Plasticity in neurogenic competence of cortical progenitors in the developing mouse neocortex

Oberst, Polina

Faculté de médecine

DOCTORAT EN NEUROSCIENCES
des Universités de Genève
et de Lausanne

UNIVERSITÉ DE GENÈVE FACULTÉ DE MÉDECINE

Professeur Denis Jabaudon, directeur de thèse

TITRE DE LA THESE

PLASTICITY IN NEUROGENIC COMPETENCE OF CORTICAL PROGENITORS IN THE DEVELOPING MOUSE NEOCORTEX

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par

Polina OBERST

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Professeur Denis Jabaudon, Faculté de médecine, Université de Genève, directeur de thèse
Professeure Marlen Knobloch, Université de Lausanne, expert
Professeur Alexandre Dayer, Faculté de médecine, Université de Genève, expert
Professeur Victor Tarabykin, Charité, Université de Berlin, expert

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Doyen

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Бабе Гале

Abstract

The distinct subtypes of cortical excitatory neurons that underlie neocortical circuit function are generated during embryonic development from progenitors located in the ventricular zone. These progenitors progress through dynamic temporal states and generate first deep- followed by superficial layer cortical neurons. While the temporal transcriptional dynamics underlying progenitor identity progression are increasingly understood (Okamoto et al., 2016; Telley et al., 2018), little is known about the plasticity of these temporal states in response to environmental cues, and the fate potential of distinct progenitor types (*i.e.* of apical *vs*. intermediate progenitors).

Previous studies have examined the aggregate fate potential of late cortical progenitors in the ferret by heterochronic transplantation into younger hosts, however, due to lack of techniques enabling the isolation of specific progenitor populations, these studies could not examine the plasticity of distinct progenitor types (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell, 1988; Mcconnell and Kaznowski, 1991).

To assess the fate potential of specific progenitor types, we used FlashTag, a technique to label isochronic populations of progenitors in the mouse neocortex (Govindan et al., 2018; Telley et al., 2016), to fate-map neuronal progeny of apical and intermediate progenitors after heterochronic transplantation into younger embryos. We find that, in contrast to intermediate progenitors which lose the ability to specify earlier-born neurons, apical progenitors remain multipotent throughout corticogenesis and are able to revert their temporal identity to specify earlier-born neuronal types when transplanted into a younger host. Thus, the temporal progression in apical progenitor identity that underlies the sequential generation of distinct neuronal types occurs without competence restriction.

These findings highlight progenitor-type specific differences in fate plasticity which could be exploited in the context of neuroregenerative applications.

Résumé

La diversité de neurones excitateurs à la base des fonctions corticales est générée au cours du développement embryonnaire par des progéniteurs neuronaux situés au contact des ventricules latéraux, dans une région appelée zone ventriculaire. Ces progéniteurs passent par différents états leurs permettant, au cours du développement, de générer successivement les neurones composant les couches profondes suivi des couches superficielles du cortex. Tandis que les dynamiques transcriptionnelles sous-tendant la progression de l'identité des progéniteurs au cours du temps a été largement étudiée (Okamoto et al., 2016; Telley et al., 2018), la capacité de ces cellules à répondre et à s'adapter à des changement de signaux environnementaux pendant le développement, ainsi que la restriction du potentiel de devenir de chaque type de progéniteur (*i.e.* soit progéniteurs apicaux *vs*. progéniteurs intermédiaires) restent mal connus.

De précédentes études, menées chez le furet, ont déterminées la capacité de cohortes de progéniteurs corticaux tardifs à changer d'identité en réponse à une transplantation hétérochronique chez un hôte jeune. Cependant, du fait de restrictions techniques ne permettant pas l'isolation de pures et spécifiques populations de progéniteurs neuronaux, ces études ne permirent pas de s'intéresser à la plasticité de sous-types distincts de progéniteurs (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell, 1988; Mcconnell and Kaznowski, 1991).

Afin de déterminer la capacité de population spécifiques de progéniteurs de changer leur identité, nous avons mis à profit l'utilisation de la technique de Flash Tag, nous permettant de marquer des populations isochroniques de progéniteurs dans le neocortex de la souris (Govindan et al., 2018; Telley et al., 2016), et d'examiner le devenir cellulaires des neurones générés par des progéniteurs apicaux et intermédiaires à la suite de transplantations hétérochroniques dans de jeunes embryons. Nous avons pu observer que, à l'inverse des progéniteurs intermédiaires qui perdent leur capacité de générer des neurones précoces, les progéniteurs apicaux conservent leur multipotencialité au cours de la corticogenèse et restent en mesure d'inverser leur identité temporelle afin de produire des types neuronaux précoces lorsqu'ils sont transplantés dans un hôte jeune. Ainsi, la progression temporelle de l'identité des progéniteurs apicaux qui sous-tend la génération séquentielle de types neuronaux distincts au cours de temps ne fait pas l'objet de restriction au cours du développement.

Ces résultats mettent en évidence les différences de plasticité de chaque type de progéniteurs spécifiques, ceux-ci pouvant être exploités dans le cadre d'études de pathologies neurodégénératives.

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Introduction

The interplay of nature and nurture, during which the action of a defined genetic framework is further altered and shaped by experience and environment, is thought to influence most aspects of our life, including our unique personalities, abilities and the predisposition to various diseases.

Interaction of genetic and environmental information also occurs at the cellular level, where cell-intrinsic and extrinsic cues together regulate important cell decisions, such as the decision to proliferate or to differentiate into specific cell types during development. The precise regulation of such decisions is crucial to the formation of complex structures, strikingly exemplified in the neocortex, where an extraordinary variety of different cell types are generated during development and assemble in an orderly manner to establish sophisticated neuronal circuits.

These neocortical circuits orchestrate numerous crucial brain functions, such as processing of sensory information, regulation of fine motor skills, cognition and consciousness. Abnormalities in neocortical development, resulting from cell-intrinsic or extrinsic processes, often translate into neurodevelopmental or neuropsychiatric disorders, such as schizophrenia, autism, and intellectual disabilities. Although highly prevalent, these disorders remain poorly understood and vastly undertreated to date, largely due to a lack of in detail understanding of brain development and circuit function.

A thorough characterization of the processes which enable the orderly generation of the various neuronal types found in the neocortex, will enhance our understanding of neocortical circuit assembly and function in health and disease, and likely open up new treatment opportunities for various neurodevelopmental and neuropsychiatric disorders.

1. Cellular organization of the neocortex

1.1 Cortical neurogenesis and neural progenitor diversity

The neocortex is derived from the telencephalic vesicles, which are formed at the anterior part of the neural tube during early stages of embryonic development. The telencephalon consists of two major compartments, the dorsal pallium (dorsal telencephalon) and the subpallium (ventral telencephalon) (Rubenstein et al., 1998). In both compartments, progenitor cells located in a germinal zone lining the lateral ventricles (ventricular zone, VZ) are dividing to self-renew and to generate neurons. Cortical excitatory neurons are generated by progenitors within the dorsal pallium and migrate radially towards the pial surface, while cortical inhibitory neurons arise from the subpallium and migrate tangentially to reach the developing cortex (Hu et al., 2017). Progenitors within the dorsal pallium appear rather homogenous, and yet it these cells that give rise to the striking diversity of highly specialized excitatory neurons found in the six horizontal layers of the postnatal neocortex (Florio and Huttner, 2014; Greig et al., 2013).

Cortical neurogenesis begins around embryonic day (E) 10.5 in the mouse, when first-born neurons migrate away from the ventricular surface of the dorsal pallium, and accumulate more basally to form the preplate. Subsequently-born neurons split the preplate in two: a superficial part termed the marginal zone, which contains Cajal-Retzius (CR) neurons and will form the future L1, and a deeper part termed the subplate (Greig et al., 2013). Neurons within the marginal zone and the subplate are the first to mature, and participate in the regulation of migration and maturation of later-born neurons (Ohtaka-Maruyama et al., 2018), which establish the cortical plate between these two structures. Deep layer (DL; L5-6) neurons are generated first (with a peak of production at E12.5), followed by superficial layer (SL, L2-4) neurons (with a peak of production at E15.5). Newly generated excitatory neurons migrate past earlier-born neurons within the developing cortical plate to reach progressively more superficial positions, resulting in an inside-first outside-last lamination of the neocortex reflecting birth order (Figure 1). At around E17.5, cortical neurogenesis ceases and gliogenesis begins, during which cortical astrocytes and oligodendrocytes are generated (Angevine and Sidman, 1961; Greig et al., 2013).

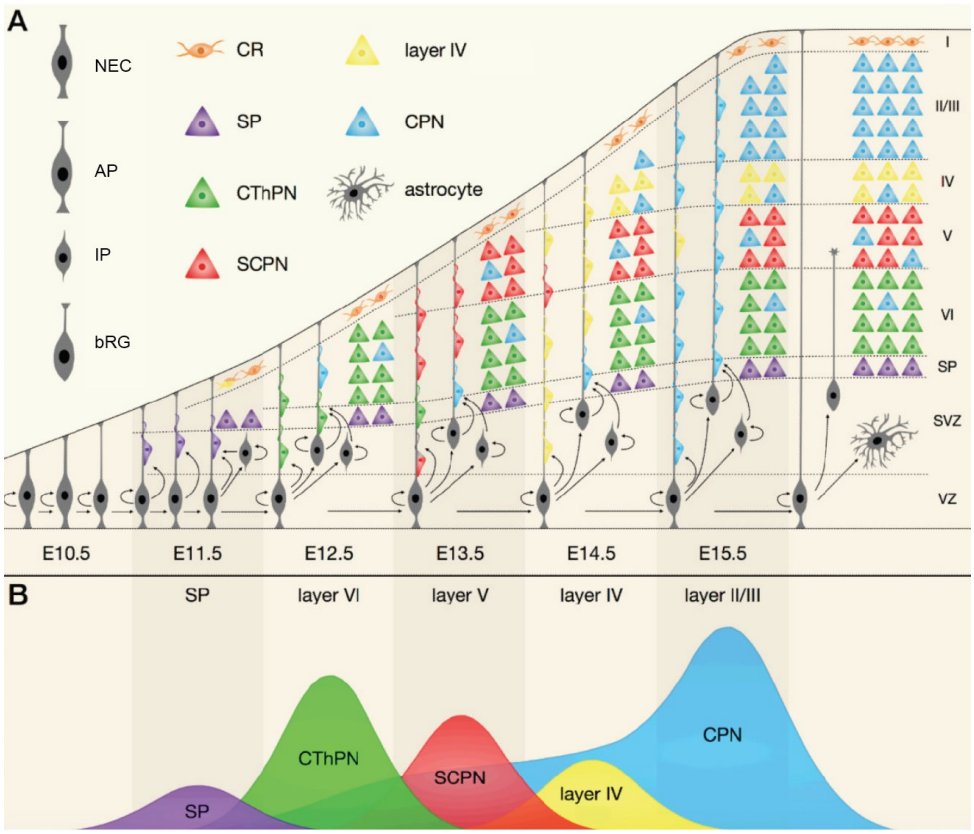

Figure 1. Cortical progenitors sequentially generate deep- and superficial layer neurons
A) At the onset of cortical neurogenesis, neuroepithelial cells transform into apical progenitors and generate first deep and then superficial layer neurons. At the same time they also generate intermediate progenitors, which form the subventricular zone and act to amplify neurogenesis. Around E16.5, cortical neurogenesis ceases and progenitors start generating glia.
B) The distinct subtypes of cortical projection neurons are born sequentially. Subplate neurons are generated around E11.5, followed by corticothalamic and subcortical projection neurons between E12.5 and E13.5, and spiny stellate neurons at E14.5. Intracortical projection neurons are generated first around E12.5 (deep layer neurons), followed by a large peak of production at E15.5 during which L2/3 neurons are generated.
NEC: neuroepithelial cell; AP: apical progenitor; IP: intermediate progenitor; bRG: basal radial glia; CR: Cajal-Retzius neuron; SP: subplate neuron; CthPN: corticothalamic neuron; SCPN: subcortical projection neuron; CPN: callosal projection neuron; SVZ: subventricular zone; VZ: ventricular zone.
Modified from: Greig *et al*, 2013

The developing pallium contains several distinct progenitor subtypes (Table 1), which composition and frequency dynamically changes in the course of corticogenesis, and which relative occurrence varies between different species (Florio and Huttner, 2014). Early in development, the neural tube is composed of one layer of neuroepithelial cells (NECs) forming the neuroepithelium. The neuroepithelium is pseudostratified (resembling a layered structure) due to segregation of nuclei in the apico-basal plane according to cell cycle stage: during G1- and S-phase the nucleus moves basally, and then re-descends apically during G2 to finally enter mitosis at the ventricular surface (Figure 2). This process is termed interkinetic nuclear migration (IKM) and occurs in progenitors located

in the ventricular zone throughout the developing central nervous system (Govindan and Jabaudon, 2017; Sauer and Walker, 1959). NECs are characterized by several key features, such as apico-basal polarity and interconnections at the apical (ventricular) surface via *adherens junctions*. They extend processes which span the entire thickness of the neuroepithelium, contacting both the apical surface and the basal lamina (pia). NECs initially divide symmetrically, leading to expansion of the neuroepithelium in both lateral and radial dimensions (Florio and Huttner, 2014).

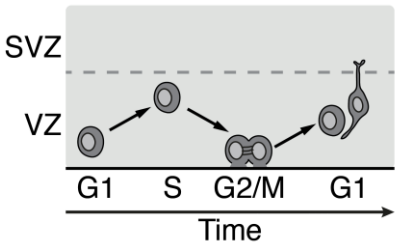

Figure 2. Interkinetic nuclear migration
Cortical progenitors exhibit apico-basal movement during different cell cycle phases. During G1-phase, cells move basally before undergoing S-phase, and descend apically during G2-phase to undergo mitosis at the ventricular border.
SVZ: subventricular zone; VZ: ventricular zone; G1: Gap 1 phase; S: S-phase; G2: Gap 2 phase.

At the onset of cortical neurogenesis, around E10.5 in the mouse, NECs transform into apical progenitors (APs; also called radial glia). APs maintain the characteristic features of NECs described above, and in addition up-regulate astroglial markers, such as GFAP (Glial fibrillary acidic protein). APs divide both symmetrically to further expand the progenitor pool, and asymmetrically to produce different daughter cells, including APs, post-mitotic neurons and intermediate progenitors (IPs; see below) (Anthony et al., 2004; Florio and Huttner, 2014; Hartfuss et al., 2001; Malatesta et al., 2000). Clonal analysis has shown that at the end of cortical neurogenesis, around E17 in the mouse, on average 1 out of 6 APs continues dividing to generate glia (Gao et al., 2014).

Another type of progenitors found in the VZ are short neural precursors (SNPs, also called apical intermediate progenitors). SNPs have a short basal process that does not reach the pia and are thought to divide just once to generate a pair of neurons (Florio and Huttner, 2014; Gal et al., 2006).

Particularly abundant in the developing mouse cortex are intermediate progenitors (IPs; also called basal progenitors). IPs are generated from APs already at the early stages of cortical neurogenesis, but are most numerous at mid- and late stages. They delaminate from the *adherens junctions* belt at the ventricular surface and migrate basally to form a second proliferative zone termed subventricular zone (SVZ) (Florio and Huttner, 2014; Haubensak et al., 2004; Noctor et al., 2004). IPs are multipolar, extend small processes which do not reach the basal or apical surfaces, down-regulate astroglial markers and up-

regulate the transcription factor TBR2 (T-box brain protein 2; also called Eomes) (Englund et al., 2005). IPs can divide both symmetrically and asymmetrically, although in rodents the majority of IPs undergo only one or two rounds of cell divisions before self-consumption by symmetric division into two neurons, thus acting primarily as transit-amplifying cells (Florio and Huttner, 2014; Haubensak et al., 2004; Noctor et al., 2004). In contrast to APs, IPs appear to only generate neurons and have not been observed to give rise to glia (Gao et al., 2014).

The SVZ also contains a second progenitor type termed basal radial glia (bRG; also called outer radial glia). Basal radial glia undergo asymmetric neurogenic divisions and express astroglial markers but not TBR2, allowing to distinguish them from IPs molecularly. They are unipolar, extend a long process that reaches the basal lamina but lack an apical process (Florio and Huttner, 2014). Initially, bRGs were thought to exist only in gyrencephalic species (Hansen et al., 2010), but a small fraction of bRGs was also found in mice (Florio and Huttner, 2014; Wang et al., 2011).

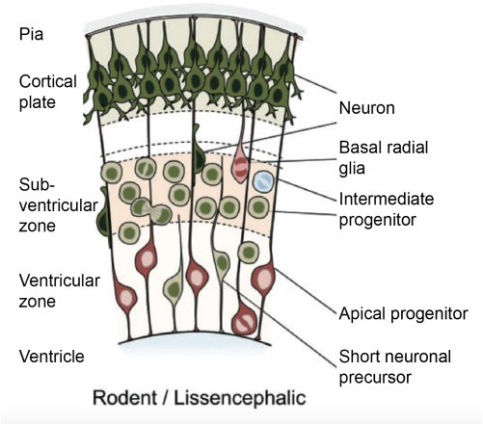

Figure 3. Progenitor subtypes in the developing mouse neocortex
At neurogenic stages, the developing mouse neocortex contains four main progenitor subtypes. Apical progenitors and short neural precursors are found in the ventricular zone, and intermediate progenitors and basal radial glia are found in the subventricular zone.
Modified from: Florio & Huttner, 2014

Table 1 Features of different progenitor subtypes in the developing mouse neocortex

Progenitor subtype	Location	Apical process	Basal process	Marker expression
Neuroepithelial cell (NEC)	VZ	Contacts apical surface	Contacts pia	PAX6
Apical progenitor (AP)	VZ	Contacts apical surface	Contacts pia	GFAP, PAX6
Short neural precursor (SNP)	VZ	Short apical process	Short basal process	GFAP, PAX6
Intermediate progenitor (IP)	SVZ	Multipolar, short processes	Multipolar, short processes	TBR2
Basal radial glia (bRG)	SVZ	No apical process	Contacts pia	GFAP, PAX6

1.2 Diversity of cortical excitatory neurons

Cortical excitatory neurons (also called pyramidal or glutamatergic neurons) account for approximately 80% of all neocortical neurons. They represent a highly diverse group of neurons that can be distinguished based on a variety of different, although often interrelated, features, such as birthdate, morphology, laminar position, molecular identity, electrophysiological properties and input-output connectivity (Greig et al., 2013; Lodato and Arlotta, 2015).

A common way to classify cortical excitatory neurons is based on their axonal projections. Two main groups exist: intracortical and corticofugal neurons. Intracortically projecting neurons reside mainly in superficial cortical layers and can be further subdivided into associative projection neurons (which extend long- or short-distance axons within one cortical hemisphere) and commissural projection neurons (which project to the contralateral hemisphere *via* the *corpus callosum* or, less often, *via* the anterior commissure). Associative neurons also include excitatory spiny stellate neurons found in L4 of primary sensory cortex, which have several dendrites of similar length, project mostly locally. Spiny stellate neurons receive input from the thalamus and are considered as the main information entry point to the neocortex (Greig et al., 2013; Jabaudon, 2017; Lodato and Arlotta, 2015).

Corticofugal projection neurons (CFPN) send their axons to targets outside of the cortex. These neurons are located mainly in deep cortical layers and can be further divided into subcerebral projection neurons (SCPN) which reside primarily in L5 and send axons to multiple targets, including different brain stem nuclei and the spinal cord, and corticothalamic projection neurons (CTPN) which are located predominately in L6 and send axons to different thalamic nuclei. However, some neurons send axonal branches to multiple targets, such as to both cortical and subcerebral targets (Cederquist et al., 2013), or to both ipsi- and contralateral cortex (Lodato and Arlotta, 2015), and thus not all neurons can be unambiguously assigned to one of the above described groups.

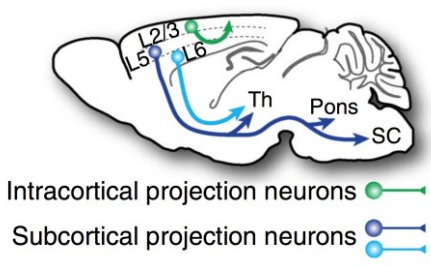

Figure 4. Main projection patterns of cortical excitatory neurons
Superficial layer neurons project intracortically, while deep layer neurons project mostly to subcerebral targets, including the thalamus, pons and spinal cord.
Th: thalamus; SC: spinal cord.
From: Jabaudon, 2017

Another parameter commonly used to classify cortical neurons is laminar position, which is strongly correlated with birthdate, connectivity pattern and expression of marker proteins. For example, neurons located in SL are likely to be generated during late stages of corticogenesis, to project intracortically and to express the transcription factors CUX1/2 (Cut Like Homeobox 1/2) and BRN1/2 (POU Class 3 Homeobox 3/2). In contrast, neurons located in DL are likely to be generated early in corticogenesis, to project mostly subcortically, and to express transcription factors such as FEZF2 (FEZ Family Zinc Finger 2), SOX5 (SRY-Box 5), TBR1 (T-box brain protein 1), CTIP2 (COUP-TF-interacting protein 2) or TLE4 (Transducin-like enhancer protein 4) (Greig et al., 2013; Lodato and Arlotta, 2015).

However, laminar position alone is not a precise parameter for classification of subpopulation of neurons, as for example highlighted by the presence of both commissural and subcerebral neurons in L5. Although born at the same developmental stage, these neurons exhibit distinct behaviors already during migration: commissural neurons extend axons towards the midline, whereas subcerebral neurons steer their axons towards the internal capsule (Koester and O'Leary, 1993, 1994; O'Leary and Koester, 1993; Richards et al., 1997). Similarly, while individual marker proteins are often used to distinguish different subtypes of cortical neurons, expression of markers might vary between cortical areas, and different subpopulations of neurons might express the same marker at different levels. For example, CTIP2 is commonly used to identify corticospinal motor neurons, but it is also expressed at lower levels in corticothalamic neurons. In addition, in many cases subtype-specific markers are lacking and additional markers or features need to be considered (Lodato and Arlotta, 2015).

With the advent of single-cell RNA-sequencing technologies, the classification of neurons based on their transcriptional identity, taking into account the expression levels of multiple genes or even entire modules of genes at the same time, has become possible (Johnson and Walsh, 2017; Poulin et al., 2016). In the neocortex, this technique has been first applied in the primary somatosensory cortex (S1) of 3-5 week old mice, identifying 7 groups of excitatory and 16 groups of inhibitory neurons, as well as many non-neuronal cell types (Zeisel et al., 2015). This study was followed by a multitude of subsequent single-cell RNA-sequencing studies examining cellular heterogeneity within the neocortex, profiling ever increasing numbers of cells and leading to the identification of multiple additional subgroups of neurons (Mayer et al., 2018; Saunders et al., 2018; Tasic et al., 2016, 2018; Zeisel et al., 2018). This complexity of neuronal subtypes is strikingly exemplified in a recent study, which identified a total of 133 cell types in two neocortical regions (primary visual cortex and anterior lateral motor cortex), comprising 56 groups of

excitatory, 61 groups of inhibitory and 16 non-neuronal cell types. Interestingly, while the vast majority of identified inhibitory neuron groups were shared between both neocortical areas examined, most groups of excitatory neurons were specific to one region (Tasic et al., 2018). Thus, it appears likely that further subgroups of excitatory cortical neurons will be identified in future studies sampling additional cortical areas.

However, a major challenge in using single-cell RNA-sequencing data to classify cell types is the potential over-splitting of groups. As such, not all transcriptionally defined clusters necessarily represent functionally distinct groups of neurons, highlighting the importance of thorough functional validation of newly identified transcriptionally defined subpopulations, and the need for integration of several cell features (Poulin et al., 2016).
Together, the comprehensive classification of cortical neurons into clearly defined subtypes has proven challenging, although significant progress has been made in recent years, particularly due to the advent of high-throughput single cell transcriptomics. Advances in sequencing technologies as well as large-scale efforts to integrate transcriptional identity and functional properties, such as electrophysiological parameters and connectivity, are likely to further improve and revise current classifications (Johnson and Walsh, 2017; Poulin et al., 2016).

1.3 Diversity of cortical inhibitory neurons

Cortical inhibitory neurons (here also referred to as interneurons (INs) for simplicity) account for around 20-30% of all neocortical neurons. These neurons are GABAergic, form local connections within the cortex and act to modulate the activity of cortical circuits. Cortical interneurons appear even more heterogeneous than cortical excitatory neurons, and many different subtypes with distinct morphologies, molecular identities, electrophysiological properties and local connectivity patterns have been described (Kepecs and Fishell, 2014; Tremblay et al., 2016).
However, three main non-overlapping groups of neocortical interneurons can be identified based on the expression of parvalbumin (PV), somatostatin (SST) or the ionotropic serotonin receptor 5HT3a (5Ht3aR) (Rudy et al., 2011). PV and SST neurons are found mainly in superficial layers, and 5Ht3aR neurons in deep layers, although this laminar allocation is less precise than that of excitatory neurons. Within these three main groups, a large variety of subtypes exist. For example, PV neurons are further subdivided into fast-spiking basket cells, which synapse onto the proximal dendrites or soma of target cells, and fast-spiking chandelier cells which target the axon initial segment. SST neurons include non-fast-spiking basket cells and burst-spiking Martinotti cells which synapse

onto distal dendrites. 5Ht3aR neurons represent a particularly diverse group of interneurons, and comprise for example bipolar vasoactive polypeptide (VIP)- and calretinin (CR) neurons, multipolar and neurogliaform interneurons and double bouquet cells (Kepecs and Fishell, 2014; Tremblay et al., 2016). As is the case for excitatory cortical neurons, single-cell RNA-sequencing technologies have greatly added to our understanding of cortical interneuron diversity and further studies unifying single-cell RNA-sequencing data and functional properties will likely lead to an improved classification of interneurons into more clearly defined subtypes (Mayer et al., 2018; Saunders et al., 2018; Tasic et al., 2016, 2018; Zeisel et al., 2018).

2. Generation of neuronal diversity

The striking diversity of different subtypes of cortical excitatory neurons are generated from a limited set of progenitors which at first glance appear rather homogenous. Although different progenitor subtypes exist, as described above, these different types *per se* do not account for the vast diversity of neurons found in the postnatal cortex. Rather, progenitors use strategies that involve spatial and temporal patterning mechanisms to generate distinct neuronal types, which are then further refined through post-mitotic mechanisms. In the following sections, I summarize the main strategies that are used during cortical development to create neuronal diversity.

2.1 Generation of neuronal diversity through spatial patterning

Spatial patterning refers to the generation of distinct types of cells at different anatomical locations. This process occurs in many regions of the central nervous system, including the spinal cord and the ventral pallium, where distinct cell types are generated in specific locations (Guillemot, 2007; Hu et al., 2017). In the neocortex, spatial patterning contributes to the generation of tangentially parcellated functional areas, which contain neurons with specialized input-output connectivity and primarily process distinct sensory modalities. Four primary areas exist within the neocortex: primary motor (M1), auditory (A1), somatosensory (S1) and visual cortex (V1). These different areas have distinct cytoarchitectures, for example, a prominent L4 in V1 and S1, which is visually not discernible in M1, and thus in the adult the transition from one neocortical area to another is readily apparent. In addition, area differences are often also mirrored in distinct neuronal morphologies and gene expression patterns. In line with this, a recent single-cell RNA-sequencing study examining neuronal diversity within the visual and lateral motor cortex found that most excitatory neuronal subtypes identified were specific to either of these two cortical regions, thus linking known functional differences between neurons of different regions with unique transcriptional profiles (Tasic et al., 2018).

Although the nature of the mechanisms underlying neocortical arealization have been intensely debated in the past (O'Leary, 1989; Rakic, 1988), it is now widely believed that the combined action of genetic and activity-dependent mechanisms together shape the specification of distinct neocortical areas. Arealization is initiated already during embryogenesis by the action of diffusible signaling molecules which are secreted from patterning centers outside of the neocortex. These diffusible factors include fibroblast growth factor 8 (FGF8) and FGF17, which are secreted rostro-medially by the

commissural plate, Wnt and bone morphogenetic protein (BMP) family members, which are secreted from the cortical hem caudo-medially, and Wnt antagonist secreted frizzled-related protein 2 (SFRP2) and epidermal growth factor (EGF) family members, which are secreted from the anti-hem laterally (Greig et al., 2013; O'Leary et al., 2007). Of these factors, only FGF8 has been shown to act as a potent organizer of cortical arealization. As such, overexpression of FGF8 leads to expansion of rostro-medial areas more caudally (Fukuchi-Shimogori and Grove, 2001; Toyoda et al., 2010), and reduction of FGF8 leads to expansion of caudal areas rostrally (Garel et al., 2003). Together, these secreted factors induce complementary and orthogonal expression of transcription factor gradients in cortical progenitors. These include high expression levels of PAX6 (paired box gene 6) rostro-laterally, EMX2 (empty spiracles homeobox 2) caudo-medially, SP8 (*trans*-acting transcription factor 8) rostro-medially, and COUPTF1 (chicken ovalbumin upstream promoter transcription factor 1) caudo-laterally (Gulisano et al., 1996; Liu et al., 2000; Sahara et al., 2007; Waclaw et al., 2006; Walther and Gruss, 1991; Zhou et al., 2001). The relative expression levels of these, and possibly other, transcription factors in a given progenitor are thought to instruct the generation of neurons with specific spatial identities which will form distinct functional areas within the neocortex (Greig et al., 2013; O'Leary et al., 2007). In line with this, a recent single-cell RNA-sequencing study examining the developing prefrontal and visual cortex found that differences in gene expression between these two cortical areas are apparent already at the progenitor level, and become more pronounced as neurons differentiate and mature (Nowakowski et al., 2017). These findings thus strongly support that cortical arealization is initiated already during early stages of corticogenesis, and at least partially stems from intrinsic differences in cortical progenitors, which are transmitted to daughter neurons and further refined at later stages.

However, activity-dependent processes have also been shown to participate in neocortical arealization. The nascent cortical plate initially appears uniform, and area-specific differences in cytoarchitecture are established only later with the arrival of thalamocortical axons, suggesting that the establishment of mature area-specific identities is at least partially regulated by input-dependent mechanisms (O'Leary et al., 2007). In addition, heterotopic transplantation of different developing neocortical areas has unveiled a striking level of plasticity in their cytoarchitecture, marker expression and projection patterns. For example, embryonic visual cortex transplanted into S1 adopted cytoarchitecture and marker expression of S1 (Schlaggar and O'Leary, 1991), and immature L5 neurons transplanted from visual to motor cortex exhibited projection patterns appropriate for their new heterotopic location, as did L5 neurons transplanted from motor to visual cortex (O'Leary and Stanfield, 1989; Stanfield and O'Leary, 1985).

Thus, it appears that genetic mechanisms act to pre-establish arealization, which is then further shaped and refined by input-dependent mechanisms, resulting in the formation of distinct functional neocortical areas (O'Leary et al., 2007) (Figure 5).

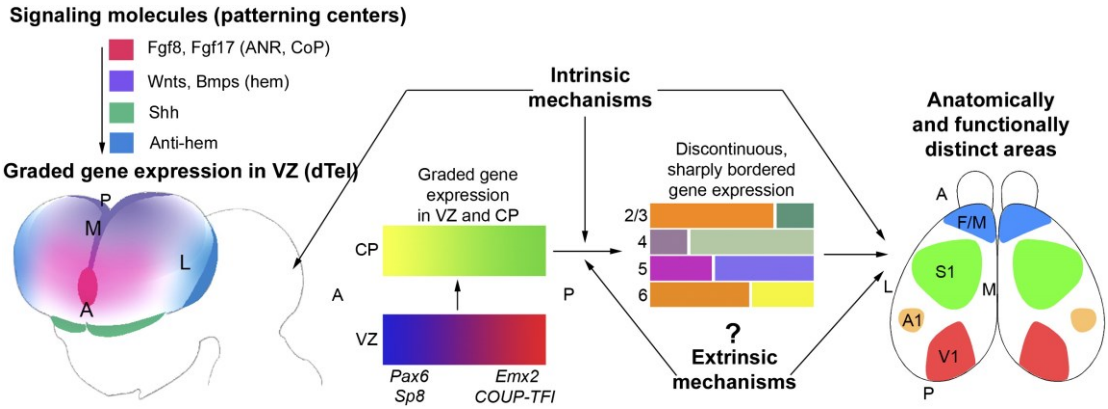

Figure 5. Spatial patterning in the developing neocortex
Signaling molecules released from patterning centers outside of the cortex induce graded expression of transcription factors in apical progenitors. The combinatorial action of these factors leads to the generation of anatomically and functionally distinct cortical areas.
VZ: ventricular zone; dTel: dorsal telencephalon; P: posterior; M: medial; L: lateral; A: anterior, CP: cortical plate. From: O'Leary, 2007

2.2 Generation of neuronal diversity through temporal patterning

Temporal patterning refers to the generation of distinct cell types from a single progenitor over time. In the neocortex, this process underlies the sequential generation of distinct neuronal subtypes from a limited set of progenitors. Clonal analysis of cortical progenitors, for example achieved by sparse labeling with retroviral vectors or using the Mosaic Analysis with Double Markers (MADM) technique, has shown that the majority of cortical progenitors labeled at early stages of corticogenesis generate approximately 8 neurons that contribute to both deep and superficial layers (Gao et al., 2014; Llorca et al., 2018). Subsets of fate-restricted progenitors, *i.e.* progenitors which although present already at early stages of corticogenesis contribute only to superficial layers, have been proposed, but appear to be rare (Franco et al., 2012). Thus, the vast majority of cortical progenitors appear to undergo temporal patterning and specify highly distinct cell types over time, comprising for example both intracortically and subcerebrally projecting daughter neurons.

Several studies have used single-cell RNA-sequencing to examine the temporal transcriptional dynamics of cortical progenitor cells at sequential stages of development to get a better understanding of the processes underlying temporal patterning (Okamoto et al., 2016; Telley et al., 2018; Yuzwa et al., 2017). A recent study from the lab found that cortical progenitors dynamically change their temporal identity in the course of corticogenesis, and that these dynamic changes in gene expression are transmitted to daughter neurons to instruct specific identities. These data thus strongly support the model that distinct neuronal subtypes emerge directly from dynamic temporal transitions in progenitor identity (Telley et al., 2018).

How are the temporal transitions of cortical progenitors achieved? Although yet incompletely understood, it appears that both cell-intrinsic and extrinsic mechanisms are involved and together orchestrate temporal transitions of cortical progenitors. In the next sections I summarize some of the key mechanisms identified so far.

2.2.1 Regulation of competence by intrinsic mechanisms

The competence of a cell is defined as its ability to respond to an intrinsic or extrinsic cue. For example, a progenitor at one developmental stage may be competent to produce a given cell type in response to a cue, but the same cell may not be competent to do so at another developmental stage. Thus, acquisition and/or loss of competence allows the generation of distinct cell types from the same progenitor exposed to the same cues, and therewith increases the diversity of neurons that can be generated. Consequently, it is not only the availability of inductive cues *per se*, but also the progenitor's responsiveness to these cues that enables the specification of different cell types from the same progenitor.

Competence can be regulated at different levels within the cell, but epigenetic mechanisms appear to play a central role. Such mechanisms refer to reversible, yet heritable, structural or chemical modifications of chromatin, DNA or histone proteins, which lead to local changes in gene expression without altering the DNA sequence itself. These modifications can lead to gene silencing by rendering gene loci inaccessible for transcription enzymes, for example by condensation of chromatin or repositioning of gene loci to silencing hubs, or in contrast enhance transcription *via* mechanisms supporting the binding of transcription enzymes (Yoon et al., 2018).

In the developing neocortex, regulation of competence by epigenetic mechanisms has probably been best studied at the transition from neurogenesis to gliogenesis at late stages of corticogenesis (MuhChyi et al., 2013; Yoon et al., 2018). Glia-inducing cues are present in the developing cortex already at early stages, yet gliogenesis is initiated only late in embryonic development, and precocious onset of gliogenesis is prevented by epigenetic mechanisms. For example, at early stages of corticogenesis the promoters of glial genes (*e.g. Gfap* and *S100β)* are hypermethylated and thus epigenetically silenced. Demethylation of these promoters at later developmental stages leads to acquisition of gliogenic competence, and in line with this, premature demethylation induces precocious onset of gliogenesis (Fan et al., 2005; He et al., 2005; Saunders et al., 2018).

Other mechanisms regulating the onset of gliogenesis include the activity of the polycomb repressive complex (PRC; consists of PRC1 and PRC2), which silences genes *via* trimethylation of histone H3 at lysine 27 (H3K27me3), leading to a change in the local chromatin configuration that suppresses transcription (Yoon et al., 2018). At late stages of corticogenesis, PRC silences the pro-neuronal gene *Neurog1*, resulting in the termination of neurogenic competence, and allowing transition to gliogenesis. In line with this, deletion of the PRC2 component *Ezh2* or knockdown of the PRC1 component *Ring1B* lengthens the neurogenic period at the expense of gliogenesis (Hirabayashi et al., 2009; Pereira et al., 2010). Interestingly, if *Ezh2* is removed before the onset of neurogenesis, corticogenesis seems to be accelerated and gliogenesis is initiated earlier on, highlighting the complex and developmentally tightly-regulated activity of PRC (Pereira et al., 2010). PRC also play important roles during the switch from deep layer to superficial layer neuron generation, and the deletion of *Ring1B* at early stages of corticogenesis leads to prolonged expression of *Fezf2* in APs, lengthens the production of DL neurons, and delays SL neuron generation (Morimoto-Suzki et al., 2014; Yoon et al., 2018).

In addition to the above-described epigenetic mechanisms, the action of several key transcription factors has been shown to also regulate competence transitions of cortical progenitors. For example, at the earliest transition from Cajal-Retzius to DL neuron generation, cortical progenitors upregulate the transcription factor FOXG1 (Forkhead box protein G1) (Hanashima et al., 2004). This upregulation is instructed by FGF8, which is secreted from the commissural plate, leading to induction of FOXG1 in a rostro-caudal manner. Expression of FOXG1 leads to rapid repression of several CR-specific genes such as *Ebf2/3* (Transcription Factor COE2/3), *Lhx9* (LIM/homeobox protein 9) and *Zic3* (Zinc finger protein 3), followed by delayed up-regulation of transcription factors instructing DL identity. In the absence of FOXG1, progenitors continuously generate CR

neurons at the expense of all other neuronal types. Similarly, conditional loss of FOXG1 at E13.5, after a period of DL neuron generation, results in heterochronic generation of earlier-born CR neurons (Hanashima et al., 2004). Thus, FOXG1-dependent repression of CR identity appears necessary to allow transition to DL neuron generation (Kumamoto et al., 2013). Interestingly, removal of FOXG1 at E15.5, a stage when progenitors generate SL neurons, does not lead to generation of heterochronic CR neurons, suggesting that at this stage progenitors do not require FOXG1 to repress CR identity.

Similarly, the transcription factor FEZF2 is expressed in cortical progenitors at early stages of corticogenesis, and its overexpression at later stages induces heterochronic generation of deep layer neurons (Molyneaux et al., 2005).

Thus, epigenetic mechanisms and the action of key transcription factors together orchestrate the competence of cells to respond to cues inducing the generation of distinct neuron types.

2.2.1.2 Cell-cycle dependent regulation of competence

In many model species and organs, exposure to the same cues at different cell cycle stages has been shown to result in the initiation of distinct fate choices, suggesting that competence to respond to specific cues is dynamically changing even within one given cell cycle. For example, pancreatic progenitors exposed to signals during early G1 exit the cell cycle through asymmetric division, whereas exposure to the same signals in late G1 leads to symmetric division and generation of two endocrine cells (Kim et al., 2015). Similarly, human embryonic stem cells in early G1 are strongly susceptible to specification cues inducing differentiation into endoderm, whereas cells in late G1 cells are especially responsive to cues inducing differentiation into ectoderm (Pauklin and Vallier, 2013). Thus, parcellation of the cell cycle, into several distinct competence windows might act to increase the generation of cell diversity.

In this context, G1 appears to play a central role in the initiation of cell fate decisions. For example, pluripotent stem cells have been shown to most rapidly respond to instructive cues when in G1 (Figure 6) (Pauklin and Vallier, 2013; Sela et al., 2012; Soufi and Dalton, 2016), and a potential link between G1 length and cell fate choice has been suggested across different systems, including in embryonic (Singh and Dalton, 2009), neural (Salomoni and Calegari, 2010) and hematopoietic (Orford and Scadden, 2008) stem cells.

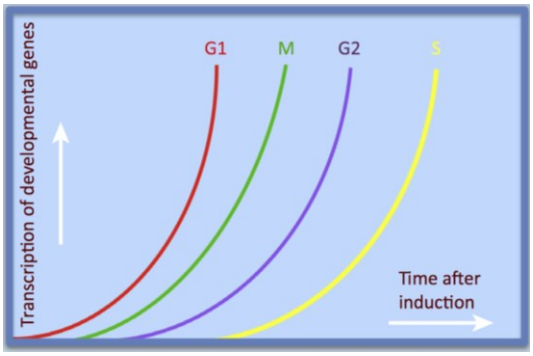

Figure 6. Kinetic of response to differentiation cues in pluripotent stem cells
Cells in G1-phase rapidly respond to differentiation cues by activation of developmental genes, whereas cells in other cell cycle phases respond with delayed kinetics.
From: Dalton, 2015

During neocortical development, progenitors exhibit a progressive lengthening of overall cell cycle duration (from 8.1 hours at E11 to 18.4 hours at E16), which largely stems from an increase in G1 duration (from 3.2 hours at E11 to 12.4 hours at E16) (Takahashi et al., 1995). Experimental lengthening of G1 by pharmacological inhibition of cdk2/cyclin E (Calegari and Huttner, 2003) or cdk4/cyclin D1 (Lange et al., 2009) induces premature neurogenesis at the expense of progenitor expansion, and, conversely, G1 shortening by overexpression of cyclin D1, cyclin E1 (Pilaz et al., 2009) or cdk4/cyclin D1 (Lange et al., 2009) leads to increased proliferation and delays neurogenesis onset.

These findings cumulated in the cell cycle length hypothesis, which proposes that G1 represents a critical window during which cell fate decisions are made, and that a short G1 promotes self-renewal by limiting the time that inductive cues can act on the cell, whereas a long G1 allows the accumulation of differentiation cues and therewith promotes differentiation (Calegari and Huttner, 2003). This model has been further supported by the observation that asymmetrically dividing neurogenic progenitors, as identified by expression of Tis21, exhibit a longer G1-phase than symmetrically-dividing proliferative progenitors (Calegari et al., 2005).

The mechanisms that predispose G1 as a stage when cell fate choice occurs are largely unknown, but it appears that changes in chromosomal architecture and epigenetic landscape just prior and during G1 might render cells particularly responsive to specification cues and enable the rapid recruitment of transcription factors and the activation of developmental genes (Dalton, 2015). For example, transition through M-phase, which occurs just prior to entry into G1, involves breakdown of the nuclear envelope, loss of interactions between nuclear lamina and genome (Kind et al., 2013) and remodeling of chromosomes including loss of cell type specific three-dimensional genome organization (Dileep et al., 2015; Naumova et al., 2013). The transition from M-phase to G1 is associated with a hyperactive, highly dynamic transcriptional state, which varies

across cells and might predispose cells to make distinct cell fate choices in G1 (Hsiung et al., 2016). Thus, erasure of epigenetic modifications and change in transcriptional state just prior to entry into G1 are thought to increase sensitivity to specification cues and together prime cells for fate decisions (Figure 7) (Dalton, 2015).

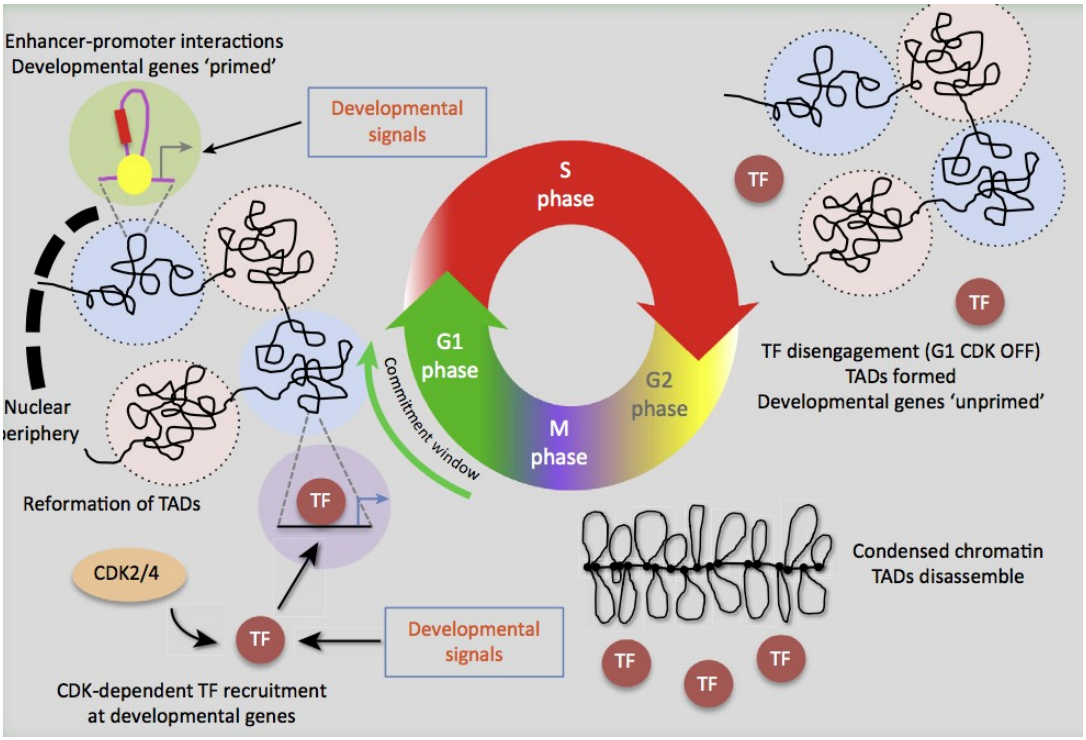

Figure 6. Epigenetic changes predisposing G1 as time window for cell fate specification
During G1, developmental genes are primed *via* enhancer-promoter interactions and the recruitment of transcription factors, allowing rapid activation of developmental programs in response to inductive signals and the initiation of cell fate choices.
From: Dalton, 2015

2.2.2 Regulation of temporal patterning by extrinsic cues

While epigenetic mechanisms and the expression of key transcription factors appear to substantially regulate temporal transitions of cortical progenitors, sole reliance on cell-autonomous factors would result in a rigid way of generating different neuronal types, and eliminate any level of adaptive plasticity which is likely to be achieved by additional integration of extrinsic cues.

To untangle the influence of intrinsic and extrinsic mechanisms underlying temporal patterning, several studies have examined the developmental potential of cortical

progenitors cultured *in vitro*. In this paradigm, progenitors are exposed solely to the extrinsic cues that are generated from the progenitor itself or from its daughter cells (*i.e.* lineage-intrinsic cues), while cues from other sources, such as the cerebrospinal fluid (CSF), blood vessels or other non-related cells (*i.e.* lineage-extrinsic cues), are excluded. Cortical progenitors derived from mouse or human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) cultured in such a paradigm recapitulate the sequential generation of first deep and then superficial layer neurons (Eiraku et al., 2008; Gaspard et al., 2008; Shi et al., 2012), suggesting that lineage-intrinsic cues are sufficient for the sequential generation of different cortical neuron types. However, an underrepresentation of late-born superficial layer neurons was reported in several studies (Eiraku et al., 2008; Gaspard et al., 2008), and transplantation of human ESC-derived cortical progenitors into a neonatal mouse strongly increased the generation of superficial layer neurons when compared to *in vitro* culture alone (Espuny-Camacho et al., 2013), suggesting that lack of lineage-extrinsic cues might result in reduced generation of SL neurons. Similarly, analysis of transcriptional profiles of *in vitro* cultured cortical progenitors has shown that while progenitors cultured in aggregates or as organoids (*i.e.* in conditions where cell-cell contacts are maintained) progressively change their gene expression profile in line with normal cortical development, clonally cultured progenitors (*i.e.* where cell-cell contacts are prevented) show only limited temporal changes in gene expression (Camp et al., 2015; Okamoto et al., 2016). Together, these findings suggest that while lineage-intrinsic cues are sufficient for specification of deep and superficial layer neurons *per se*, the finely balanced generation of these cell types in correct numbers might require additional lineage-extrinsic cues.

Such cues include feedback signaling from post-mitotic neurons to apical progenitors which are involved at the transition from DL to SL competence as well as at the transition to gliogenesis (Parthasarathy et al., 2014; Seuntjens et al., 2009; Wang et al., 2016). For example, deletion of the transcription factor *Sip1* in young cortical neurons induces precocious generation of SL neurons through mechanisms involving NTF3 (Neurotrophin 3) feedback signaling (Parthasarathy et al., 2014; Seuntjens et al., 2009). Similarly, embryonic ablation of DL neurons (using Neurog2$^{CreER/+}$ mice) results in prolonged generation of DL neurons at the expense of SL neurons (Toma et al., 2014). Together, these findings suggest that feedback signaling from post-mitotic neurons to apical progenitors is a critical contributor to the regulation of competence transitions in the latter. Another source of extrinsic signaling involved in the regulation of progenitor competence is β-catenin signaling, which activity is temporally-regulated during corticogenesis and highest at early stages during the generation of DL neurons. Overexpression of β-catenin

increases the generation of DL neurons, and conversely, inhibition of β-catenin signaling leads to increased generation of SL neurons. In addition, overexpression of β-catenin at late stages of corticogenesis results in partial restoration of DL generation, suggesting that high levels of β-catenin activity might induce DL competence (Mutch et al., 2009).
Finally, progressive hyperpolarization of apical progenitors during corticogenesis has been implicated in the regulation of neurogenic competence progression through Wnt-dependent mechanisms, and hyperpolarization of progenitors at mid-stages of corticogenesis leads to generation of later-born neuron types (Vitali et al., 2018).

Other potential sources of extracellular signaling molecules affecting temporal transitions of cortical progenitors include the CSF, which contains a large set of dynamically changing molecules including known regulators of cell survival, proliferation and differentiation (Chau et al., 2015; Lehtinen and Walsh, 2011; Lehtinen et al., 2011; Zappaterra et al., 2007), factors derived from invading (*e.g.* thalamocortical) axons (Dehay et al., 2001), and factors released from blood vessels, as for example described for neural crest stem cells, where BMP signaling factors produced by the dorsal aorta instruct progenitor migration and lineage identity (Saito et al., 2012).

2.3 Refinement of neuronal diversity post-mitotically

Manipulation of sensory inputs at early postnatal stages has long been shown to induce changes in connectivity and electrophysiological properties of young neocortical neurons (Sur et al., 1988; Van der Loos and Woolsey, 1973), demonstrating that at these early post-mitotic stages final neuronal identity is still plastic.

Supporting this notion, young neurons often co-express markers of different neuronal subtypes. For example, early born neurons that settle in the cortical plate between E12.5 and E14.5 (*i.e.* future corticothalamic and subcortically projecting neurons), initially strongly co-express CTIP2 and TBR1. Acquisition of appropriate deep layer subtype identity relies on the reciprocal regulation of these and other subtype-specific proteins (Greig et al., 2013). For example, CTIP2 and FEZF2 are normally strongly expressed in corticospinal neurons, and in absence of either of these factors neurons fail to extend axons via the corticospinal tract, but instead acquire phenotypes characteristic of DL callosal neurons (Chen et al., 2008). In callosal neurons, the DNA-binding chromatin remodeling protein SATB2 represses expression of CTIP2, and in the absence of SATB2, these neurons ectopically up-regulate CTIP2 and extend subcortical projections (Alcamo

et al., 2008; Lodato and Arlotta, 2015). Similarly, the transcription factor TBR1 is normally strongly expressed in subplate and corticothalamic neurons, and in its absence these neurons upregulate FEZF2. In addition, TBR1-deficient corticothalamic neurons fail to project to the thalamus but instead send axons to subcerebral targets (Han et al., 2011; McKenna et al., 2011). Thus, a regulatory gene work comprising SATB2, FEZF2, TBR1 and CTIP2, and perhaps additional proteins, acts in post-mitotic deep layer neurons to establish final subtype identity.

In line with the importance of these factors in establishing neuronal subtype identity, overexpression of FEZF2 in early postnatal superficial layer neurons induces their reprogramming into L5 neurons with appropriate molecular identity, morphology, connectivity and electrophysiological properties despite their superficial layer location. This reprogramming is most efficient at very early postnatal stages and is eventually lost (De la Rossa et al., 2013; Rouaux and Arlotta, 2013), further supporting the existence of an early critical period during which neuronal identity is still plastic.

3. Transplantation approaches to probe cell fate potential

3.1 Probing cell fate

Cell fate is defined as the phenotype that a cell will acquire during the course of its normal, unperturbed, development. However, a chosen fate does not necessarily reflect the entire fate potential of a cell, but rather only one of possibly several outcomes. While some progenitors may indeed be unipotent (sometimes also referred to as "restricted" or "committed"), other cells might be multipotent (sometimes also referred to as "unrestricted", "plastic", "uncommitted") and able to generate several different phenotypes. In the latter case, the chosen fate is likely to depend on cues, which can be both cell-intrinsic and extrinsic. Thus, there is an important distinction between the progenitor itself being fate-restricted (*e.g.* even in the presence of instructive cues, the cell is not competent to respond to the cues, for example due to epigenetic mechanisms), or whether the fate potential is restricted by the microenvironment (*e.g.* the cell is in principle competent to respond to the cue, but such cues are missing) (Brüstle et al., 1995; Gaiano and Fishell, 1998). Thus, while approaches such as fate mapping and lineage tracing can reveal the normal developmental outcome of a cell, they do not necessarily address the cell's full potential.

A means to probe the fate potential of cells is to expose them to different inductive cues and to assess the type of cells they give rise to. This can be done *in vitro*, by addition of defined cues, or *in vivo* by transplantation into hosts to expose the cells to the entire set of cues present in the host environment. In this context, transplantation studies have been widely used for over a century to examine the developmental potential of progenitors across different anatomical regions, and at different stages of development. In the following sections, I will discuss several key experiments and findings of both heterotopic (*i.e.* transplantation to another anatomical location) and heterochronic (*i.e.* transplantation in a host of a different age) transplantation studies, focusing on studies in the developing telencephalon.

3.2 Heterotopic transplantations

Heterotopic transplantations have been used to examine the presence of instructive local environmental cues, and to probe the regional fate potential of cells from different anatomical regions (Gaiano and Fishell, 1998).
For example, conditionally immortalized stem cells transplanted into the early postnatal hippocampus and cerebellum (where neurogenesis still occurs postnatally) were shown to adopt morphologies and axonal projections similar to neighboring neurons in their respective host locations, suggesting that the postnatal hippocampus and cerebellum contain local instructive cues which can induce the generation of region-specific neurons (Renfranz et al., 1991).

Other studies examined the fate potential of telencephalic cells by heterotopic transplantation into distinct brain regions. Earlier experiments used transplantation of entire tissue fragments, and predominantly found that cells within these grafts maintained a donor-specific phenotype, even when transplanted into heterotopic positions (Barbe and Levitt, 1991). However, it was later suggested that the local microenvironment of the tissue fragment could instruct maintenance of donor-specific identity, and thus prevent the cells from adopting host-specific phenotypes by shielding them from exposure to host-specific cues.

Subsequent studies examined the fate potential of dissociated heterotopically transplanted mouse dorsal (Brüstle et al., 1995; Fishell, 1995) and ventral (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995) telencephalic progenitors, which were individually exposed to the host environment upon transplantation. These cells were injected into the ventricles of rat host embryos, from where they successfully integrated into various brain regions and followed local migration streams to reach distinct areas (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995). Independent of donor- and host-region, these studies showed that transplanted cells adopted phenotypes strikingly similar to that of neighboring host cells. Striatal progenitors that integrated into the host cortex adopted pyramidal morphologies, acquired expression of host region-specific markers and extended axons *via* the *corpus callosum* and the corticospinal tract (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995). Similarly, transplanted cortical progenitors that integrated into the host striatum adopted local striatal phenotypes (Brüstle et al., 1995). An interesting side observation in several studies was that integration patterns differed depending on the host developmental stage (Brüstle et al., 1995; McMahon and McDermott, 2007), and integration appeared to be higher in areas where active

neurogenesis was taking place at the time of transplantation. Strikingly, one study using E16 and E18 host rats found that the differences observed in integration pattern of transplanted cells correlated with the endogenous host neurogenic gradient (Brüstle et al., 1995). Together, these studies suggest that progenitors within the telencephalon are not committed to a dorsal *vs.* ventral fate early on, but instead maintain competence to respond to local environmental cues instructing either of these fate choices and guiding migration in a region-specific manner.

In addition, it was found that primarily mitotic cells (as assed by ^{3}H-thymidine labeling) integrated into different host regions, suggesting that the ability to integrate into distinct brain regions and/or to be respecified into host-appropriate neuronal types might be compromised at post-mitotic stages (Campbell et al., 1995; Fishell, 1995). In line with this, another study used expression of LAMP (limbic-system-associated membrane protein), which is expressed by post-natal neurons in the perirhinal but not in the sensory cortex, to test the commitment of rat cortical cells to a limbic vs. non-limbic phenotype at different stages of development. When donor tissue was isolated at very early stages of development when the vast majority of cells are mitotic, both cells derived from the sensory and perirhinal cortex adopted phenotypes appropriate for their new heterotopic environment: sensory cells acquired expression of LAMP when integrated into the perirhinal cortex, and perirhinal cells failed to upregulate LAMP when integrated into the sensory or motor cortex. In contrast, tissue fragments isolated at later developmental stages when the majority of cells are post-mitotic, did not change their original donor phenotype after heterotopic transplantation, as assessed by on/off expression of LAMP, suggesting that commitment to limbic *vs*. non limbic fate occurs at early post-mitotic stages (Barbe and Levitt, 1991). However, in this study entire tissue fragments were transplanted directly into cortical regions of P1 rats, rather than into the ventricle or ventricular zone, and thus it is possible that donor cells were not sufficiently exposed to instructive developmentally regulated cues.

While the above described studies showed that telencephalic progenitors exhibit a striking level of fate plasticity, closer examination of ventral telencephalic progenitors that integrated into more distant structures, such as the diencephalon and mesencephalon, showed that these cells maintained expression of the telencephalic marker BF-1 despite their heterotopic location (Na et al., 1998), thus identifying a potential limit to the plasticity of these progenitors. This finding suggests that commitment to a telencephalic identity occurs already at the progenitor stage, and that mere exposure to environmental

cues is not sufficient to induce respecification of telencephalic progenitors to di- and mesencephalic phenotypes.

Together, similar results obtained across different studies found that telencephalic progenitors are multipotent and can generate distinct telencephalic (*e.g.* ventral and dorsal) phenotypes when exposed to corresponding local environmental cues (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995). However, to date no detailed molecular characterization of heterotopically transplanted progenitors and their daughter cells exists, and thus, although it appears that these progenitors are highly plastic and can adopt their neurogenic output when exposed to region-specific cues within the telencephalon, further more in-depth studies are needed to confirm the full acquisition of region-specific identity of transplanted progenitors and daughter neurons. In addition, as indicated in several studies (Barbe and Levitt, 1991), it is likely that a heterogeneous population of cells was transplanted, and thus further studies focusing on specific progenitor populations are needed to fully characterize the fate potential of different telencephalic progenitor types.

3.3 Heterochronic transplantations

Heterochronic transplantations have been used to examine dynamic changes in environmental factors over developmental time, and to test the temporal fate potential of cells at distinct developmental stages.

In a series of pioneering studies, McConnell performed transplantations of cortical progenitors at distinct developmental stages and assessed their ability to generate neurons of different cortical layers. These experiments were done in the ferret (*Mustela furo*), in which cortical neurogenesis continues into postnatal stages (from E20 to P14 in the visual cortex (Jackson et al., 1989)). Cells were dissociated into single-cell suspensions, labeled with ^{3}H-thymidine and then injected into the proliferative and lower intermediate zone of hosts, where they were individually exposed to any environmental cues present in the developing host cortex.

In a first set of experiments, cells were transplanted into same-age hosts (isochronic transplantations). The migration pattern of such isochronically-transplanted cells closely resembled that of endogenous host cells born at the time of transplantation, as did their morphology when examined six weeks after transplantation. Similarly, the final laminar position of transplanted cells was identical to that of endogenous cells labeled with ^{3}H-thymidine at similar time points, and retrograde labeling experiments showed that at least

some cells extended axons to appropriate targets (McConnell, 1988). Together, these experiments showed that donor cells were able to migrate within the host tissue, and that the transplantation procedure *per se* did not alter their normal cell fate.

In subsequent studies, McConnell probed the fate potential of early and late cortical progenitors by transplanting them into older or younger hosts (heterochronic transplantations) (Desai and McConnell, 2000; Frantz and McConnell, 1996; McConnell, 1988; Mcconnell and Kaznowski, 1991). These studies revealed that cortical progenitors isolated at early stages of corticogenesis (*i.e.* during formation of DL; E22-E38 in the ferret (Jackson et al., 1989)), were not committed to generating DL neurons, but instead could produce SL neurons when transplanted into older hosts (McConnell, 1988; Mcconnell and Kaznowski, 1991). This ability to be respecified was found to depend on the cell cycle stage at which progenitors were transplanted: transplantation of S-phase cells allowed respecification, but transplantation of cells at different time-points after S-phase labeling, invariantly resulted in generation of DL neurons, and a complete loss of fate plasticity was observed as early as 4 hours after S-phase labeling. This abrupt drop in competence to respond to environmental cues is striking, considering that subsequent analyses found that the average S-phase duration of different progenitors types in the P0 ferret cortex ranges from approximately 14-22 hours (Reillo and Borrell, 2012). Nonetheless, these findings suggested that during S-phase cortical ferret progenitors are particularly sensitive to environmental cues instructing cell fate, and that such sensitivity is lost during other cell cycle stages (Mcconnell and Kaznowski, 1991).

In contrast, when progenitors were isolated at late stages of corticogenesis (*i.e.* during formation of SL; E32-P14 in the ferret (Jackson et al., 1989)), these cells appeared to be committed to a SL fate, even when transplanted into a younger host. This commitment to a SL fate was observed even when S-phase cells were transplanted, suggesting that these progenitors were restricted to generating SL neurons (Frantz and McConnell, 1996). Finally, mid-stage cortical progenitors (normally generating only L4 neurons) transplanted into younger hosts (where L6 neurons were being generated), gave rise to neurons located in L4 (*i.e.* their normal donor identity) and L5 (*i.e.* neither host nor donor identity). These findings suggest that at mid-corticogenesis progenitors are partially fate-restricted and cannot give rise to L6 neurons anymore, but are still competent to specify earlier-born L5 neurons (Desai and McConnell, 2000). Together, these studies lead to the proposal of the progressive fate restriction model, which postulates that in the course of corticogenesis cortical progenitors are becoming progressively more fate restricted and gradually lose the ability to specify earlier-born neuron types (Desai and McConnell, 2000).

However, due to technical limitations at the time when these studies were performed, it was not possible to isolate specific populations of cells for transplantation, and thus a heterogeneous pool of cells comprising different subtypes of progenitors (*e.g.* apical and intermediate progenitors) were likely transplanted. In addition, recent technological advances now allow more-in-depth characterization of heterochronically-transplanted progenitors and their daughter neurons, for example by using subtype specific molecular markers and single-cell RNA-sequencing.

4. Aim of the project

During cortical neurogenesis, apical progenitors give rise to the striking diversity of different excitatory neuronal types found in the postnatal neocortex (Florio and Huttner, 2014). To achieve the specification of distinct subtypes of neurons, apical progenitors dynamically progress in their temporal identity and first generate deep layer neurons, followed by the generation of superficial layer neurons (Okamoto et al., 2016; Telley et al., 2018).

The temporal progression of apical progenitor identity, which underlies the generation of cortical excitatory neuron diversity, is thought to result from the interplay of intrinsic changes in progenitor competence, achieved for example through the action of transcription factors and epigenetic regulators (Yoon et al., 2018), and environmental cues, which modulate temporal identity transitions and ensure sufficient plasticity in the generation of distinct neuronal types (Toma and Hanashima, 2015).

While the transcriptional events underlying the progression in apical progenitor temporal identity are increasingly understood (Okamoto et al., 2016; Telley et al., 2018), the plasticity of these temporal states in response to environmental cues, as well as progenitor subtype-specific differences in temporal plasticity and fate potential remain unknown and are the subject of the current thesis project.

Summary of the results

In this study, we find that apical progenitors are multipotent and remain competent to specify early-born deep layer neurons throughout cortical neurogenesis. In contrast, intermediate progenitors isolated at late stages of corticogenesis lack the competence to specify early-born deep layer neurons and instead appear committed to the generation of superficial layer neurons.

Apical progenitors isolated at a late stage of cortical neurogenesis when only superficial layer neurons are being generated, were found to generate both deep and superficial layer neurons after transplantation into an E12.5 host. These daughter neurons expressed lamina-appropriate molecular markers, and were found to extend both intracortical and subcerebral projections.
The re-emergence of neurons with deep layer identities from heterochronically transplanted E15.5 apical progenitors was found to result from a molecular and physiological respecification of apical progenitor temporal identity to an E12.5-like state.

Together, these findings reveal progenitor subtype-specific differences in temporal plasticity, and demonstrate a striking level of plasticity in the temporal identity of apical progenitors.

Discussion

1. Considerations on the use of transplantations to probe cell fate potential

Heterotopic and heterochronic transplantations have been widely used in the past to probe the fate potential of progenitors across different brain regions and at different developmental stages (Brüstle et al., 1995; Campbell et al., 1995; Desai and McConnell, 2000; Fishell, 1995; Frantz and McConnell, 1996; McConnell, 1988). While these studies provided important insights into progenitor plasticity and fate commitment, several important aspects need to be addressed when analyzing transplantation experiments.

A first concern relates to the possibility that the transplantation procedure itself could induce a change in progenitor behavior or daughter neuron identity. To examine this possibility in our study, we first performed isochronic transplantations of E12.5 ($AP_{12\rightarrow12}$) and E15.5 ($AP_{15\rightarrow15}$) apical progenitors. These control experiments revealed that transplanted progenitors integrated into the developing host cortex rapidly (within less than 6 hours), exhibited typical apical progenitor morphology one day after transplantation, and generated daughter neurons which followed a time course of migration highly similar to that of non-transplanted endogenous cells. When examined at postnatal stages once cortical lamination is complete, daughter neurons of $AP_{15\rightarrow15}$ were found specifically in superficial cortical layers, whereas neuronal progeny of $AP_{12\rightarrow12}$ were found in both deep and superficial layers. These laminar distributions of daughter neurons were replicated by *in utero* electroporation of a piggyBac transposon construct (allowing fate mapping of progenitors by stable integration of a GFP transgene into the genome of electroporated cells) (Chen and LoTurco, 2012). In addition, patch-clamp recording of isochronically transplanted E15.5 apical progenitors showed that the Vm values of these transplanted cells did not differ from their non-transplanted host counterparts.
Together, no confounding effect on progenitor behavior or daughter neuron identity resulting from the transplantation procedure itself was detected in these control experiments, and instead isochronically transplanted progenitors were found to generate neurons which were essentially undistinguishable from endogenous non-transplanted cells generated at the same time.

A second concern relates to the potential preferential selection of (potentially normally rare) subsets of progenitors as a result of the transplantation. Such selection could be particularly relevant when cells are injected into the ventricles of embryos and only a subset of cells is found to successfully integrate into the developing host cortex (Brüstle et al., 1995; Campbell et al., 1995; Fishell, 1995). Thus, small differences in cellular features such as robustness, ability to attach to the ventricular surface, or ability to successfully integrate into and migrate within the tissue, could result in the preferential selection of a specific (potentially rare) progenitor type. Thus, while isochronic control experiments show that the migration pattern and daughter neuron identity of transplanted cells is essentially undistinguishable from that of native cells, a potential selection for a particularly plastic progenitor type in the heterochronic transplantations cannot be fully excluded. However, such selection appears unlikely, since the efficiency of the transplantation procedure (*i.e.* the number of cells that integrated into the host cortex) did not differ markedly between isochronic and heterochronic transplantations and in addition, single-cell RNA-sequencing analysis of E15 progenitors has not identified any subgroups of apical progenitors (Telley et al., 2018). The existence of subsets of fate-restricted apical progenitors, which despite being present already at early stages of corticogenesis only contribute to superficial layers, has been proposed in a previous study (Franco et al., 2012), however, such fate-restricted progenitors are likely to be rare, since single-cell RNA-sequencing analysis has not until now identified such progenitor subtypes (Telley et al., 2018; Yuzwa et al., 2017).

Injection of cells directly into the host ventricular zone, which would decrease the likelihood for selection events to occur, was not attempted since it would likely result in contamination of the subventricular or intermediate zone. Instead, through the use of intraventricular injections, integration of all cells necessarily occurs through the ventricular zone, which might be important, since the ventricular zone is the location where apical progenitors normally reside, and is likely to contain niche-specific cues, such as factors derived from the adjacent CSF (Chau et al., 2015; Lehtinen et al., 2011) or from neighboring cells. An additional advantage in using intraventricular transplantation as opposed to intraparenchymal injection is that integration of donor cells into the host tissue is found to be sparse, and thus transplanted progenitors are surrounded almost exclusively by host cells, limiting potential confounding effects from co-transplanted donor cells.

2. Daughter neuron identity of heterochronically transplanted apical progenitors

To examine the fate potential of heterochronically transplanted E15.5 apical progenitors ($AP_{15\rightarrow12}$), we assessed several features of daughter neuron identity, such as laminar position, the expression of molecular markers and the presence of intracortical and subcortical projections.

We first examined the laminar fate of daughter neurons in $AP_{15\rightarrow12}$, and compared their radial distribution to that of neuronal progeny from isochronic control transplantations ($AP_{12\rightarrow12}$ and $AP_{15\rightarrow15}$) and piggybac electroporations. These experiments revealed that $AP_{15\rightarrow12}$ generated daughter neurons that settled in both deep and superficial cortical layers, which was in striking contrast to the almost exclusive superficial layer location of $AP_{15\rightarrow15}$. Instead, the radial distribution of $AP_{15\rightarrow12}$ daughter neurons was practically indistinguishable from that found in control $AP_{12\rightarrow12}$ or E12.5 piggybac electroporations, suggesting that E15.5 apical progenitors remain competent to specify early-born deep layer neurons.

To rule out a potential mismigration which could account for the presence of $AP_{15\rightarrow12}$ daughter neurons in deep layers, we next examined the molecular identity of daughter neurons using markers for deep (TBR1 and CTIP2) and superficial layer (CUX1) neurons (Greig et al., 2013). Expression of these molecular markers was harmonious with the laminar position of daughter neurons: neurons located in superficial layers expressed CUX1, while neurons located in deep layers expressed CTIP2 or TBR1. In addition, examination of the axonal projections of $AP_{15\rightarrow12}$ daughter neurons revealed the presence of both intracortical and subcerebral projections, which was in contrast to isochronically-transplanted $AP_{15\rightarrow15}$, which sent axons only to intracortical targets. Together, these findings reveal the respecification of the laminar, molecular and projection identity of $AP_{15\rightarrow12}$ daughter neurons, strongly suggesting that $AP_{15\rightarrow12}$ remain competent to specify early-born deep layer neurons.

3. Respecification of progenitor temporal identity

Deep layer neuron identity found in $AP_{15\rightarrow12}$ could in principle also result from a post-mitotic rather than pre-mitotic process. In such a scenario, mismigration of daughter neurons to deep cortical layers could be followed by their post-mitotic reprogramming to a layer-appropriate (*i.e.* deep layer) neuronal identity. Such post-mitotic reprogramming of neuronal identity by the surrounding environment has recently been described in mispositioned future L4 neurons, which were found to lose L4 characteristics and acquiree L2/3 characteristics upon mismigration to L2/3 (Oishi et al., 2016).
However, examination of the small fraction of transplanted post-mitotic neurons (identified as RFP^+ EdU^- cells) in our study showed that these nascent neurons migrated specifically to the superficial layers, indicating that they were committed to a superficial layer fate already at the time of transplantation. Thus, the emergence of deep layer neurons in $AP_{15\rightarrow12}$ appears to result from a pre-mitotic progenitor respecification, rather than post-mitotic reprogramming of neuronal identity.

To directly investigate if respecification of cell identity occurs already at the progenitor level, we examined the transcriptional identity of $AP_{15\rightarrow12}$ one day after heterochronic transplantation using patch-seq RNA sequencing and compared it to that of native E12.5 and E15.5 apical progenitors. Strikingly, we found that $AP_{15\rightarrow12}$ repressed the transcriptional programs normally present in E15.5 APs, and re-induced E12.5-like transcriptional programs. Thus, it appears that exposure to an E12.5 cortical environment induces the rapid and global molecular respecification of E15.5 apical progenitors to an earlier E12.5-like temporal state.
Such rapid temporal respecification was also found when examining temporally-regulated progenitor features, such as the relative occurrence of neurogenic divisions. While at early stages of corticogenesis proliferative divisions predominate, the occurrence of neurogenic divisions increases at late stages of corticogenesis (Haubensak et al., 2004). In line with the temporal respecification of $AP_{15\rightarrow12}$ to an E12.5-like identity, proliferative divisions (as assessed by the fraction of cells labeled by the progenitor marker SOX2) increased, and the fraction of neurogenic divisions (as assessed by the fraction of cells labeled by the neuronal marker NEUROD2) decreased to values normally found at E12.5.
In addition, examination of the resting membrane potential of $AP_{15\rightarrow12}$ revealed that Vm values were reset to those normally found in E12.5 apical progenitors (Vitali et al., 2018).

Together, these findings reveal that E15.5 apical progenitors undergo a temporal respecification of their molecular and physiological properties to an E12.5 like state upon heterochronic transplantation, and that this temporal respecification results in the re-emergence of deep layer neuronal progeny. The potential mechanisms underlying this rapid temporal respecification of apical progenitor identity will be discussed in the section "Potential mechanisms underlying apical progenitor respecification".

4. Cell-type specific differences in progenitor plasticity and differences with previous heterochronic transplantation studies in the ferret

The temporal respecification of $AP_{15\rightarrow12}$ observed in this study contrasts with previous transplantation studies examining the fate potential of late cortical progenitors in the ferret. In these previous experiments it was found that late stage cortical progenitors had lost the competence to specify early-born deep layer neurons, and instead were restricted to generating superficial layer neurons, even when transplanted into a younger host (Frantz and McConnell, 1996).

In these previous studies, cells were labeled by use of radiolabeled thymidine (3^{H}-thymidine), which labels cells in S-phase regardless of their anatomical location, and thus results in labeling of cortical progenitors in both the ventricular and subventricular zone (Angevine and Sidman, 1961). Thus, it is likely that in these previous studies a heterogeneous pool of different progenitor types, comprising for example apical progenitors, intermediate progenitors and basal radial glia cells was transplanted (Reillo and Borrell, 2012). In addition, the developing ferret pallium contains a thick subventricular zone (comprised of an inner and outer part) which harbors large numbers of intermediate progenitors and basal radial glia, which collectively are thought to account for the majority of neuronal types produced at late stages of ferret corticogenesis (Reillo and Borrell, 2012). Thus, the predominant presence of intermediate progenitors at late stages of corticogenesis might occlude the temporal plasticity of apical progenitors identified here.

To investigate potential cell type-specific differences in progenitor fate potential, and more specifically to examine the fate potential of intermediate progenitors, we performed FlashTag labeling at E15.5 and waited 10 hours before collection of cells, allowing daughter intermediate progenitors to differentiate. At this stage, the vast majority (> 75%) of FT-labeled progeny co-expressed TBR2 and KI67, the molecular signature of intermediate progenitors (Vitali et al., 2018). Heterochronic transplantation of these cells resulted in the predominant generation of superficial layer neurons, and only a small number of neurons were found in deep layers. In line with their superficial layer location, daughter neurons expressed the superficial layer marker CUX1, but not the deep layer marker CTIP2.

Together, these findings demonstrate that in contrast to apical progenitors, intermediate progenitors at late stages of corticogenesis are committed to generating superficial layer neurons and are not competent to specify earlier-born deep layer neurons. Thus, the

differences between this study and previous findings (Frantz and McConnell, 1996) could result directly from progenitor subtype-specific differences in fate plasticity.

However, while not examined in this study, it is possible that some of the differences between the present and previous studies could also result from broader species-specific differences, as several aspects of corticogenesis differ markedly between mouse (the species used in this study) and ferret (the species used in previous studies).
For example, ferret cortical neurogenesis is protracted, extends for over two weeks and continues into postnatal stages (Jackson et al., 1989), whereas corticogenesis in the mouse extends for only around one week and is completed by the end of embryogenesis (Greig et al., 2013). Thus, it is possible that the relatively longer period of cortical neurogenesis found in the ferret could lead to the accumulation of larger differences in early *vs.* late progenitor identity, potentially affecting the competence to specify earlier-born neuronal types.
Finally, in the mouse differences in cell cycle length between distinct progenitor populations (e.g. proliferative vs. neurogenic progenitors) stem from differences in G1-length, highlighting the importance of G1 in the regulation of cell fate decisions (Calegari et al., 2005; Dehay and Kennedy, 2007). In contrast, in the ferret, G1 length is rather constant across different progenitor types, and major changes in cell cycle length between progenitor types instead result from differences in the length of the S-phase (Turrero García et al., 2016). Thus, cell fate decisions might be regulated differently in ferret and mouse, potentially leading to differences in the regulation of competence.

5. Potential mechanisms underlying apical progenitor respecification

What are the mechanisms that lead to the respecification of heterochronically transplanted apical progenitors?

The respecification of heterochronically transplanted apical progenitors could in principle result from the absence of extracellular cues normally present in the late developing cortex, and/or the presence of new sources of environmental cues found in the young host environment.

At late stages of corticogenesis, apical progenitors are thought to become increasingly more exteroceptive, as exemplified by the increased expression of transcripts involved in ion-transport related processes and cell-cell interactions (Telley et al., 2018). This increase in environment-sensing capabilities is compatible with the fact that at these late developmental stages more sources of extrinsic factors are present in the developing cortex, for example thalamocortical axons which start invading the developing cortex from around E13.5 (Auladell et al., 2000), abundance of early-born post-mitotic neurons which have been shown to provide feedback cues to apical progenitors (Parthasarathy et al., 2014; Seuntjens et al., 2009; Toma et al., 2014), differentiation of the choroid plexus in the lateral ventricles around E14.5 (Sturrock, 1979), and a progressively increasing concentration and repertoire of proteins found in the CSF (Chau et al., 2015; Lehtinen et al., 2011).

Thus, extrinsic signaling is likely involved in the emergence of late-born superficial layer neurons from apical progenitors, as for example experimentally shown for feedback signaling from early-born neurons (Parthasarathy et al., 2014; Seuntjens et al., 2009; Toma et al., 2014). Together with the observation that late-born superficial layer neurons appear underrepresented in *in vitro* conditions (Eiraku et al., 2008; Gaspard et al., 2008), and the finding that apical progenitors cultured at clonal density appear to undergo only limited changes in their temporal transcriptional profile over time (Okamoto et al., 2016), it appears likely that the generation of superficial layer neurons from apical progenitors at least in part relies on the presence of environmental factors at late stages of corticogenesis.

However, mere deprivation of such cues appears not to be sufficient to induce the respecification of apical progenitors to an earlier temporal state. E15.5 apical progenitors cultured *in vitro* at low density, where all sources of extracellular factors (apart from cues produced by the progenitors themselves or their progeny) are lacking, do not generate deep layer neurons, as assessed by staining for the deep layer marker CTIP2 (Telley et al.,

2018). Thus, a simplistic model wherein E15.5 apical progenitor identity results from extrinsic cues present in the late cortical environment and absence of such cues leads to the respecification of transplanted progenitors to an E12.5-like "ground state" appears not to be true, but rather temporal respecification likely depends at least partially on the presence of instructive cues present in the early cortical environment.

Such cues present in the early developing cortex could for example include β-catenin signaling, which has been shown to be higher at early stages of cortical neurogenesis when deep layer neurons are generated, and decreases as corticogenesis proceeds (Mutch et al., 2009). In line with an instructive role of β-catenin signaling in the generation of early-born *vs*. late-born neurons, over-activation of β-catenin signaling at mid stages of corticogenesis leads to the increased production of deep layer neurons, and overexpression at late stages induces the partial re-emergence of deep layer neuron generation (Mutch et al., 2009). Similarly, inhibition of β-catenin signaling was found to induce the generation of later-born, more superficially located neuronal types (Mutch et al., 2009; Vitali et al., 2018). Thus, the temporal respecification of heterochronically transplanted E15.5 apical progenitors, could at least partially result from increased levels of β-catenin found in the E12.5 ventricular zone.

In line with a role of β-catenin signaling in the temporal respecification of $AP_{15\rightarrow12}$, we found that the resting membrane potential of heterochronically transplanted apical progenitors was reset to values normally found at E12.5 (*i.e.* values appropriate for the new host). Progressive hyperpolarization of the V_m has been shown to regulate progression in neurogenic competence of apical progenitors through a Wnt-dependent mechanism (Vitali et al., 2018), and thus resetting of Vm values found in the heterochronically transplanted progenitors might contribute to their temporal respecification, for example by regulating their responsiveness to β-catenin signaling. However, the nature of the source inducing the resetting of Vm values in the heterochronically transplanted apical progenitors remains to be identified.

6. Transplantation of E12.5 apical progenitors into E15.5 hosts

To assess the temporal plasticity of young (E12.5) apical progenitors, we heterochronically transplanted these cells into older E15.5 hosts ($AP_{12\rightarrow15}$) (see additional data). These experiments revealed that despite their new host environment in which only superficial layer neurons were still being generated, transplanted E12.5 apical progenitors still generated both deep and superficial layer neurons. Strikingly, and in contrast to both isochronic control E15.5 and E12.5 transplantations, the majority of $AP_{12\rightarrow15}$ daughter neurons (> 80%) were located in deep layers.

Examination of the molecular identity of these daughter neurons revealed that while the expression of the deep layer markers TBR1 and CTIP2 was congruent with daughter neuron laminar location, daughter neurons expressing the superficial layer marker CUX1 were found in both superficial and deep layers, suggesting the mismigration of a subset of daughter neurons normally destined for the superficial layers.
What could be the mechanism underlying the potential mismigration of late-born $AP_{12\rightarrow15}$ daughter neurons? The majority of cortical excitatory neurons have been shown to use the radial glia scaffold as a locomotion guide for migration (Nadarajah and Parnavelas, 2002), and perturbation of this scaffold was found to lead to mismigration (Liu et al., 2015; Nadarajah and Parnavelas, 2002). In the context of $AP_{12\rightarrow15}$, developmentally regulated changes in the radial-glia scaffold at the transition from neurogenesis to gliogenesis (*i.e.* changes occurring around E17.5 in the host, corresponding to "E14.5" $AP_{12\rightarrow15}$) could lead to the mismigration of late-born $AP_{12\rightarrow15}$ daughter neurons.
To test this possibility, we are currently examining potential developmental changes in the radial glia scaffold occurring at the end of cortical neurogenesis, and are performing EdU pulse-labeling experiments in $AP_{12\rightarrow15}$. In these latter experiments, we administer a pulse of EdU at E18.0, a stage when host cortical neurogenesis is complete, to assess if heterochronically transplanted E12.5 apical progenitors are still dividing, and if daughter neurons born at this time-point correspond to the fraction of mis-positioned CUX1-expressing daughter neurons.

However, independent of a potential mismigration effect of late-born $AP_{12\rightarrow15}$ daughter neurons, the presence of CTIP2 and TBR1 expressing daughter neurons in deep cortical layers indicates that E12.5 apical progenitors do not undergo immediate temporal respecification upon heterochronic transplantation, but instead initially generate deep layer neurons, as they would normally do in the donor. In addition, the relative fraction of

TBR1, CTIP2 and CUX1-positive daughter neurons is essentially the same as in isochronic control $AP_{12\rightarrow12}$, strongly suggesting that $AP_{12\rightarrow15}$ are not temporally respecified to an E15.5-like state upon transplantation. Thus, it appears that while late E15.5 apical progenitors can revert their temporal identity to an earlier state, early E12.5 progenitors cannot immediately change their temporal identity to that of a later E15.5-like state. To confirm the lack of temporal respecification of $AP_{12\rightarrow15}$, we are currently analyzing several temporally-regulated progenitor identity features, such as the resting membrane potential (Vitali et al., 2018), and the relative occurrence of proliferative *vs*. neurogenic divisions (Haubensak et al., 2004).

What could explain the potential absence of apical progenitor temporal respecification in $AP_{12\rightarrow15}$?

Studies examining competence transitions in apical progenitors have found that the transcriptional repressor FOXG1 is required at the initial competence transition from the generation of first-born Cajal-Retzius neurons to deep layer neuron generation, and that in the absence of FOXG1 apical progenitors only generate Cajal-Retzius neurons (Hanashima et al., 2004). Interestingly, when Foxg1 expression is experimentally delayed until E14.5 or E15.5 (a stage at which progenitors normally generate only superficial layer neurons), progenitors initially generate deep layer neurons despite the "late" environment, and only later transit to generating superficial layer neurons, suggesting that acquisition of superficial layer competence requires an initial transition through deep layer competence (Toma et al., 2014).

Similarly, while progenitors cultured *in vitro* generate appropriate numbers of deep layer neurons, the efficient generation of superficial layer neurons appears to require additional extrinsic cues (Espuny-Camacho et al., 2013; Gaspard et al., 2008). In line with the progressively increasing role of extrinsic cues in the specification of late-born neuronal types, apical progenitors have been shown to become progressively more exteroceptive as corticogenesis proceeds (as assessed by the progressively increased expression of transcripts involved in ion-transport related processes and cell-cell interactions) (Telley et al., 2018). These findings indicate that over the course of corticogenesis apical progenitors progressively acquire competence to respond to novel sources of extrinsic signaling (such as cues from invading thalamocortical axons (Auladell et al., 2000), early-born post-mitotic neurons (Parthasarathy et al., 2014; Seuntjens et al., 2009; Toma et al., 2014), the choroid plexus (Sturrock, 1979), or progressively increasing repertoire of proteins found

in the CSF (Chau et al., 2015; Lehtinen et al., 2011)), and that E12.5 progenitors simply lack the competence to respond to these cues.

Together, it appears that although young apical progenitors indeed have a broad fate potential and give rise to both deep and superficial layer neurons over time (Gao et al., 2014), the specification of distinct neuronal types might require the sequential transition through different progenitor states during which progenitors progressively acquire the competence to respond to novel intrinsic or extrinsic cues, and that these progenitor states cannot be simply skipped by transplantation into an older environment.

7. Conclusions and perspectives

In this thesis project, we examined the fate potential of distinct progenitor subtypes present in the late developing mouse neocortex through heterochronic transplantation into a younger host. These experiments revealed that apical progenitors are multipotent and remain competent to specify early-born deep layer neurons throughout cortical neurogenesis. In contrast, late stage intermediate progenitors lack the competence to specify early-born neurons, and instead appear to be committed to the generation of superficial layer neurons independent of their environment.

The generation of deep layer neurons from heterochronically transplanted apical progenitors resulted from a temporal respecification of apical progenitor identity to that of an earlier, E12.5-like temporal state. This temporal respecification was reflected in an increase in proliferative divisions, a more depolarized resting membrane potential, and the re-induction of E12.5-like transcriptional programs.

In future studies, it will be important to understand the precise nature of the factors enabling and instructing the respecification of apical progenitor identity, which could provide important insight into the molecular mechanisms regulating the generation of distinct neuronal types, and aid the optimization and refinement of neuroregenerative approaches.

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Articles

Additional Data

Appendix

Appendix 1

Title:
Progenitor Hyperpolarization Regulates the Sequential Generation of Neuronal Subtypes in the Developing Neocortex

Authors:
Ilaria Vitali,[1,4] Sabine Fièvre,[1,4] Ludovic Telley,[1,5] Polina Oberst,[1] Sebastiano Bariselli,[1,6] Laura Frangeul,[1] Natalia Baumann,[1] John J. McMahon,[2] Esther Klingler,[1] Riccardo Bocchi,[1,7] Jozsef Z. Kiss,[1] Camilla Bellone,[1] Debra L. Silver,[2] and Denis Jabaudon[1,3,8,*]

Affiliations:
[1]Department of Basic Neurosciences, University of Geneva, 1 Rue Michel Servet, 1211 Geneva, Switzerland
[2]Department of Molecular Genetics and Microbiology, Duke University Medical Center, 224 Carl Building, Durham, NC 27710, USA
[3]Department of Neurology, Geneva University Hospital, 4 Rue Gabrielle-Perret-Gentil, 1205 Geneva, Switzerland
[4]These authors contributed equally
[5]Present address: Department of Fundamental Neurosciences, University of Lausanne, Rue du Bugnon 9, 1005 Lausanne, Switzerland
[6]Present address: National Institute of Diabetes and Digestive and Kidney Diseases, NIH, 9000 Rockville Pike, Bethesda, MD 20892, USA
[7]Present address: Department of Physiological Genomics, Ludwig-Maximilians University, Grosshaderner Strasse 9, 82152 Martinsried-Planegg, Germany
[8]Lead Contact
*Correspondence: denis.jabaudon@unige.ch

Appendix 2

Title:
In vivo pulse labeling of isochronic cohorts of cells in the central nervous system using FlashTag

Authors:
Subashika Govindan[1,3], Polina Oberst[1] and Denis Jabaudon[1,2]*

Affiliations:
[1]Department of Basic Neurosciences, University of Geneva, Geneva, Switzerland.
[2]Clinic of Neurology, Geneva University Hospital, Geneva, Switzerland.
[3]Present address: Interfaculty Institute of Bioengineering, EPFL, Lausanne, Switzerland.
*e-mail: denis.jabaudon@unige.ch

Appendix 3

Title:
Single-cell transcriptional dynamics and origins of neuronal diversity in the developing mouse neocortex

Authors:
L. Telley[1,3]*†, G. Agirman1,4†, J. Prados[1], S. Fièvre[1], P. Oberst[1], I. Vitali[1], L. Nguyen[4], A. Dayer[1,5], D. Jabaudon[1,2]*

Affiliations:
[1]Department of Basic Neurosciences, University of Geneva, Geneva, Switzerland.
[2]Clinic of Neurology, Geneva University Hospital, Geneva, Switzerland.
[3]Current address: Department of Basic Neuroscience, University of Lausanne, Switzerland.
[4]GIGA-Neurosciences, University of Liège, C.H.U. Sart Tilman, Liège, Belgium.
[5]Department of Psychiatry, Geneva University Hospital, Geneva, Switzerland.
† equally contributed to this work.
* Correspondence to: ludovic.telley@unil.ch; denis.jabaudon@unige.ch

Status: in revision

Appendix 4

Title:
Temporal patterning of progenitors in the developing invertebrate and vertebrate nervous system

Authors:
Polina Oberst[1]*, Gulistan Agirman[1,2]*, Denis Jabaudon[1,3]

Affiliations:
[1] Department of Basic Neurosciences, University of Geneva, Switzerland
[2] GIGA-Neurosciences, University of Liège, C.H.U. Sart-Tilman, Liège, Belgium
[3] Department of Neurology Geneva University Hospital, Geneva, Switzerland
* Contributed equally to this work.

Status: in revision