

## Forebrain neurogenesis: From embryo to adult

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### ABSTRACT

A satellite symposium to the Canadian Developmental Biology Conference 2016 was held on March 16-17, 2016 in Banff, Alberta, Canada, entitled *Forebrain Neurogenesis: From embryo to adult*. The *Forebrain Neurogenesis* symposium was a focused, high-intensity meeting, bringing together the top Canadian and international researchers in the field. This symposium reported the latest breaking news, along with 'state of the art' techniques to answer fundamental questions in developmental neurobiology. Topics covered ranged from stem cell regulation to neurocircuitry development, culminating with a session focused on neuropsychiatric disorders. Understanding the underlying causes of neurodevelopmental disorders such as autism spectrum disorder (ASD) and attention deficit/hyperactivity disorder (ADHD) is of great interest as diagnoses of these conditions are climbing at alarming rates. For instance, in 2012, the Centers for Disease Control reported that the prevalence rate of ASD in the U.S. was 1 in 88; while more recent data indicate that the number is as high as 1 in 68 (Centers for Disease Control and Prevention MMWR Surveillance Summaries. Vol. 63. No. 2). Similarly, the incidence of ASD is on the rise in Canada, increasing from 1 in 150 in 2000 to 1 in 63 in 2012 in southeastern Ontario (Centers for Disease Control and Prevention). Currently very little is known regarding the deficits underlying these neurodevelopmental conditions. Moreover, the development of effective therapies is further limited by major gaps in our understanding of the

fundamental processes that regulate forebrain development and adult neurogenesis. The *Forebrain Neurogenesis* satellite symposium was thus timely, and it played a key role in advancing research in this important field, while also fostering collaborations between international leaders, and inspiring young researchers.

**KEYWORDS:** forebrain, neurogenesis, stem cell, neurodevelopment, neuropsychiatric disorders

### 1. Introduction

The evolution of the cerebral cortex is believed to be one of the most critical developmental processes underlying cognitive differences between humans and lower mammals. Indeed, defective development of the cerebral cortex is a major cause of intellectual disability disorders. In order to understand the molecular basis of these disorders and to develop future therapies to treat them, a thorough understanding of cerebral cortex development is required. The satellite symposium *Forebrain Neurogenesis: From embryo to adult*, focused on highlighting recent advances in the basic principles of cerebral cortex development. The cortex is a laminar structure that develops from a single layer of neuroepithelial progenitor cells. As such, the basic principles regulating stem cell proliferation, cell fate specification and regionalization are critical to proper development and were key topics of discussion. In addition, understanding how these processes go awry in developmental disorders and how we can induce adult neural stem cells to repair

or treat such disorders were also key topics in the symposium. As a prelude, we provide a brief description of how the forebrain develops before summarizing the highlights of talks presented in the symposium.

Early regionalization of the neural tube gives rise to three vesicles: the rhombencephalon, which gives rise to the hindbrain; the mesencephalon, which gives rise to the midbrain; and the prosencephalon, which gives rise to the forebrain. The forebrain, which was the subject of this meeting, first becomes a distinct region at E8.5 in mice [1, 2]. The forebrain is further subdivided into the diencephalon, which later forms the retina, thalamus, subthalamus, hypothalamus, and epithalamus, and telencephalon, which later forms the basal ganglia, amygdala, and cerebral cortex. The cerebral cortex is further subdivided into the hippocampus, piriform cortex, and neocortex. The establishment of these distinct structures starts with ligand gradients in the rostral-caudal and dorsal-ventral axis. These ligand gradients confer regional identities onto the embryonic neural stem cells that line the ventricles of the forebrain, resulting in the regional-specific expression of transcription factors that confer a spatial identity. Once a regional identity is specified, the factors that promote neurogenesis come into play. A mixture of transcription factors, signaling pathways, and epigenetic factors combine to control neurogenesis, and later gliogenesis. Notably, neurogenesis is not confined to embryonic time-points. Two sources of neural stem cells persist into adulthood. These adult neural stem cells reside in the subventricular zone and subgranular zone of the forebrain and contribute to neurogenesis throughout the lifespan of the organism.

The satellite symposium was organized into three sessions, discussing recent findings in three areas of forebrain development: stem cell regulation, both embryonic and adult; forebrain development and neural fate specification, including regionalization and subtype specification; and understanding neurodevelopmental/neuropsychiatric disorders. In the following sections we present a summary of talks presented in the three sessions at the symposium.

## **2. Session 1: Stem cell regulation**

Proper development of the nervous system relies on a delicate equilibrium between neural stem cell (NSC)

proliferation and differentiation. In the forebrain, differentiation is temporally regulated, with NSCs gradually becoming restricted in their developmental potential, first generating neurons, followed by astrocytes and finally oligodendrocytes. By first generating neurons, neural circuits are established before the formation of glial cells, which support the circuitry. Several factors influence the decision by NSCs to proliferate or differentiate and the temporally-regulated choice of neural progeny, including length of the cell cycle, mode of cell division, and extrinsic and intrinsic determinants. The opening session was focused on understanding the molecular mechanisms underlying the regulation of NSC and progenitor cell fate decisions in embryogenesis, as well as the early development of adult NSCs. Several key mechanisms were addressed, including the regulation of quiescence, size of the stem cell pool, and self-renewal versus commitment decisions. Key findings relevant to this theme are discussed below.

### **2.1. Regulation of neural stem and progenitor cells in embryogenesis**

During early development, the telencephalon is a pseudostratified neuroepithelium that is comprised of a single layer of neural progenitor cells termed neuroepithelial cells. Neuroepithelial cells have long processes that extend to, and make contact with, both the ventricular (apical) and pial (basal) surfaces of the embryonic neural tube. Between embryonic day (E) 10 and E12 in the mouse brain, neuroepithelial progenitors give rise to a different type of neural progenitor called a radial glial cell (RGC), which, much like its neuroepithelial forebearers, attaches long processes to the apical/ventricular and basal/pial surfaces (Figure 1) [3]. RGCs maintain many properties of neuroepithelial cells while also gaining some astroglial properties, expressing glial proteins such as GLAST, BLBP and RC2 [3]. RGCs either continue to proliferate, differentiate into neurons, or give rise to intermediate neuronal progenitors (INPs), either through symmetric or asymmetric cell divisions [4]. INPs are a type of neural progenitor that is more restricted in developmental potential, normally undergoing only 1-2 divisions before differentiating. When INPs form, they translocate basally, and during this process, lose their adherens junctions and retract their apical processes [4]. The basal migration of INPs results in the formation of

a new progenitor layer called the subventricular zone (SVZ). INPs also differ from RGCs in that they lack polarity and their mode of division is almost exclusively symmetric neuronal [4].

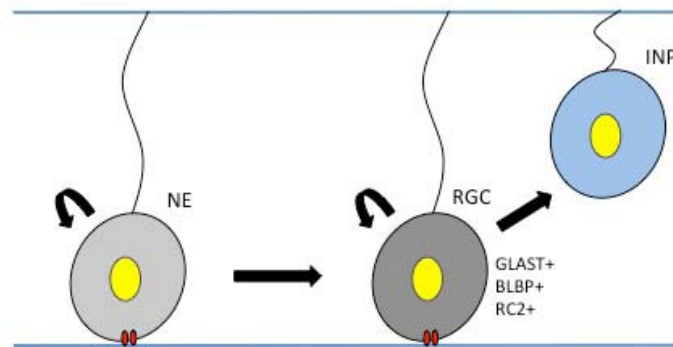
In the developing neocortex, the decision to proliferate or differentiate is regulated at multiple levels, including epigenetic changes that influence gene regulation. Polycomb group proteins modulate chromatin structure to confer long term transcriptional repression. The Ezh1 (Enhancer of Zeste homolog) and Ezh2 methyltransferases are part of the Polycomb repressive complex 2 (PRC2), and they catalyze H3K27 trimethylation (H3K27me3), which is a repressive heterochromatin mark [5]. PRC2 also recruits PRC1, which recognizes H3K27me3 marks, and PRC1 then further modifies chromatin into a repressive state *via* Ring1a-mediated monoubiquitination of H2AK119 [6]. Yukiko Gotoh demonstrated that polycomb group proteins regulate cortical neurogenesis by repressing the expression of *Neurog1* [7], a proneural gene encoding a basic-helix-loop-helix (bHLH) transcription factor that acts redundantly with *Neurog2* to promote the differentiation of deep-layer neurons in the neocortex [8]. She observed increases in H3K27me3 marks over the period of corticogenesis at the *Neurog1* locus. A conditional mutation in *Ring1b* blocked this process, resulting in the derepression of *Neurog1* expression. As a consequence, the neurogenic phase was elongated and astrocyte differentiation was delayed in *Ring1b*-null cortices [7].

To further explore the molecular mechanisms by which neuronal differentiation is regulated in the developing neocortex, Stefano Stifani and colleagues showed that the NF- $\kappa$ B pathway has an important role in inhibiting progenitor differentiation into neurons [9]. Using *NF- $\kappa$ B-LacZ* reporter mice, the group showed that *NF- $\kappa$ B* was expressed in radial glia during corticogenesis. Blocking this pathway with a dominant negative form of *IKK $\beta$*  resulted in fewer proliferative (Ki67<sup>+</sup>) cells and premature neuronal differentiation. Knock-down with *RelA* siRNA also caused a decrease in progenitor cells and differentiation of supernumerary neurons, while expression of exogenous *RelA* expanded the progenitor population. Using protein-protein interaction and transcription assays they revealed that *Hes6* could inhibit *NF- $\kappa$ B* by repressing *RelA*. In agreement with these competing biochemical functions, *Hes6*

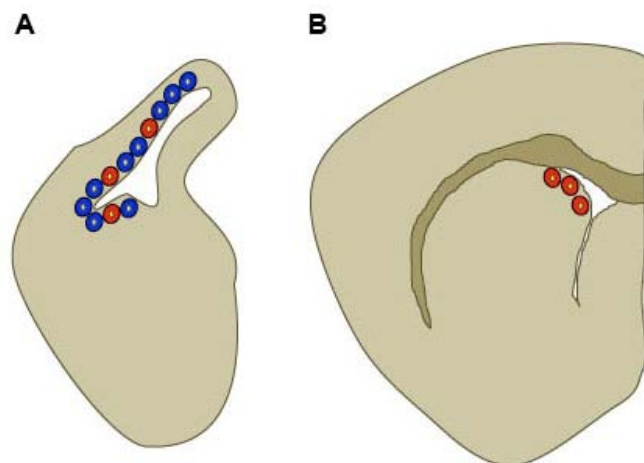
and *NF- $\kappa$ B* play opposing roles during cortical neuronal differentiation. These studies revealed that the NF- $\kappa$ B pathway, acting at least in part through antagonistic interactions with *Hes6*, plays an important role in the regulation of progenitor cell fate during cortical neurogenesis.

Studies presented by Carol Schuurmans revealed further insight into the mechanisms by which forebrain progenitors are maintained. In the cortex, a common progenitor pool gives rise to different cell types in sequence, and hence control mechanisms must exist to maintain some progenitors for later differentiation events [10]. *Neurog2* and *Ascl1* encode bHLH transcription factors that are traditionally considered to have proneural functions, promoting cell cycle exit and the acquisition of opposing neuronal fates - glutamatergic and GABAergic neuronal phenotypes, respectively [11, 12]. The Schuurmans' lab found non-traditional roles for these genes when they are co-expressed in cortical progenitors (unpublished). They demonstrated that *Neurog2* and *Ascl1* proteins physically interact *in vivo*, and antagonize each other at the level of expression and function. Time-lapse imaging revealed that *Neurog2-Ascl1* double-positive progenitors resolve over time into single-positive cells, but as double positive cells, they are molecularly distinct, undergo self-renewing divisions, and preferentially differentiate into oligodendrocytes, the last-born neural cell type. This data implicates proneural genes in multi-lineage priming and stem cell maintenance, a novel function for these genes.

Mitochondria are critical components of cellular metabolic function. Mitochondria dynamically change their shape in response to the functional demands of the cell. In *Sox2*-expressing apical progenitors, mitochondria have an elongated shape, but as cells commit to differentiation and become *Tbr2*-expressing progenitors, mitochondria become fragmented. Ruth Slack showed that disrupting mitochondrial shape in *Sox2*-expressing progenitors through deletion of *Mfn1/2* led to mitochondrial fragmentation, and resulted in a depletion of *Sox2*<sup>+</sup> cells and an increase in the *Tbr2*<sup>+</sup> committed population [13]. Forced mitochondrial fragmentation by knock-down of fusion proteins *Mfn1/2* or *Opa1* caused an increase in mitochondrial reactive oxygen species, which induced a nuclear transcriptional program to suppress stem cell self renewal and



**Figure 1. Embryonic progenitor transitions in the neocortex.** Between E10.5 and E12.5, neuroepithelial (NE) cells transition to radial glial cells (RGC), upregulating the expression of glial factors (GLAST, BLBP, RC2). Radial glia can self-renew, differentiate, or give rise to basally-localized intermediate neuronal progenitors (INP). Curved arrows represent self-renewal, while straight arrows represent transitions to other cell types.



**Figure 2. Embryonic and adult neural stem cells.** (A) Schematic of a frontal hemi-section of the embryonic mouse telencephalon. Embryonic neural stem cells are heterogeneous, with both active (blue) and quiescent (red) cells lining the lateral ventricles. (B) Schematic of a frontal hemi-section of an adult mouse cerebral cortex. Neural stem cells that were quiescent during embryogenesis now comprise the adult neural stem cell niche (red) in the subventricular zone.

enhance differentiation. A key mechanism underlying this process was the activation of the oxidative stress response transcription factor, *Nrf2*, which triggered a dual transcriptional program to inhibit stem cell self renewal and promote differentiation. Thus, changes in mitochondrial dynamics can signal retrogradely to the nucleus to modify critical self-renewal vs commitment decisions, thereby modifying the fate of neural stem cells [13].

In the embryonic forebrain, RGCs have been identified as having stem cell-like activity [14]. In addition, RGCs serve as a scaffold for glial-guided neuronal locomotion, extending processes that contact both the basal and ventricular surfaces of the brain. Deborah Kurrasch has found that these RGC projections are not ubiquitous in the tuberal hypothalamus, and has uncovered a novel mechanism controlling their pruning within a distinct region.

Previous work in the Kurrasch Lab showed that the tuberal hypothalamic progenitor zone is subdivided into distinct domains responsible for giving rise to discrete populations of neurons [15]. It is the dorsal-most domain that is particularly intriguing, since this region seems to give rise not only to neurons, but also to macroglia, tanycytes, and ependymal cells (unpublished). She also noticed that in the embryonic brain, microglia congregate around this same progenitor domain and appear to be interacting with RGC projections. A functional relationship between the two cell types was further elucidated by depleting microglia in the brain by feeding mice a chow treated with the *Csf1r* antagonist PLX5622 ([www.plexxikon.com](http://www.plexxikon.com)). Strikingly, in the absence of microglia, RGC fibers were more abundant in the tuberal hypothalamus. Furthermore, using time-lapse imaging, Dr. Kurrasch was able to show that microglia make contact with RGC fibers, surround them, and then sever these projections. This clipping behaviour is associated with the accumulation of RC2<sup>+</sup> RGC debris within microglia, consistent with active phagocytosis. While the significance of microglia-mediated RGC clipping is not known, an intriguing possibility is that the removal of RGC fibers makes room for tanycyte projections, which are specialized, RGC-derived cells that play an important role in the release of hormones. While RGCs are present in the embryonic brain, tanycytes only appear in the last few days before birth. Microglia-clipping of RGCs may leave ‘space’ for the tanycytes to project their fibers, suggesting that there may be a finite room for projections from these two cell types.

Taken together, key decision points involving self-renewal and commitment are regulated by a complex network of intrinsic factors that respond to cellular metabolism, epigenetic regulation as well as environmental changes. Importantly, the NSCs that will occupy the adult brain are generated during forebrain development. Thus, future studies examining the early regulation and expansion of this population will contribute greatly to efforts for mobilizing this stem cell pool for cellular repair.

## 2.2. Adult neural stem cell regulation

Recent studies presented by Yukiko Gotoh revealed that adult stem cells are generated during embryogenesis and that these embryonic cells, which

reside in the SVZ, remain quiescent into adulthood (Figure 2). Examination of the underlying mechanisms revealed that the cyclin-dependent kinase inhibitor *Cdkn1c* (p57Kip2) was crucial for the maintenance of stem cell quiescence, as deletion of *Cdkn1c* resulted in loss of quiescence and the depletion of adult NSCs [16].

Given that most adult NSCs are quiescent and very few progress through the cell cycle, the question as to how entry and return to quiescence is regulated remains a topic of intense interest. Recent studies presented by Francois Guillemot explored the mechanisms by which subgranular zone (SGZ) stem cells within the hippocampus regulate the transition from activated to quiescent states. Their studies revealed that in SGZ stem cells, the E3-ubiquitin ligase *Huwe1* [17] becomes activated and destabilizes *Ascl1*, a factor that is required for stem cell activation. When *Ascl1* is degraded, stem cells remain quiescent. In search of the underlying mechanism, *Ascl1* was found to bind to enhancers for *Ccnd2*, *Rrm2*, *Skp2*, and *Fbl*, all cell cycle regulators, while *Ascl1* loss resulted in depletion of D cyclins and entrance into a quiescent state. Thus, *Ascl1* is an activation factor that must be suppressed to enable stem cells to enter a resting state, which is essential for their long term maintenance in the adult brain.

Ion channel *Pannexin 1* (*Panx1*) has been previously shown to play a role in growth in the VZ. Studies from the lab of Leigh Anne Swayne (presented by Leigh Wicki-Stordeur) demonstrated that *Panx1* plays an important role in adult neural precursor cell (NPC) maintenance [18]. When tracking NPC numbers in the VZ, *Panx1*-null progenitors were less abundant than *Panx1*-expressing progenitors. After stroke, NPC numbers were increased, but this increase required *Panx1* expression, suggesting that *Panx1* is important to mediate progenitor expansion after stroke. However, in peri-infarct cortices, *Panx1*-null cells had improved NPC survival. *Panx1*-expressing cells are believed to release ATP that acts as an “eat-me” signal to microglia in the peri-infarct cortex. Thus, the role of *Panx1* in the adult brain is context-specific, suggesting that ion channels are critical regulators of neural stem/progenitor population size, which will be an important focus of future studies.

In summary, key questions regulating the adult stem cell pool include uncovering the mechanisms by which stem cell quiescence and activation are controlled, defining intrinsic and extrinsic factors that regulate population size, and identifying the signaling pathways that determine their ultimate fate in the healthy and injured brain, all of these will be subjects for intensive investigations.

### 3. Sessions 2 and 3: Forebrain development and neural cell fate specification

The central nervous system (CNS) is characterized by an astounding diversity of neuronal phenotypes that are generated in a region-specific manner in the neural tube. In the last few decades, great strides have been made towards understanding how neurons acquire their specific identities during development, revealing a central role for both intrinsic and extrinsic factors, including multiple transcription factors, growth factors, and their downstream signal transduction molecules. The lab of Pierre Drapeau has revealed that glycine is a novel signal that also controls neurogenesis, or the decision by neural progenitor cells to differentiate into neurons [19]. Eric Samarut from his lab presented mechanistic insights into this process, revealing that glycine binds to its receptor, GlyR, to hyperpolarize progenitor cells by inducing Cl<sup>-</sup> influx through NKCC1 channels, leading to neurogenesis. Consequently, if GlyR signaling is blocked, neurogenesis is decreased, but interestingly, only interneuron numbers and not motor neuron numbers decline in the spinal cord. Eric went on to perform a transcriptomic analysis of GlyR-deficient NSCs in zebrafish, and identified five different signaling pathways that may mediate the effects of glycine on neurogenesis [20]. Further studies will help to clarify how these pathways mediate the effects of glycine on neurogenesis, and will also address whether these signaling pathways are similarly at play in the forebrain.

Much of the work on neuronal fate specification has been conducted in the telencephalon. The embryonic telencephalon is divided into distinct dorsal and ventral domains that give rise to the cerebral cortex and basal ganglia (striatum & globus pallidus), respectively. The cerebral cortex is comprised of two neuronal populations; excitatory, glutamatergic projection neurons and a smaller number of inhibitory,

GABAergic interneurons. Cortical projection neurons are born from progenitor cells situated in the ventricular zone (VZ) of the dorsal telencephalon over 11 cell divisions, differentiating between embryonic day (E) 10.5 and E17 [21, 22]. As projection neurons differentiate, they migrate radially to generate the six neuronal layers of the mature cortex. In the first wave of neurogenesis, presumptive layer I and transient layer VII (subplate) are formed, followed by the inside-out and sequential generation of layer VI, V, IV and finally II/III neurons. Interspersed amongst the six cortical layers are GABAergic interneurons, which are derived from progenitors in the medial and caudal ganglionic eminences in the ventral telencephalon [23]. GABAergic interneurons born in ventral domains migrate tangentially to reach the cortex [24-26]. We describe the work presented in the symposium that provided new insights into how cell fate specification is regulated in this region of the neural tube.

#### 3.1. Regionalization of the forebrain

Cellular differentiation requires that neural progenitors first acquire a regional identity, which is often conferred by homeodomain transcription factors, followed by subtype specification and differentiation, which are induced by other transcription factors and signaling molecules. A great deal of work has gone into elucidating the patterns of transcription factor expression that help establish regional identity, identifying homeodomain transcription factors such as *Emx2* and *Pax6* as important determinants of a rostrocaudal identity [27]. The identification of new factors involved in the acquisition of regional identity was described in this meeting. John Rubenstein and colleagues showed a role for *Pbx1*, a TALE-homeodomain transcription factor, in establishing a frontal regional identity in the cerebral cortex [28]. *Pbx1* conditional knock-outs (cKO) were generated with an *Emx1-cre* driver specific to cortical progenitors, or a *Nex1-cre* driver specific to cortical neurons. In *Pbx1* cKO cortices generated with *Emx1-cre*, *Lmo4*<sup>+</sup> and *Nt3*<sup>+</sup> frontal domains were lost, while caudal *Lmo4*<sup>+</sup> domains shifted rostrally, and dorsal *Nt3*<sup>+</sup> domains shifted ventrally. *Pbx1* cKOs generated with *Nex-Cre* also had reduced expression of frontal domain markers, albeit to a lesser extent compared to those generated with *Emx1-cre*. Cortical defects associated with the loss of *Pbx1* were more severe in a *Pbx2*<sup>+/-</sup> background,

indicating that there is some functional redundancy amongst genes in the *Pbx* family. *Pbx* genes are thus required in both progenitors and neurons to establish a frontal identity, while the repression of dorsal gene expression is a progenitor-specific function. Further studies were performed on *Pbx1;Emx1-Cre* mice, revealing that Reelin was ectopically expressed in frontal domains, leading to inversion of the cortical layers. RNA array analyses identified several deregulated genes in the E15.5 *Pbx1* cKO cortex. To determine which genes were *Pbx1* targets, *Pbx1* ChIP-Seq was performed. Repressive binding sites for *Pbx1* were found in several genes, including *Lhx2* and *Emx2*, known cortical patterning genes, and *Reelin*, a regulator of neuronal migration.

Another important gene for forebrain patterning is the zinc finger protein *Prdm15*, a potential transcriptional regulator. *Prdm15* is of interest as it maps to the Down syndrome trisomy region in humans and mice. Landry Nfonam from Monica Justice's lab at the Hospital for Sick Children generated *Prdm15* knockout mice and reported that *Prdm15* is also responsible for regulating rostral-caudal patterning in the forebrain, with mutant embryos developing truncated fore and mid-brain structure and a rostral expansion of caudal regions of the brain. Given the link to Down syndrome, this study provides important insights into what might go awry in the forebrain of children afflicted with this neurodevelopmental disorder.

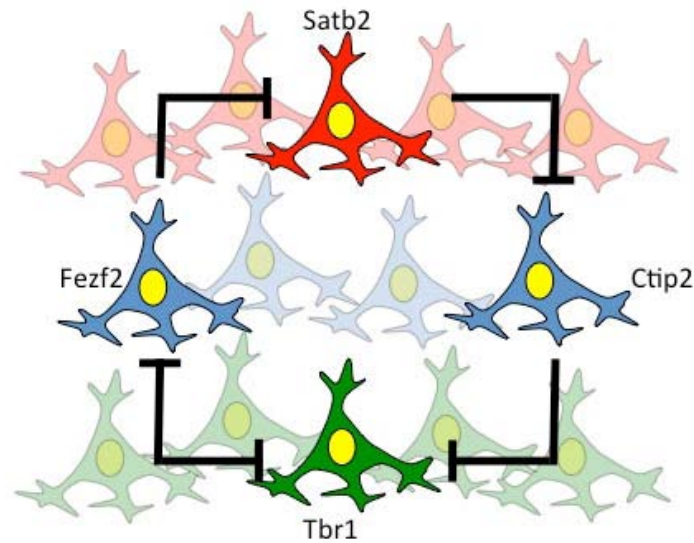
### 3.2. Laminar fate specification

In the neocortex, the laminar fates of glutamatergic projection neurons are specified by a depression circuit involving the transcription factors *Tbr1*, *Fezf2*, *Satb2* and *Ctip2*, which are each expressed in different neuronal layers (Figure 3). *Tbr1*, a T-box transcription factor, is highly expressed in layer VI, where it specifies a corticothalamic neuronal identity [29]. Within layer VI, *Tbr1* is also required to repress an alternative layer V subcerebral identity by inhibiting the expression of *Fezf2*, a zinc finger transcription factor required to specify a layer V fate [30]. Within layer V, *Fezf2* also represses *Tbr1* expression to prevent the acquisition of a corticothalamic fate in layer V [31, 32]. *Fezf2* also represses *Satb2* expression [33], which is a nuclear matrix DNA-binding protein that specifies a layer II-III callosal identity [34, 35]. In layer II-III callosal neurons, *Satb2* represses the expression of *Ctip2*,

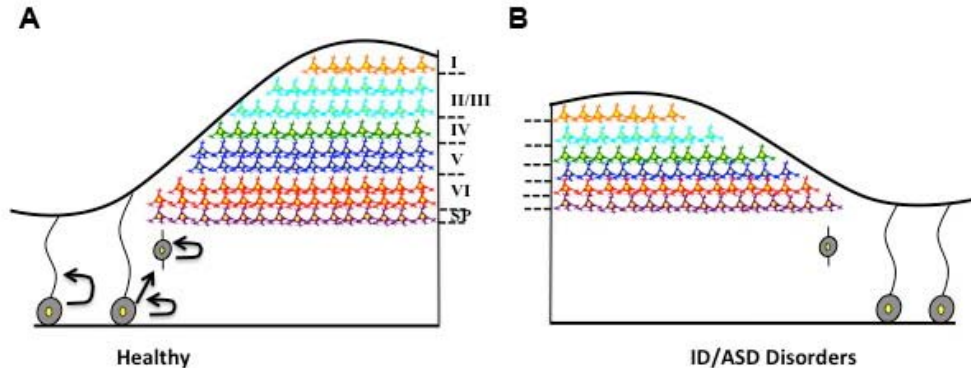
a zinc finger transcription factor required for the formation of layer V subcerebral axon projections [36, 37]. *Ctip2* also represses *Tbr1* expression in layer V, preventing layer VI neurons from being generated in their incorrect position [38].

While the cortical derepression circuit has been well described, inputs into this circuit are poorly understood. Angelo Iulianella presented unpublished work demonstrating an important role for *Mllt11* in laminar fate specification. *Mllt11* is a conserved, poorly characterized 90 amino acid protein containing a nuclear export signal that is upregulated in aggressive leukemias, but which has not been studied in the nervous system [39]. The Iulianella group identified *Mllt11* in a screen for *Cux* factor-interacting proteins, which regulate the decision by neural progenitors to proliferate or differentiate in the CNS [40]. In the neocortex, they found that *Mllt11* was highly expressed in upper layer neurons and lower in deep layer neurons [41]. In *Mllt11* mutant mice, cortical progenitor cells were normal; however the expression of laminar-specific neuronal markers was altered. *Cux2*, *Brn2* and *Satb2* expression was highly reduced in upper layers of the neocortex, while corticothalamic projection neuron identity was lost. Strikingly, the number of *Ctip2*<sup>+</sup> neurons was normal. Importantly, *Mllt11* loss led to the abnormal accumulation of *Cux1* and *Reelin* cells in the ventricular region, while *Mllt11* overexpression promoted neuronal migration to the superficial cortical plate. Collectively this data suggests that *Mllt11* is required for migration of superficial neurons and the establishment of cell interactions with *Reelin*-expressing cells in the marginal zone, highlighting the complexity of laminar fate specification in the neocortex.

Nenad Sestan and colleagues identified an important cis-regulatory element 7.3 kb downstream of *Fezf2*'s transcriptional start site, which they named the E4 enhancer [42]. When E4 was deleted, fluorescence was lost from a 200 kb *Fezf2-GFP* BAC transgene that recapitulates the endogenous expression profile of *Fezf2* in the cortex [43]. Strikingly, knock-out mice lacking E4 displayed a striking decrease in *Fezf2* expression levels, phenocopying the defects in corticospinal projections observed in *Fezf2* mutant mice. Based on the identification of 8 Sox-binding sites in the E4 enhancer, they screened for potential Sox factors that would bind to E4 based on overlap



**Figure 3. Derepression circuit for neocortical sub-type specification.** *Satb2* specifies a callosal projection neuron identity (red), *Fezf2* and *Ctip2* specify a subcerebral projection neuron fate (blue), and *Tbr1* specifies a corticothalamic projection neuron identity (green). *Satb2* represses subcerebral factor *Ctip2* and is in turn repressed by subcerebral factor *Fezf2*. *Ctip2* represses corticothalamic factor *Tbr1* and is repressed by callosal factor *Satb2*. *Fezf2* represses callosal factor *Satb2* and has reciprocal repression with corticothalamic factor *Tbr1*.



**Figure 4. Impaired proliferation in neurodevelopmental disorders.** (A) Schematic of normal neocortical lamination arising from the controlled decision by cortical progenitor cells to self-renew, proliferate or differentiate, leading to the formation of the six layers of the mature neocortex and the subplate. (B) Schematic of defects in neocortical development in children with neurodevelopmental disorders. Neurodevelopmental disorders often have defects in progenitor cell self-renewal and proliferation, resulting in reduced neuronal production and thinning of the cortical layers.

in their spatiotemporal expression profiles. The screen identified 3 *Sox* transcription factors that converge onto E4, including *Sox4*, *Sox11*, and *Sox5*. While *Sox5* repressed *Fezf2* expression, *Sox11* or *Sox4*

were able to alleviate this repression, indicating that *Sox4* and *Sox11* compete with *Sox5* for E4 enhancer binding [42, 44]. Accordingly, *Fezf2* expression was lost in *Sox4;Sox11*-null mice, which also



displayed perturbations in their corticospinal axon tracts. The regulation of *Fezf2* expression is thus a central control mechanism for the formation of corticospinal axon tracts.

Yukiko Gotoh also demonstrated that Polycomb group proteins are important for laminar fate specification. They found that *Ring1b*, which is part of *Prc2*, associates with the promoter of the subcerebral fate determinant *Fezf2*. In *Ring1b*-null mice there was increased production of subcerebral (*Ctip2*<sup>+</sup>) cells [45].

### 3.3. Specification of a GABAergic interneuron fate

*Dlx* genes (*Dlx1/2*, *Dlx5/6*) are key transcriptional regulators involved in the specification of a GABAergic interneuron identity in the ventral telencephalon [24, 26, 46]. Marc Ekker's group has been instrumental in identifying the regulatory mechanisms governing the expression of these genes, which share intergenic enhancers comprised of conserved regulatory elements between mouse and zebrafish [47]. Understanding the regulation of *Dlx* gene expression is critical, since these genes are required for the generation of forebrain GABAergic interneurons, and an imbalance between excitatory and inhibitory neurotransmission is associated with autism. Consistent with these findings, a SNP has been identified in an autistic proband in the middle of the 156i enhancer, which was identified by Dr. Ekker as being essential for the expression of *Dlx5/6* in cells of the ventral telencephalon. Interestingly, mice in which the 156i enhancer has been deleted show altered fear conditioning, and increased socialization, which reflects a potentially related condition in which affected kids are friendlier to strangers. These studies highlight the medical relevance of basic biological studies on transcriptional regulatory elements in model systems.

David Eisenstat reported on downstream targets of *Dlx1/2* in the ventral forebrain, revealing that these transcription factors bind to the promoter of *neuropilin-2* (*Nrp2*), which encodes a Semaphorin co-receptor (unpublished and [48]). In *Dlx1/2* double knockouts, *Nrp2* is ectopically expressed, indicating that *Dlx1/2* act as transcriptional repressors at this locus, which was confirmed using *Dlx1/2*-VP16 and *Dlx1/2*-Engrailed fusion proteins. Dr. Eisenstat hypothesized that the ectopic *Nrp2* expression would

contribute to the blockade of tangential migration of interneurons into the forebrain in *Dlx1/2* double knock-outs. Indeed, in *Dlx1/2*;*Nrp2* triple knockouts, there was a partial rescue of the migration of somatostatin<sup>+</sup> interneurons into the neocortex. *Nrp2* is thus an essential downstream target of *Dlx1/2* for the guidance of interneuron migration in the neocortex.

*Ctcf* is an insulator protein that helps organize the 3D looping structure of chromatin. *Ctcf*-binding sites are enriched at the borders of topologically associated domains (TADs). Since knocking out *Ctcf* leads to massive cell death and a loss of the forebrain [49], Nathalie Berube and colleagues used a *Nestin-Cre* driver to inactivate *Ctcf* at a slightly later, less harmful, time-point. The mutant forebrain had increased DNA damage in proliferative zones and evidence of DNA replication stress. Since the lethality of *Nestin-Cre*;*Ctcf*<sup>fllox</sup> mice precluded postnatal analysis, *Ctcf* deletion was driven by *Nkx2.1-Cre* mice, where deletion was specific to the MGE. While these mice survived postnatally, they had a loss of somatostatin<sup>+</sup> and parvalbumin<sup>+</sup> GABAergic interneurons caused by a defect in early fate specification of MGE neural progenitors.

## 4. Session 4: Understanding neurodevelopmental/neuropsychiatric disorders

One important reason a thorough understanding of neurogenesis is required is to help delineate the pathogenesis associated with cognitive impairment. Neurodevelopmental/neuropsychiatric disorders is a collective term encompassing intellectual disability (ID) and autism spectrum disorders (ASD) that have an estimated prevalence of ~2% of the population, and a prevailing underlying genetic predisposition [50, 51]. Diagnostically, neurodevelopmental disorders present with a heterogeneous clinical presentation that is confirmed at the molecular level with over 700 genes identified [52]. Analyses of gene function have identified several common pathways that may alter brain development/function, including impaired proliferation of neural progenitors (Figure 4), defects in neural migration, synaptogenesis and synaptic signalling defects, and epigenetic regulation [53-55]. More importantly, animal models are facilitating the identification of dysregulated pathways [56], some of which are proving to be amenable to therapeutic strategies, a proposition that was

considered to be unattainable a decade earlier. The final session of the symposium highlighted some recent advances in our understanding of these complex disorders.

Prader-Willi syndrome (PWS) is a rare multigene disorder characterized by developmental delay, low muscle tone, and hyperphagia leading to obesity at approximately 8 years of age [57]. In children with PWS, inactivation of *MAGEL2* is believed to be a critical component of the PWS phenotype. Indeed, *de novo* protein-truncating mutations in *MAGEL2* were recently identified and result in a PWS-like phenotype with neonatal hypotonia, developmental delay, autism, and variable hyperphagia and obesity, known as Schaaf-Yang syndrome [58, 59]. In mice, *Magel2* is highly expressed in the arcuate nucleus (ARC), with disruption of *Magel2* leading to PWS-like symptoms, including increased adipose tissue. Interestingly, the onset of obesity in PWS patients is later than other forms of genetic obesity suggesting that there may be a postnatal developmental or degenerative component to obesity in PWS patients. Rachel Wevrick and colleagues showed that young (P10) *Magel2*-null mice have normal numbers of pro-opiomelanocortin (POMC) neurons in the ARC nucleus, and that the mutant POMC neurons activate Stat3 in response to leptin injection. To perform a broader postnatal analysis they used hypothalamic slice preparations and cytosolic calcium imaging with an EGFP-reporter to mark POMC neurons [60]. This study demonstrated that leptin insensitivity is not congenital, but occurs progressively, with normal leptin responses still observed in *Magel2*-null mice at 4 weeks old. However, at 6 weeks of age the proportion of leptin responsive POMC neurons had diminished in mutant mice. *Magel2* interacts with E3 ligases to modulate endosomal protein trafficking and protein degradation, while protein ubiquitination and recycling are also linked to autophagy. Autophagy is mediated by the p62 protein and *Magel2*-null mice showed aberrant levels of ubiquitylated p62 in POMC neurons and muscle, suggesting that *Magel2* is required for normal levels of autophagy in these tissues. Moreover, aberrant p62 levels in muscle were associated with impaired treadmill running and reduced grip strength, suggesting that muscle atrophy in *Magel2*-null mice is caused by altered autophagy. Overall, these results highlight *Magel2*-null mice

as a good pre-clinical disease model, opening an avenue for treatment of hyperphagia in children with Prader-Willi or Schaaf-Yang syndrome.

The alpha thalassemia mental retardation X-linked (ATR-X) syndrome is characterized by severe intellectual deficits, facial dysmorphia, microcephaly, and alpha-thalassemia that are caused by mutations in the *ATR-X* gene [61, 62]. The ATRX protein interacts with the histone chaperone Daxx and is critical for loading the histone variant H3.3 into chromatin, primarily at telomeres and repetitive sequences [63, 64]. Despite this knowledge, it is unknown how *ATR-X* mutations lead to the ATR-X syndrome phenotype. David Picketts and colleagues presented data characterizing the mechanism underlying the reduced forebrain development of *Atrx*-null mice [65]. In humans, loss of *ATR-X* is associated with reduced forebrain size, reduced production of late-born neurons, and activation of the DNA damage response in neural progenitors [66]. Accordingly, *Atrx*-null mouse cells were sensitive to hydroxyurea-induced replication stress and showed Parp-1 hyperactivation, which suggested that stalled replication forks were not sufficiently protected. DNA fiber assays showed that replication fork degradation was rampant and mediated by Mre11, while upregulation of Parp-1 was a compensatory protective mechanism. This data is indicative of an important role for *Atrx* in protecting rapidly proliferating cells against replication stress, thus limiting the accumulation of DNA damage.

While *Atrx* is an intrinsic regulator of neural progenitor numbers, David Kaplan and colleagues addressed whether extrinsic factors that altered NSC pools could impair cognition. To address this question, they focused on a maternal cytokine surge that occurs during first and second trimester maternal infections and is linked to schizophrenia and ASD [67, 68]. In mice, interleukin-6 (IL-6) release during maternal infection is associated with behavioral aberrations and hence they asked whether IL-6 also had effects on progenitor cells. Mice were given a single injection of IL-6 at E13.5, followed by analysis of the stem cell niche of adult mice. They observed expansion of the adult forebrain but not the hippocampal NPC pool, resulting from increased self-renewal of embryonic forebrain precursors. They went on to show that embryonic forebrain precursors expressed the IL-6

receptor and concluded that IL-6 signalling at E12-14 determines the size of the stem cell pool [69]. In an unpublished work, Kaplan and colleagues also discussed a link between low levels of the methylglyoxal (MG) detoxifying enzyme Glyoxylase 1 (Glo1) and ASD. Accumulation of MG would be of particular concern during gestational diabetes, when MG levels are already elevated. In mice, maternal exposure to MG resulted in adult progeny with diminished progenitor cells and increased neuronal number. These MG-treated progeny exhibited defects in fear conditioning.

Despite recent advances in ASD gene discovery by exome sequencing, most patients still lack a genetic identification. Flora Vaccarino and colleagues obtained induced pluripotent stem cells from families affected by idiopathic ASD and created telencephalic 3D-organoids to model early cortical development in ASD patients [70]. While they did not observe any specific genomic mutation, transcriptome and gene network analyses revealed changes in genes involved in proliferation, neuronal differentiation, and synaptic assembly. Organoids showed an increase in synaptic connections, as measured by increases in Synapsin-I<sup>+</sup> puncta. Increases in GABAergic (VGAT<sup>+</sup>) but not glutamatergic (VGLUT1<sup>+</sup>) puncta collectively suggested an increase in inhibitory synapses. Indeed, immunostaining for DLX1-2 and GAD1/GAD67 showed an increase in GABAergic fate determinants. Transcription factor *FOXG1* was identified as a consistently upregulated factor in ASD-derived organoids. Accordingly, *FOXG1* was targeted by shRNA constructs in ASD-derived organoids, resulting in a downregulation of GABAergic lineage factors. FOXG1 dysregulation leading to increased GABAergic neurons may be an important developmental event for ASD.

### 5. Synopsis and future perspectives

Proper functioning of the nervous system relies on a delicate equilibrium between excitatory and inhibitory neurotransmission. Indeed, in the neocortex, the region of the brain responsible for higher cognitive functioning and sensory processing, defects in the balance of excitatory and inhibitory signals are associated with severe neuropsychiatric disorders, including schizophrenia, bipolar disorder and autism. One of the challenges of the next century is to fully understand how the brain develops,

processes, stores and recalls information. With an increasing incidence of ID/ASD, and the cognitive decline associated with an aging population, a thorough understanding of these processes is critical for brain health. The satellite symposium on Forebrain Neurogenesis showcased a wide range of topics that indicated the complexity involved in answering such questions, but at the same time highlighted significant advances in defining the factors critical for manipulating neural stem cells, generating neuronal fate decisions, and delineating the mechanisms underlying ID/ASAD disorders. With predictions of ~2000 ID/ASD causative genes, there remains a bright future for the next generation of upcoming neurodevelopmental scientists and clinicians in the field.

### ACKNOWLEDGEMENTS

This symposium was co-organized by CS and RSS. CS is the Dixon Family Chair in Ophthalmology Research at Sunnybrook Research Institute and RSS holds a University of Ottawa Research Chair. We would like to thank Jason MacLaurin for his assistance in organizing the meeting. We would also like to thank several external sources for financial support of this symposium, including the Canadian Institutes of Health Research, International Society for Developmental Neuroscience, Campus Alberta Neuroscience, and the Company of Biologists. We would also like to thank internal sources of support, including the Hotchkiss Brain Institute, Alberta Children's Hospital Research Institute, and Departments of Biochemistry and Molecular Biology and Cell Biology and Anatomy, all at the University of Calgary, as well as the University of Ottawa Research Chair to RSS.

### CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

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