in the specification of the distinct neuronal subtypes.

The **MGE** is characterised by the expression of Nkx2.1, which is required for the generation of GABAergic cortical interneurons and for the suppression of alternative cell fates ^{144, 145}. Gene expression profiling of the MGE reveals a high degree of heterogeneity of the Nkx2.1 positive domain, indicating that further subdivisions of the MGE exist ^{146, 147}. It is possible that distinct combinations of transcription factors in each of these MGE subregions may define fate-restricted progenitors that will give rise to specific interneuron subpopulations. For example, coexpression of Nkx2.1 with Nkx6.2 in the most dorsal part of

the MGE, has been shown to induce the specific generation of calretinin (CR)+ and somatostatin (SST)+ non-fast spiking Martinotti interneurons ^{148, 149}.

Within the single population of cortical GABAergic interneurons derived from the Nkx2.1-positive and Nkx6.2-negative domain, there is an astonishing variety of subtypes, distinguished by unique combinations of neurochemical, morphological and electrophysiological properties ^{150, 151}. As in the neocortex, we have little understanding of how this high degree of neuronal diversity is achieved in the ventral telencephalon.

Progenitor types with distinct morphologies have been identified in the subpallium (RGC, SNP, IP and the newly described SAP ¹⁵²) (for recent reviews refer to: ¹⁰⁸). However it is currently unknown whether these represent distinct subtype-restricted progenitors. Fate mapping studies and electrophysiological analysis point towards the existence of one progenitor that changes fate potential for discrete interneuron subtypes over time ^{142, 153}. This is consistent with early birth-dating experiments, showing that early born interneurons preferentially populate deep cortical layers where age-matched cortical projection neurons are located, whereas later-born interneurons mainly settle into more superficial layers¹⁵⁴⁻¹⁵⁶. Recent clonal analyses also support the temporal generation of distinct interneuron subtypes ^{157, 158}. Interestingly, clonally related or synchronously born interneurons tend to cluster together once they reach the cortex, suggesting that some aspects of the final functional organization of inhibitory interneurons in the cortex are established at the progenitor level.

A key transcription factor known to act downstream of Nkx2.1 in the regulation of cortical interneuron specification and migration is the homeodomain protein Lhx6 ¹⁵⁹⁻¹⁶¹. Similar to Nkx2.1 deletion, loss of Lhx6 results in a reduced number of MGE-derived GABAergic interneurons in both the cortex (parvalbumin-PV+ and SST+) and striatum ^{159, 161}. Moreover, Lhx6 expression in the MGE has been shown to identify a bi-potent common precursor for both GABAergic and cholinergic striatal interneurons ¹⁶². As development progresses, subsequent expression of Lhx7 and Isl1 down-regulates Lhx6 and specifies cholinergic identity, whereas the maintenance of Lhx6 expression induces GABAergic fate. Lhx7 and Isl1 interact to bind to cholinergic genes, such as acetylcholine synthesizing enzyme (AChE) and transporters (ChAT) ¹⁶³ and the striatum of Lhx7 null mutants exhibits a strong reduction in ChAT+ Isl1+ cells, with a concomitant increase in GABAergic Lhx6 positive interneurons ¹⁵⁹. Indeed, in both full ¹⁵⁹ and ChAT-Cre ¹⁶⁴ conditional Lhx7 knockouts, the committed precursors for cholinergic interneurons switch fate in the absence of Lhx7 and differentiate towards GABAergic interneurons of the striatum. Not only does this suggest the

presence of a common bipotent progenitor that is biased towards the GABAergic fate, but also emphasises the plasticity of progenitor fate specification after initial commitment.

Progenitors located in the **LGE** are instead characterised by high expression of Gsx2, which has distinct temporal and spatial roles in the production of striatal projection neurons and olfactory bulb interneurons¹⁶⁵. Gain and loss of Gsx2 function experiments performed at different embryonic time points, reveal that Gsx2 promotes the acquisition of striatal interneuron identity at early stages, and that of olfactory interneurons at later stages. Moreover, the combinatorial expression of Gsx2 with other transcription factors that are spatially segregated in subregions of the LGE ultimately define the transcriptional code required for the specification of these two molecularly distinct neuronal subtypes¹⁴⁶.

For example, the most dorsal part of the LGE produces olfactory interneurons and exhibits the highest Gsx2 expression together with selective expression of Er81. In contrast, ventral LGE progenitors express lower Gsx2 levels together with the transcription factor Isl1, predominantly forming striatal interneurons^{165, 166}.

In addition to Gsx2, the related protein Gsx1 contributes to establish LGE identity, as shown by the more severe disruption in striatal and olfactory development in the double Gsx1/Gsx2 mutants, in comparison to Gsx2 single mutant¹⁶⁷. However, Gsx1 and Gsx2 exhibit distinct roles in the balance between proliferation and differentiation of LGE progenitors; Gsx2 is uniquely required for the expansion of LGE progenitors, thus coordinating the growth of the progenitor pool in the LGE with the specification of its progeny¹⁶⁸.

The molecular identity of the **CGE** has been more difficult to define, since the CGE represents an extension of the LGE in the more caudal brain regions without a clear anatomical boundary. Similar to the LGE, the CGE also expresses transcription factors such as Gsx2 and Er81, but in contrast to the LGE, where Gsx2 specifies striatal or olfactory interneurons, in the CGE, Gsx2 positive progenitors give rise to a discrete subpopulation of interneurons destined to the cerebral cortex¹⁴⁸. In fact, the CGE contribute to 30% of all cortical interneurons, although the major source remains the MGE^{142, 169}. CGE-derived interneurons express different markers than MGE-derived cells and the majority of these neurons migrate to more caudal cortical regions. Interestingly, neural precursors in the CGE lack the expression of the MGE-specific factor Lhx6 and instead selectively express COUPTFII, which has been shown to also impart caudal migratory properties¹⁷⁰. The identification of

additional CGE-restricted factors will contribute to our understanding of cortical interneuron diversification.

Altogether, these studies demonstrate the existence of several discrete domains of gene expression in the subpallial VZ that identify distinct types of fate-committed progenitors. Such domains of gene expression create molecular boundaries that go beyond the primary anatomical divisions and further subdivide the classical MGE, LGE and CGE into subregions expressing distinctive combinations of fate-determining transcription factors.

4.2 Mechanisms of subpallial progenitor diversification: a role for Shh

A question that still attracts the interest of many researchers is how distinct gene expression domains and diversification among neural progenitors arise. Once again, we revisit the concept of morphogen signals, secreted from early patterning centres, which may contribute to the formation of specific molecular identities that are then maintained by cross-repressive interactions among transcription factors.

As discussed above, one of the most studied factors in DV patterning is Shh. Early expression of Shh in the prechordal plate underlying the telencephalic primordium, initially leads to the formation of a sharp boundary between the dorsal Pax6 domain and the ventral Nkx2.1 domain, by repressing Pax6 and by promoting Nkx2.1 expression in the ventral forebrain^{12, 39, 171}. Later in development, around E10 in the mouse, Shh-dependent and independent mechanisms induce the expression of the transcription factor Gsx2 between the Pax6 and the Nkx2.1 domains, thus marking the prospective LGE/CGE regions¹⁷². In addition, Shh expression in the MGE domain is required to prevent ectopic expression of Gsx2, thus preventing LGE identity conversion in MGE progenitors³⁷.

Continued Shh signaling is required to maintain Nkx2.1 expression in MGE progenitors. In turn, Nkx2.1 and the downstream transcriptional cascade mediated by Lhx6 and Lhx7 subsequently contribute to maintenance of Shh expression in the mantle zone of the ventral MGE, thus creating a positive feedback that promotes MGE progenitor development^{36, 173}.

It has been proposed that the levels of Shh influence the diversity of MGE-derived interneurons³⁷. *In vitro* stimulation of MGE explants with high Shh concentrations leads to the generation of SST positive cells, whereas lower Shh concentrations induce PV fate. These results are consistent with earlier transplantation studies demonstrating the existence of a spatial bias within the MGE for the production of SST and PV positive interneurons¹⁴⁷.

However, a major difference arises from these studies, since PV cells that are produced *in vitro* at low Shh concentrations, are preferentially generated *in vivo* from the ventral MGE, which would likely be exposed to higher Shh levels due to Shh expression in the most ventral regions of the forebrain. Similarly, SST positive cells that originate under stimulation with high Shh concentrations *in vitro* are predominantly produced *in vivo* in the dorsal MGE, which is located at a greater distance from the area of higher Shh expression. Interestingly, in contrast to Shh expression itself, it has been shown that the downstream activation of Shh effectors and target genes is higher in the more dorsal part of the MGE, in the interganglionic sulcus^{146,147}. This anomaly can be explained by potential differences in Shh availability and diffusion, which may be distorted by the presence of specific extracellular matrix components and/or by differences in progenitor competence to respond to Shh signalling. Indeed, progenitors in the ventral MGE lack the expression of the Gli activators and it is possible that Shh stimulation of these progenitors dampens responsiveness to Shh itself to prevent a self-reinforcing loop³³.

CONCLUSION

The neurodevelopmental processes that transform the single layered neuroepithelium into the complex structure of the adult brain have been subject of fascination and research investigation for many years now. Since the first beautiful drawings of Ramon y Cajal we have come a long way in describing the variety of neuronal types that populate the CNS and connect to each other to form functional circuitries. Technical advances in lineage tracing analysis and in genetic manipulation of the embryonic brain *in vivo* via *in utero* electroporation contributed strongly to the rapid accumulation of knowledge on how neuronal diversity is achieved. Similar mechanisms of embryonic patterning are sometimes shared in different CNS regions, such as the ventral telencephalon and spinal cord, where clear boundaries of gene expression mark distinct progenitor domains that will give rise to specific neuronal subpopulations. However, distinct and still poorly understood mechanisms determine neuronal diversity in the dorsal pallium, where the presence of fate-restricted progenitors has just begun to be explored.

Specification of cell fate requires strict coordination of extrinsic positional information and intrinsic state of progenitor competence, both of which may undergo temporal changes as development progresses.

Studies performed on neural progenitors cultured *in vitro* have challenged some of the key concepts of neural differentiation; reprogramming of fibroblast cells into neurons revealed a high degree of plasticity in cell fate choices, which were initially believed to be irreversible^{174, 175}. Also, *in vitro* cultures of cortical neuron precursors have been shown to recapitulate the sequential generation of projection neurons observed *in vivo*, suggesting that environmental cues of the *in vivo* niche may play only secondary roles in cell fate switches in progenitor cells¹⁷⁶. Future studies will be aimed at understanding the contribution and coordination of genetic and environmental factors in neuronal specification, in order to exploit this knowledge to drive pluripotent stem cells towards therapeutically relevant neuronal subtypes. The intersection between basic developmental neurobiology and stem cell biology will be an exciting field of research that to our view holds the key for regenerative medicine in neurodegenerative diseases.

FIGURE LEGENDS

Figure 1: Early telencephalic patterning and patterning centres

(A) Schematic view of the developing neural tube, showing the main subdivisions. A series of vesicles later give rise to the structures within the forebrain, midbrain and hindbrain, with the telencephalic territory being specified in the most rostral regions of the prosencephalon.

(B) Expansion and folding of the cephalic end of the neural tube determine the formation of the cerebral hemispheres. Neural progenitor cells within the telencephalon acquire a positional identity through the concerted action of patterning centres that release various morphogen signals. The Hem, Anti-Hem, Prechordal plate and Anterior Neural Ridge (ANR) are illustrated on the diagram, along with examples of secreted morphogens as described in the main text.

(C) Coronal section of the forebrain, illustrating the location of the Hem and Anti-Hem in relation to the developing dorsal and ventral telencephalon. These regions are characterised by differential expression of transcription factors that are indicated on the right.

Figure 2: Cortical arealisation and graded expression of transcription factors

(A) Schematic representation of the relative size and position of the four main functional areas of the cortex. M1: motor; S1: somatosensory; A1: auditory; V1: primary visual cortex.

(B) Schematic view of the developing telencephalon, illustrating the gradient of expression of four transcription factors with patterning activity. *Emx2-Pax6* and *COUPTFI-SP8* exhibit

overlapping counter-gradients that establish cortical area identities along the major spatial axes. A: anterior; P: posterior; L: lateral; M: medial.

(C) Summary of the changes in area patterning observed in genetic knock-out animals of the specified transcription factors. See text for details and references.

Figure 3: Development of the ventral forebrain

(A) Basic organization of the embryonic brain to illustrate the spatial location of the medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminences. Anterior and posterior cross-sections of the forebrain are shown in B and C, respectively.

(B) Coronal view of one cerebral hemisphere at a rostral level, illustrating the medio-lateral position of the LGE and MGE. Differential expression of transcription factors between GEs and within GEs is illustrated by different colour codes. These regions ultimately generate different neuronal populations, as indicated in the text below the figures.

(C) Cross sectional view of one cerebral hemisphere at a caudal level, showing the site of origin of 30% of cortical interneurons.

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Figure 1

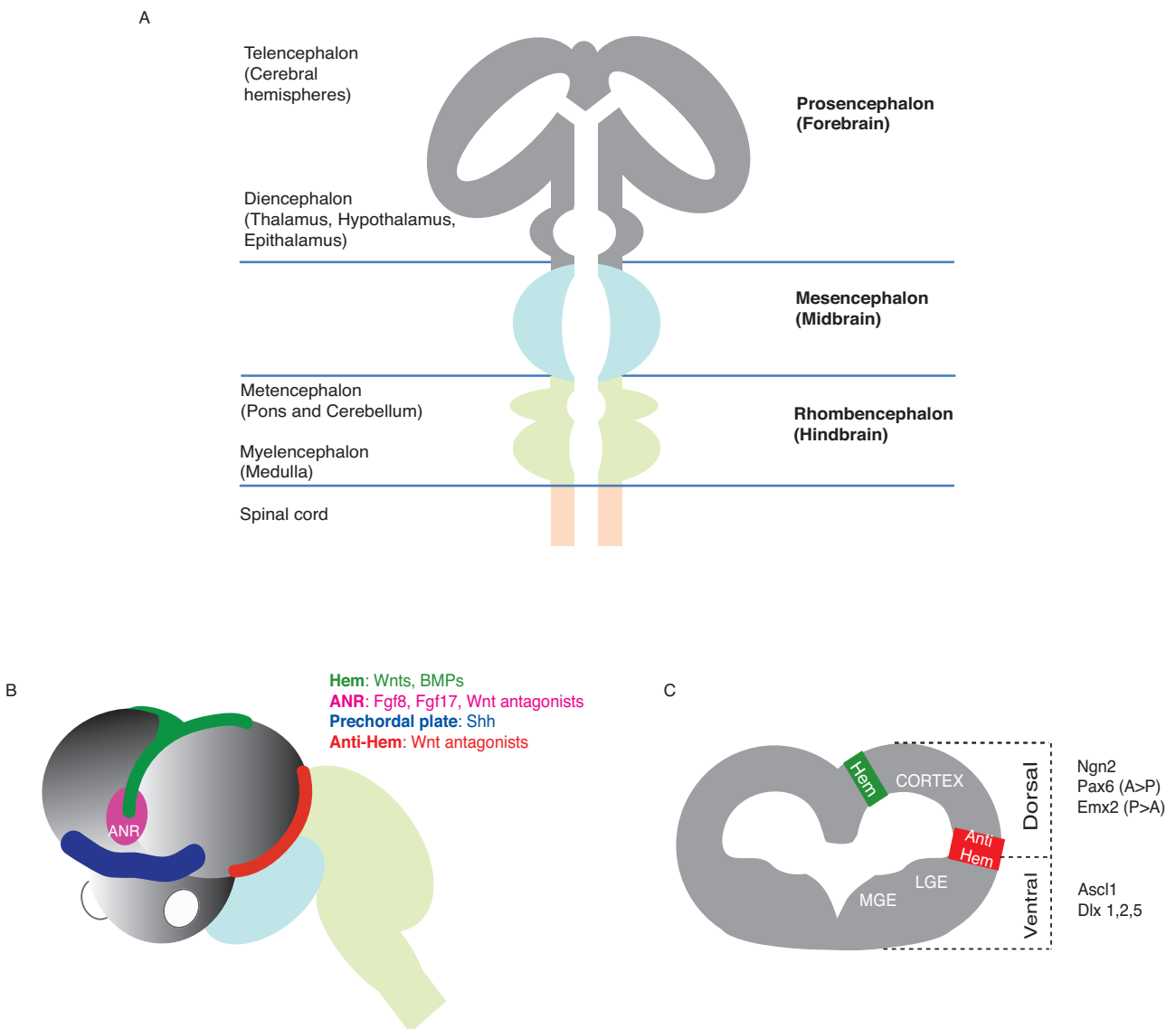


Figure 2

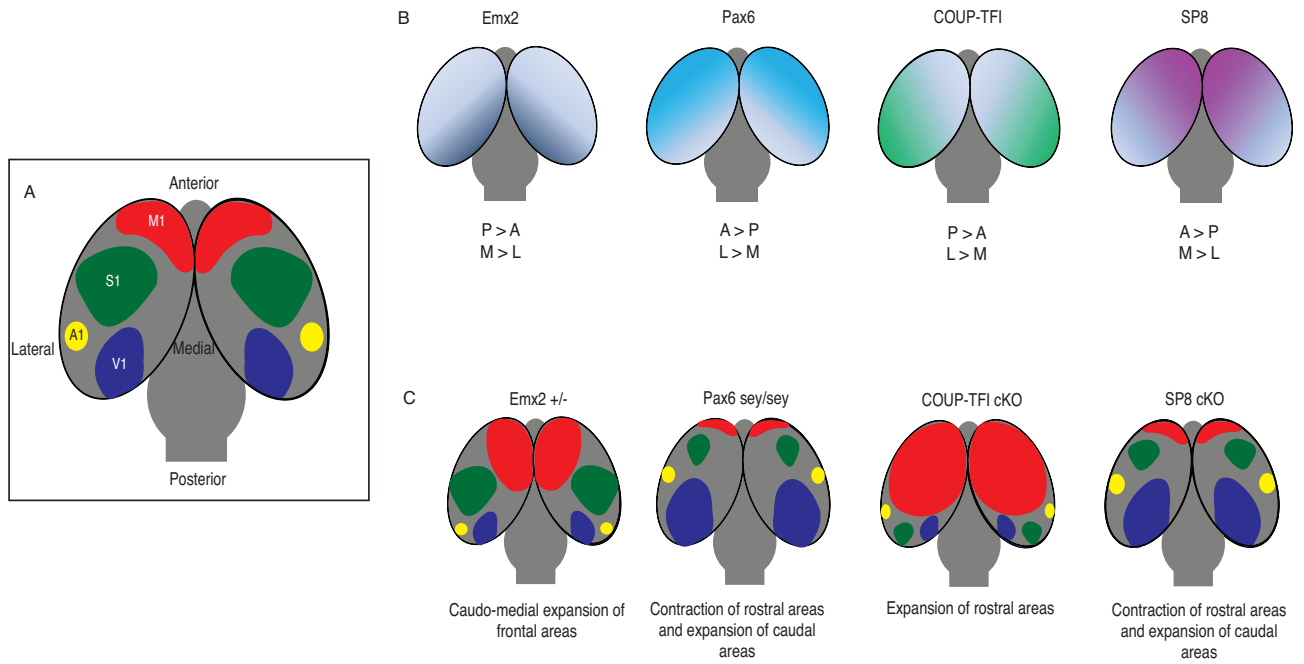


Figure 3

