A Postnatal Progenitor Cell Population in the Mouse Cerebral Cortex Negatively Regulated by *Fgf10*.

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“If the brain were so simple that we could understand it, we would be so simple that we couldn’t.”

Emerson M. Pugh

For my dearest Grandad

1 ABSTRACT

There is growing evidence that neurogenesis is widespread within the postnatal/adult brain, in areas overlapping with expression of Fibroblast growth factor 10 (*Fgf10)* which is postulated as consistent with neural progenitor cell identity. To test this hypothesis in the postnatal cortex, 4-day old *Fgf10*$^{CreERT2::Rosa26\text{-}tdTomato}$ double-transgenic pups were tamoxifen-treated to activate tdTomato expression (Tom), and brains analysed at multiple stages thereafter. At each postnatal age Tom$^+$ cells were sparsely scattered throughout the cortex forming isolated 'clones'. The Tom$^+$ cells were scrutinised for expression of markers of neurons, intermediate progenitors, and proliferating cells, and scored for clone size, laminar and rostro-caudal distribution. To understand the role of *Fgf10*, parallel experiments were performed after its conditional deletion in *Fgf10*$^{CreERT2/floxed::Rosa\text{-}tdTomato}$ triple-transgenic mice. The 'clone' size, distribution and identity of *Fgf10*-deficient Tom$^+$ cells was then compared to the *Fgf10*$^{CreERT2/+}$ condition.

In both models, Tom$^+$ cells amplified and differentiated into both pyramidal and non-pyramidal neurons, and rare glial cells. Moreover, Tom$^+$ cells were distributed within all cortical laminae and across the rostro-caudal axis, with a majority populating the deep cortical layers and caudal regions. Strikingly, *Fgf10*-deficiency enhanced the amplification of Tom$^+$ cells resulting in more two-cell and greater Tom$^+$ clones, suggesting that loss of *Fgf10* induces at least one extra cell division. This was corroborated by detection of PCNA$^+$ Tom$^+$ cells. Surprisingly, no FGF10$^+$ cells were observed within the embryonic cortical germinal zones but were evident within the meninges after birth. Finally, FGF10's cognate receptors – *Fgfr1-IIIb* and *Fgfr2-IIIb* isoforms were not expressed in the postnatal cortex, indicating a non-canonical mode of FGF10 action, possibly involving its recently-identified intracellular function.

This indicates that postnatal cortical FGF10$^+$ cells exhibit characteristics of quiescent neural stem/progenitors, and that FGF10 normally represses neurogenesis. The novel cell populations identified here may be beneficial for approaches in prompting brain repair after injury or ageing.

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2 INTRODUCTION

2.1 The Mammalian Cerebral Cortex

The rodent cerebral cortex is the outermost layer of neural tissue surrounding the cerebrum, comprised of the neocortex and allocortex, and split into symmetrical left and right hemispheres in mammals. The neocortex is organised horizontally into 6 layers depending on cellular composition, whereas the allocortex has a variable lamination pattern, comprised of less than 6 distinct layers. In rodents, cortical layers II and III hold no architectural boundary, so are often referred to collectively as laminar II/III. The cerebral cortex is a vastly interconnected region, responsible for a range of functions. On a macroscale, such functions are localised to specific cortical regions. For example, the visual cortex and auditory cortex are concerned with the processing of visual and auditory stimulus respectively; the motor cortex, functions primarily to control voluntary movement; and the prefrontal cortex in humans is involved in complex cognitive processes such as working memory, reasoning and decision making, with some functions shared in rat models (Brown and Bowman, 2002; Kesner, 2000). Throughout evolution the neocortex has increased in size relative to other brain regions, characterised by an increase in neurogenesis, in line with the evolutionary increase in the complexity of cognitive function (Finlay and Darlington, 1995).

On a cellular level the cerebral cortex is comprised of two main heterogenous neuronal populations, interneurons and pyramidal projection neurons. Interneurons make local inhibitory connections within the cortex whereas pyramidal neurons are capable of projecting to distant targets (intracortical, subcortical and sub-cerebral). Broadly speaking, a majority of pyramidal neurons are glutamatergic, excitatory and the major output neurons of the cerebral cortex, whereas interneurons can be principally defined as inhibitory regulators of cortical circuitry, with GABA (γ-aminobutyric acid) as their predominant neurotransmitter (Molyneaux et al., 2007; Tan et al., 1998). Pyramidal neurons may be distinguished from interneurons by their dendritic morphologies, specifically their long apical projecting dendrite, and pyramidal shape of their soma (Spruston, 2008). Interneurons may be identified by axons that arborize within a cortical column, capable of projecting laterally, with their axons and dendrites restricted locally within the cerebral cortex (Markram et al., 2004).

Neurons residing within different layers of the neocortex send and receive inputs from intra-cortical locations, or other distant brain regions such as the thalamus and spinal cord. Broadly, the layer VI pyramidal neurons project to the thalamus, layer IV to the basal ganglia, thalamus, midbrain and brain stem. Glutamatergic neurons within layer V receive projections from the thalamus, and neurons residing in layers II/III send

projections to other cortical areas (Koester and O'Leary, 1993).

2.1.1 Embryonic Development

As mentioned previously, the cerebral cortex comprises of two main neuronal cell types, glutamatergic pyramidal neurons and GABAergic interneurons. The cerebral cortex contains a complex compliment of glial cells (astrocytes, oligodendrocytes and microglia) which populate the cortical plate during embryonic and postnatal development.

Embryonically the cerebral cortex originates from a thin proliferative layer of the dorsal telencephalon, derived from the prosencephalon, which is the most anterior vesicle of the neural tube. Expression of the genes *Paired box 6* (*Pax6)* and *empty-spiracles homeobox* (*Emx3)* is essential for specification of the dorsal telencephalon (pallium). In their absence a striatum-like structure forms in place of the cerebral cortex as a result of re-specification of pallial (dorsal telencephalon) structures to subpallial (ventral telencephalon) (Muzio et al., 2002). Neuroepithelial cells within the ventricular zone (VZ) are the earliest neural stem cells (NSCs) within the embryonic cerebral cortex. They initially divide symmetrically to expand the progenitor pool, and subsequently asymmetrically to give rise to cells called radial glia (RGCs) (Noctor et al., 2002). Both NSCs and RGCs extend radial processes from the ventricular lumen to the basement membrane, and may be distinguished from each other by the expression of glial specific markers, such as the astrocyte-specific L-glutamate/L-aspartate transporter (GLAST) expressed by RGCs (Hartfuss et al., 2001).

In early embryonic development, NSCs transition into RGCs which are restricted to give rise to neurons, and later astrocytes and oligodendrocytes as determined by *in vitro* fate mapping studies (Malatesta et al., 2000). RGCs have their cell bodies within the VZ, an apical process which contacts the ventricular surface, and a basal process terminating at the pial surface. The onset of neurogenesis, a hallmark of which is the generation of post-mitotic neurons, occurs at approximately E10.5 as projection neurons in the mouse and form a transient structure called the preplate at E11.5. Generation of new neurons and migration of them into the preplate splits this structure into the superficial marginal zone (presumptive layer I) and subplate, between which the cortical plate develops (**Fig. 2-1**). As neurogenesis proceeds a second proliferative germinal zone develops above the VZ known as the embryonic subventricular zone (eSVZ) at approximately E13.5 and contains basal intermediate progenitors (IPCs) derived from RGCs, which divide terminally in mid-late neurogenesis. By E19 the eSVZ is the primary germinal zone within the developing brain, cells produced by this

region from E17 though postnatal life are destined glial cells (Levison et al., 1993; Parnavelas, 1999). At approximately E12 the first cohort of interneurons innervate the preplate derived from the medial ganglionic eminences and migrating tangentially into their position (Métin et al., 2006). The following section will give an overview into the process of embryonic cortical neurogenesis, in terms of the broad subtypes that comprise this brain region, the accompanying complement of glial cells and genetic regulators.

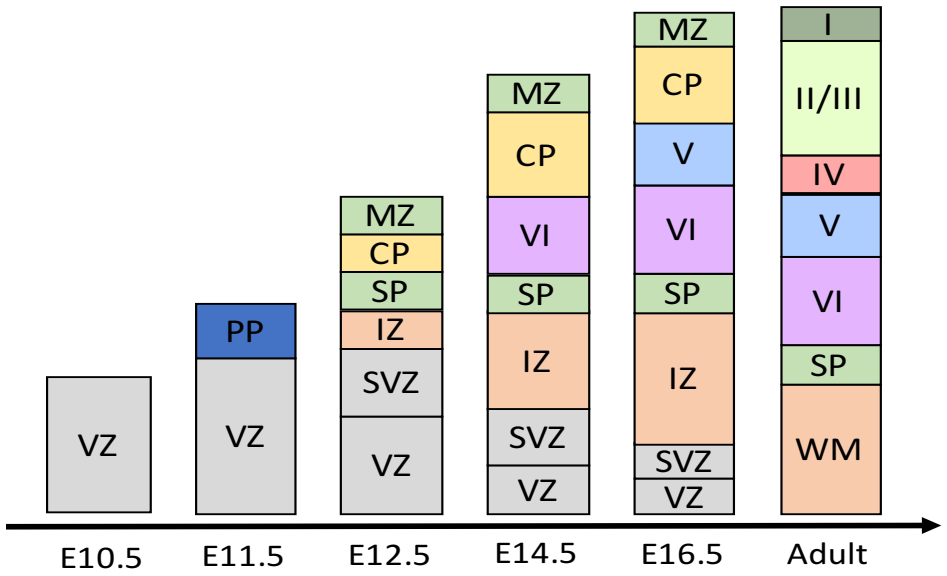

Figure 2-1 Growth and establishment of layers in the embryonic mouse cerebral cortex: The splitting of the cortical plate through mouse embryonic development into the mature adult laminar structure as identified by roman numerals I-VI. Adapted from Molyneaux et al., 2007. VZ, ventricular zone; PP, Preplate; SVZ, Subventricular zone; IZ, Intermediate zone; SP, Subplate; CP, Cortical Plate; MZ, Marginal zone; WM, White Matter.

2.1.1.1 Pyramidal Neurons

In mouse cortical development, projection neurons are born within the eSVZ and VZ, either directly or indirectly derived from RGCs between mouse embryonic day 10.5 and 17.5 (Caviness et al., 1995; Rakic, 1990). RGCs are first identified within the murine cerebral cortex at mouse embryonic day 10.5, which coincides with Notch signalling within the dorsal telencephalon, as determined by detection of the Notch effectors *Hes1* and *Hes5* (Hatakeyama, 2004; Mission et al., 1988). Premature activation of Notch pathways has been shown to strongly activate expression of RGC markers, highlighting its role in this transition (Gaiano et al., 2000).

New-born neurons from the proliferative zone (VZ) migrate radially into the cortical plate, perpendicular to the vesicular surface, using RGCs as a scaffold (**Fig. 2-2A**).

Waves of post-mitotic new-born neurons bypass earlier generated neurons to find their place within the cortical plate, generating cortical laminae in an inside-out pattern, with the deepest layers of the cerebral cortex containing the earliest born neurons, and layer 2 containing the latest born neurons (Jun and Sidman, 1961). Amplification of embryonic neurogenesis is achieved by the existence of basal transit amplifying cells or intermediate progenitor cells (IPCs). During neurogenesis RGCs divide asymmetrically to give rise to either a single IPCs or a neuron, ensuring self-renewal of RGC progenitors at the apical surface (**Fig. 2-2C**).

The IPCs divide within the eSVZ and basal VZ, as committed neural progenitors, producing pyramidal neurons across all cortical layers. In the embryonic dorsal telencephalon two types of IPCs exist, one more neuronally committed than the other, identifiable by morphology as ventricular intermediate progenitors (vIPs) and outer intermediate progenitors (oIPs). The vIPs comprise the less differentiated subtype on a molecular level, retaining apical contact with the ventricle. Despite their location, division of vIPs rarely occurs within the VZ and more often vIPs divide away from the ventricular surface, able to migrate into the SVZ without mitosis and covert to cells with an oIP morphology or neurons directly (Gal et al., 2006; Tabata et al., 2009). The oIP cells are observed as multipolar, and are more aligned to neuronal differentiation, capable of symmetrical cell division to give rise to two neurons or two like oIPs (Hevner, 2019; Pontious et al., 2008) (**Fig. 2-2B**). In mice, IPCs undergo at most two rounds of further divisions within the SVZ, whereas in gyrencephalic species such as humans undergo many more (Jiang and Nardelli, 2015; Noctor et al., 2004).

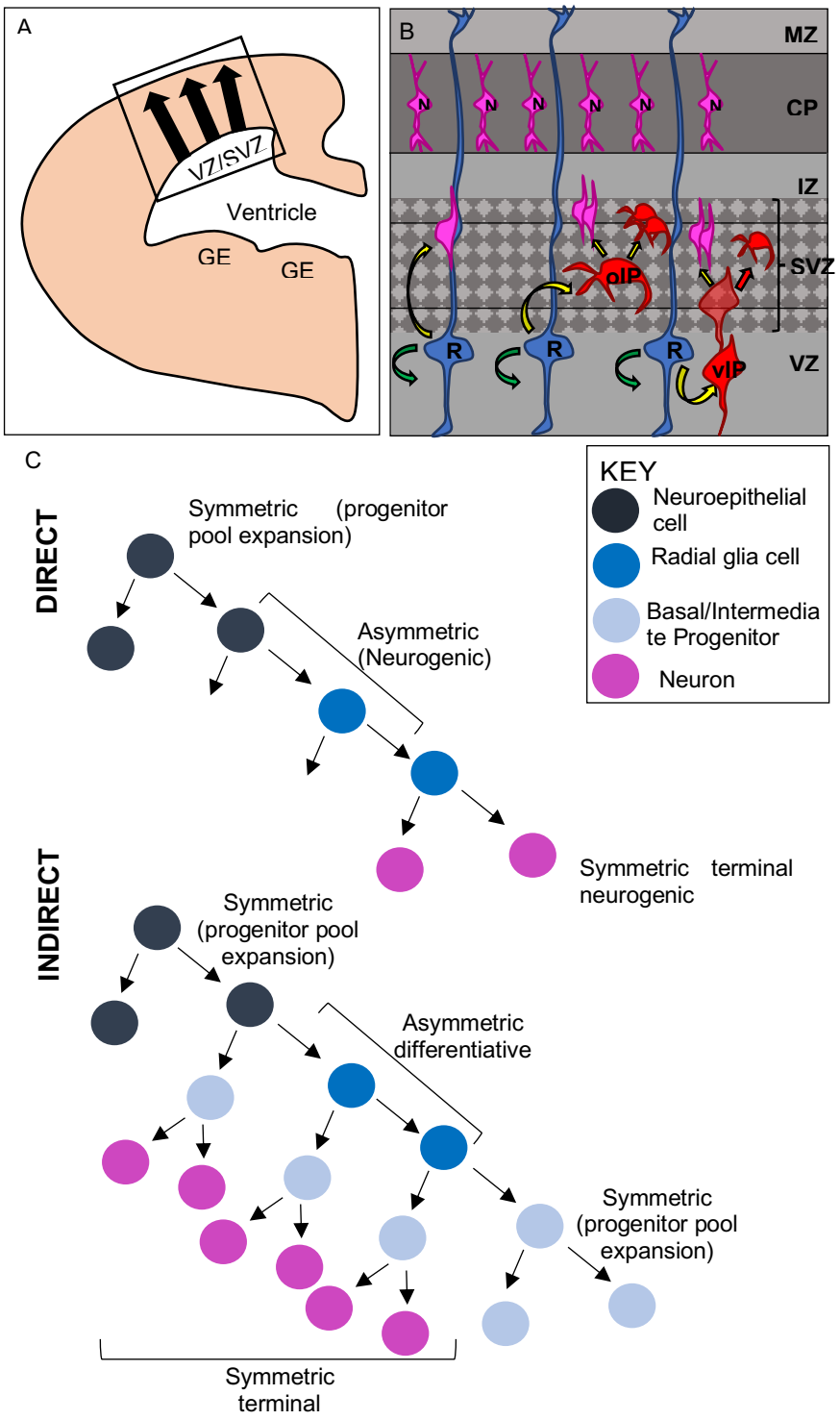

Figure 2-2 Embryonic Development of glutamatergic pyramidal Neurons: Pyramidal neurons enter the cortical plate radially from the ventricular zone (VZ) and subventricular zone (SVZ) (A). Radial glia cells (R) can give rise to intermediate progenitor cells (oIP and vIP) which divide within the SVZ before giving rise to neurons (N) within the cortical plate. Adapted from Kreigstein and Noctor 2002 (B). Neuroepithelial cells and radial glia can give rise to neurons both directly and indirectly, by the generation of intermediate progenitors, Adapted from Gotz and Huttner 2005 (C). GE, ganglionic eminences; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, Medial Zone.

IPCs within the SVZ have been shown to contribute to mainly late born upper layer neurons. Actively proliferating cells within the SVZ but not the VZ express the transcript for *Subventricular expressed transcript 1 (Svet1)* whereas the VZ is marked by expression of the homeobox transcription factor *Orthodenticle Homeobox 1 (Otx1).* Subsequent lineage tracing studies showed that later in development expression of *Svet1* and *Otx1* is found in subsets of upper (II-IV) and deep (V-VI) layer neurons respectively. Suggesting that deep layer neurons are specified in the VZ, whilst upper layer neurons are specified in the SVZ (Tarabykin et al., 2001).

Analysis of sequential marker expression can enable the lineage progression of RG cells, IPCs and post-mitotic neurons to be identified. Specifically, IPCs express the transcription factor *T-Box brain protein 2* (*Tbr2)*, derived from RGCs which express *Pax6*. The resulting post-mitotic projection neurons within the cortical plate are identifiable by expression of the transcription factor *T-Box brain protein 1 (Tbr1)*. The early embryonic lethality of *Tbr2* knockout poses an obstacle to determining the extent of its influence in cortical neurogenesis via IPCs, which occurs much later (Russ et al., 2000). Analysis of a broad range of conditional knock-out mice lines identified that inactivation of *Tbr2* in early brain development causes microcephaly, through reduced numbers of SVZ progenitors (Arnold et al., 2008). Additionally, *Tbr2* expressing intermediate progenitors are able to attract GABAergic neurons from the ventral telencephalon in part by mechanisms involving c-x-c motif chemokine 12 (Cxcl12) chemokine signalling. Its knockout not only severely reduced glutamatergic neuron production but also the contribution of GABAergic interneurons (Sessa et al., 2010).

The proneuronal basic-helix-loop-helix (bHLH) transcription factors neurogenin 2 (Ngn2) and neurogenin 1 (Ngn1) directly control neurogenesis in the embryonic cerebral cortex and are activated by *Pax6,* which establishes the cortical territory in early embryonic development (Scardigli, 2003). In early neurogenesis, Ngn1 and Ngn2 specify glutamatergic cortical neurons and repress genes that specify GABAergic neurons and subcortical areas, producing neurons that contribute to deep layer neurons. The later born neurons destined for upper cortical layers are specified by mechanisms independent of Ngn1 and Ngn2 (Schuurmans et al., 2004). Not only do neurogenins have a role in influencing specification of deep layer neurons, specifically Ngn2 also has an indirect role in cortical neuron migration. In migrating cortical neurons and intermediate progenitors, Ngn2 induces transient expression of the small GTP-binding protein Rnd2. Silencing of Rnd2 by electroporation of siRNA at E12.5 resulted in defects in radial migration through from the ventricular zone to the cortical plate, whilst proliferation of cortical progenitors, the integrity of the RGC

scaffold and specification of cortical neuron identity remained unaffected (Heng et al., 2008).

2.1.1.2 Interneurons

A vast majority of interneurons are not born locally within the dorsal telencephalon, and are instead derived from ventral telencephalon or subpallium, specifically the ganglionic eminences and to a lesser extent the preoptic area (Anderson et al., 1997; Gelman et al., 2011a). The ganglionic eminences (GE) may be subdivided into three transitory structures; the medial ganglionic eminence (MGE), the caudal ganglionic eminence (CGE) and the lateral ganglionic eminence (LGE) based on their location along the rostral-caudal axis (DeDiego et al., 1994; van Eden et al., 1989). During embryonic rodent development the ganglionic eminences arise between E12 and E13, and are restructured to form structures that comprise the basal telencephalon by E17 (Jiménez et al., 2002).

Collectively the MGE and CGE are thought to give rise to vast majority of cortical interneurons, with the origin of the remaining population being a source of much conflict in the field (Rubin et al., 2010). The contribution of the LGE to the cortical interneuron population is also the subject of debate. A transplantation and slice culture study determined that both the MGE and LGE produce cortical interneurons by use of fate mapping (Anderson et al., 2001). Further evidence utilised explants which ensure the LGE and MGE are definable show robust migration of cells from the LGE early in embryonic development (Jiménez et al., 2002). Another recently identified contributor to the interneuron population within the cerebral cortex is the pre-optic area (PoA), The PoA contributes a relatively minor population of diverse interneurons, accounting for 5-10% of interneurons within the cerebral cortex (Gelman et al., 2011b, 2009).

Interneurons migrate tangentially from the GE into the dorsal telencephalon and then change direction to enter the cortical plate radially from both above and below (**Fig. 2-3A-B**) (Tan et al., 1998; Tanaka, 2006). Tangential migration of interneurons is a process that is independent of the radial glia scaffold, it has been suggested that interneurons may use corticofugal fibres as a scaffold as they migrate toward the cortical plate, by use of the neural adhesion molecule TAG-1 (Denaxa et al., 2001). Additionally interneurons are attracted to Cxcl12 chemokine signalling, produced by *Tbr2* expressing basal progenitors, which to some degree have an influence on the migratory route and number of GABAergic interneurons in the cerebral cortex (Sessa et al., 2010).

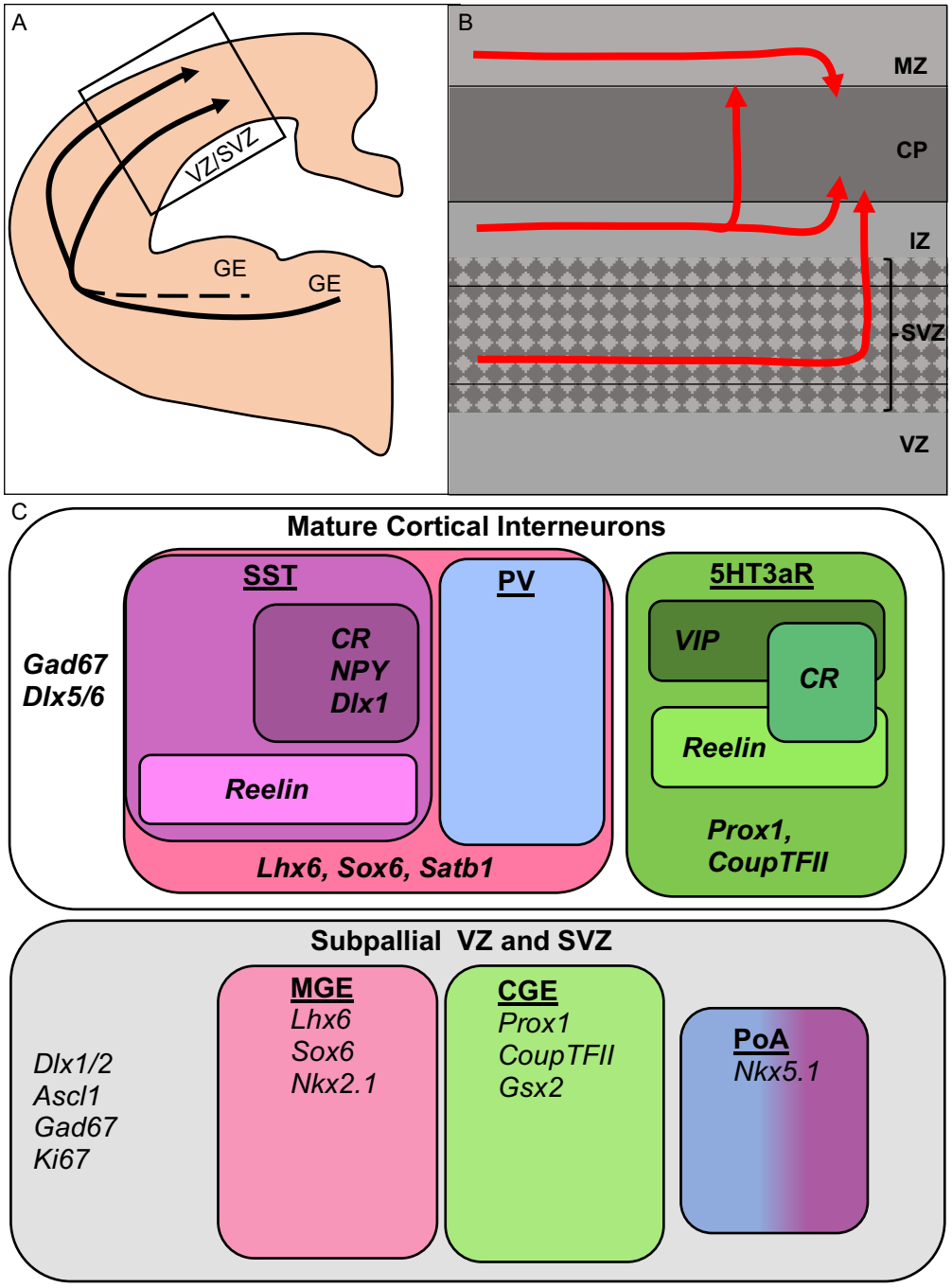

Figure 2-3 Embryonic Development of GABAergic interneurons: Interneurons enter the cortical plate tangentially from the ganglionic eminences (GE) and preoptic area (not shown) (A). Tangentially migrating interneurons enter the cortical plate from above or below, and switch to radial migration to reach their final cortical positions. Adapted from Kreigstein and Noctor *et al.* 2004 (B). Interneurons are a highly heterogenous population derived from varied anatomical origins and progenitors, which may be identified by expression of specific markers as outlined, and broadly by expression of Gad67 (C). Adapted from Laclef and Métin 2018. GE, ganglionic eminences; VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate; MZ, Medial Zone.

GABAergic interneurons subtypes may be broadly characterised by their expression of calcium binding proteins. The three predominant cortical interneuron classes are parvalbumin (PV)-expressing interneurons, somatostatin (SST)-expressing interneurons, and a recently identified population of the ionotropic serotonin receptor 5HT3aR (5HT3aR)-expressing interneurons. Together, these subtypes account for all neocortical GABAergic interneurons, 70% of which are of the SST and PV subtypes (Rudy et al., 2011). A majority of PV and SST interneurons are derived from the ventral and dorsal MGE respectively, whereas most interneurons of the 5HT3aR subtype originate from the CGE. It should be noted that these three broad subtypes are large and extremely heterogenous populations, and their expression of other proteins and neuropeptides may be used to characterise them further (Lee et al., 2010; Xu, 2004). The preoptic area (PoA) contributes a highly diverse population of interneurons to the cerebral cortex, expressing PV, SST and reelin, with a very small number expressing Calretinin (CR), Neuronal nitric oxide synthase (NOS) or Vasoactive Intestinal polypeptide (VIP) (Gelman et al., 2011b). Interneurons are extremely heterogenous populations, as shown by their large variation in marker expression (**Fig. 2-3C**) (Broadly, all mature cortical interneurons are identifiable by their expression of Glutamate decarboxylase isoform 67 (Gad67), regardless of their origin.

Progenitors within the embryonic subpallial CGE, MGE and PoA are identifiable by their expression of the transcription factors *Dlx1/2* and *Ascl1.* Mice that lack *Dlx1/2* or *Ascl1* display a 50-80% decrease in cortical GABAergic neurons confirming their crucial role (Anderson et al., 1997; Casarosa et al., 1999). The Dlx homeobox genes are widely expressed throughout the subpallium and are required for differentiation of the GE. Transition of progenitors from the subpallilal VZ to SVZ coincides with *Dlx1/2* expression which decreases as interneurons differentiated. *In vitro* analysis shows that ectopic expression of *Dlx2* and *Dlx5* which acts downstream is sufficient to induce expression of glutamate decarboxylase (Nery et al., 2003; Stühmer et al., 2002). Several other markers are restricted to progenitors within specific regions of the subpallium, and hence it is possible to lineage trace the progeny of specific populations in terms of the various interneuron subtypes they generate (**Fig. 2-3C**) (Flames et al., 2007; Laclef and Métin, 2018)

An additional tangentially migrating population are the transient Cajal-Retzuis cells, which arrive at the preplate in early embryonic development, specifically populating the marginal zone and secreting the glycoprotein Reelin. The origin of Cajal-Retzuis cells appears complex, with various studies indicating derivation from the cortical hem and ganglionic eminences (García-Moreno et al., 2007; Hevner et al., 2003). Cajal-

Retzuis cells and reelin have an important role in laminar organisation of the cerebral cortex. One such example is that of Reeler mutant mice, in which Cajal-Retzuis cells are unable to synthesise/secrete Reelin. As a result, migrating neurons are incapable of splitting the preplate into marginal zone (MZ), cortical plate (CP) and subplate (SP), instead accumulating beneath it and resulting in an inverted cortical lamination pattern (D'Arcangelo et al., 1997; D'arcangelo et al., 1995).

2.1.1.3 Cortical Patterning

The cerebral cortex is patterned into not only a laminar arrangement of neurons, but along the rostral caudal axis, and into functional subunits. A plethora of transcription factors and signalling gradients are crucial to establish the proper development of this complex cortical map (**Fig. 2-4**). For example, sonic hedgehog (*Shh*) expression is restricted to the ventral telencephalon and hypothalamus, and has an essential role in specification of the subpallium and pallium thus establishing a dorso-ventral pattern (Rallu et al., 2002). Bone morphogenetic protein (BMP) and Wnts secreted from the dorsal midline specify the pattern of the medial and dorsal pallium regions including the hippocampus and neocortex. Synergistically, FGF8, SHH and BMP4 in the chick embryo have been shown to regulate the morphogenesis and regional specification of the telencephalon. For example, implantation of BMP4 beads in ectopic positions represses *Fgf8* expression in the commissural plate but not the mid-hindbrain boundary (isthmus). It was also shown that BMP4 beads potently repressed expression of *Shh* within ventral regions such as the hypothalamus (Ohkubo et al., 2002)

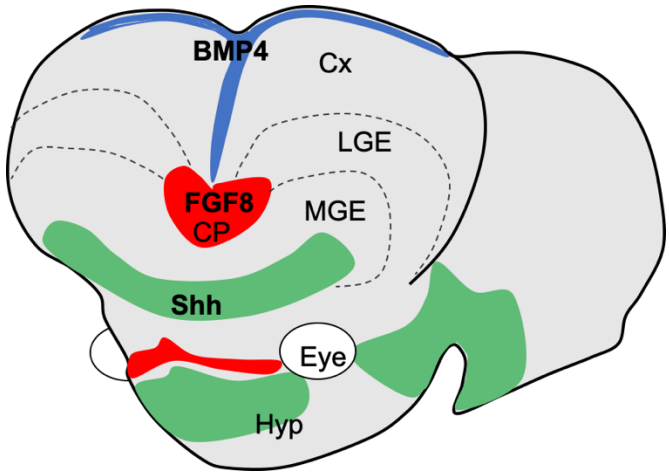

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Figure 2-4 Organizing centers of early cortical patterning in embryonic development: In embryonic development opposing gradients are established of dorsal BMP signaling and ventral sonic hedgehog (Shh), with *Fgf8* expressed within the commissural plate (rostral signaling center). Cp, commissural plate; Hyp, hypothalamus; Cx, cortex; LGE, lateral ganglionic eminences; MGE medial ganglionic eminences. Adapted from Rubenstein (2011).

Regional identity of different cortical functional areas also requires complex mapping and specification, likely determined by vast overlapping roles of numerous gradients and mechanisms. One such determinant is the gene *Emx2*, expressed throughout the dorsal telencephalic neuroepithelium, in a gradient of high caudal expression and low in rostral regions. Knockout of the transcription factor *Emx2* resulted in mice with a normal compliment of cortical functional regions, although the areas of caudal-medial identities were reduced and those with anterior-lateral identities were expanded (Mallamaci et al., 2000). By use of specific area markers that have regional expression patterns such as the type II classical cadherins Cad6 and Cad8 to identify the motor area and somatosensory area respectively, it was determined that the motor and somatosensory was expanded, whereas visual areas are reduced (Bishop, 2000). The transcription factor *Pax6* is expressed throughout the neuroepithelium and has an opposing gradient to *Emx2* (high rostral, low caudal) and its knockout results in an opposite effect (reduction in motor and somatosensory area and expansion of visual areas) (Bishop et al., 2002).

2.1.1.4 Astrocytes

Astrocytes of the CNS are an extremely heterogenous population, broadly subdivided into protoplasmic astrocytes of the grey matter, and fibrous astrocytes of the white matter, with vital and diverse functions in brain homeostasis. Through close associations with synapses astrocytes are capable of monitoring and altering synaptic transmission (Chung et al., 2015). Through interactions with endothelial cells astrocytes upregulate many features of the blood brain barrier, leading to a tighter barrier by reinforcement of endothelial cell tight junctions, and a role in regulating blood flow (Abbott et al., 2006; Petzold and Murthy, 2011). In response to injury in the adult, astrocytes undergo a process named reactive astrogliosis, becoming hypertrophic in morphology and upregulating expression of glial fibrillary acidic protein (GFAP). In severe cases, this causes the formation of a glial scar as a result of re-entry of mature astrocytes into the cell cycle and production of new astrocytes within the lesion (Buffo et al., 2008). During development, generation of astrocytes within the cerebral cortex peaks between postnatal day 0 and 21 (P0 and P21), in a temporal sequence preceded by neurogenesis (Levison et al., 1993; Parnavelas, 1999; Qian et al., 2000). In contrast to the complex multiple origins of cortical neurons, cortical astrocytes are exclusively derived from precursors within the dorsal telencephalon (pallium) as determined by lineage tracing of cells expressing the dorsal telencephalon restricted transcription factor *Emx1* (Gorski et al., 2002).

Following completion of neurogenesis, RGCs shift their fate to generating astrocytes.

By fluorescence activated cell sorting (FACs), RGCs were isolated at various points at embryonic development and cultured. In late embryonic development, E14-E18 there was a reduced propensity for RGCs to produce neuronal clones. By E18, their progeny were almost exclusively astrocytes (Malatesta et al., 2000). As part of this process it has been shown that a subset of radial glia lose their basal contact with the ventricle surface, translocate into the CP and transform into astrocytes following their final asymmetric cell division (Noctor et al., 2004). As in neurogenesis, radial glia can give rise to astrocyte/oligodendrocyte committed basal progenitors within the SVZ (Levison and Goldman, 1993). Postnatally, there is a 6-8 fold expansion of the glial cell population within the rodent brain, which is attributed to the local symmetrical division of astrocytes ($GFAP^+$ and $Ki67^+$) within the postnatal cerebral cortex (Ge et al., 2012a).

2.1.1.5 Oligodendrocytes

Oligodendrocytes are responsible for the myelination of axons within the central CNS and have an asymmetrical distribution in the postnatal/adult cerebral cortex. A majority of oligodendrocytes in the rodent cerebral cortex reside in the deep layers and white matter, with very few observed in the more superficial layers. The reasons for this graded distribution of oligodendrocytes within cortical laminae is unknown. It has been hypothesised that a majority of oligodendrocytes reside in the deeper cortical layers to enable close proximity to the long axons of the projection neurons that inhabit these layers. However pyramidal neurons are found within all cortical layers of the cerebral cortex, with the exception of layer I (Tan et al., 2009). Another possibility is that oligodendrocyte positioning is regulated by cell autonomous factors inherent to the oligodendrocyte programme of development.

Unlike astrocytes, oligodendrocytes have diverse origins including the MGE, LGE, dorsal telencephalon (SVZ) and, potentially the thalamus (Kessaris et al., 2006). Oligodendrocytes populate the mouse cerebral cortex in three distinct waves. The first ventral wave of oligodendrocyte production arises from *Nkx2.1* expressing precursors within the ventral medial ganglionic eminences and anterior entopeduncular area, migrating, and eventually entering the developing cerebral cortex at E11.5-E12.5. This lineage derived from *Nkx2.1* expressing cells are at first, the only oligodendrocyte precursors (OLPs) and oligodendrocytes (OLs) in the cerebral cortex. By postnatal day 10 (P10) *Nkx2.1* derived OLPs and OLs are gradually lost and replaced by other populations (i.e. the second and third wave). The second wave of OLPs and OLs into the cerebral cortex originates from the lateral and/or caudal ganglionic eminences from precursors expressing the homeobox gene *Gsx2* at approximately E15. The third wave

arises from within the postnatal cortex itself postnatally, from precursors expressing the homeobox gene *Emx1,* in a process directed by *Olig2* expression, which directs SVZ progenitors to glial fates (Marshall et al., 2005; Rowitch and Kriegstein, 2010). When any one of these OLP/OL waves is destroyed at its origin, OLPs/OLs from the remaining waves spread into territory and result in a normal complement of oligodendrocytes and myelin (Kessaris et al., 2006).

Cortical OLPs remain abundant within the adult CNS and can be identified by expression of the NG2 proteoglycan on their surface, platelet derived growth factor receptor-α (PDGFRA) and the transcription factor Olig2 (Rivers et al., 2008). Through adult life $NG2^+$ cells contribute newly generated OLs to the cerebral cortex, which may be required for adult remodelling or maintenance of myelin (Young et al., 2013). As OLPs differentiate into OLs PDGFRA is downregulated following cell cycle exit. In terminal differentiation expression of *Sox10* is required, as shown by studies of Sox10-deficiency OLPs still persist but differentiation into oligodendrocytes is disrupted (Solt et al., 2002).

2.1.1.6 Cortical Invasion of Microglia

Microglia are the macrophages of the central nervous system (CNS) and are crucial not only for immune response, but also have roles in brain development (neurogenesis and differentiation) and homeostasis. In the adult brain microglia constantly survey their environment for signals of invading pathogens or those released by damaged or dying cells. Upon detection of such signals, microglia become activated to protect the CNS from insult (Hanisch and Kettenmann, 2007). The origin of microglia is one of conflict, however largely studies agree that rodent microglia are derived from primitive haematopoiesis within the yolk sac (Alliot et al., 1999). In mouse embryonic development microglia are born within the yolks sac between E8.5 and E9.0 and begin to accumulate at the pial surface and within the lateral ventricle, by approximately E11.5 and invade the cortical parenchyma through until E17.5 (Ginhoux and Prinz, 2015; Swinnen et al., 2013). In the adult, upon completion of the blood-brain-barrier the microglia population is retained within the CNS by lifelong self-renewal without any significant contribution from circulating cells (Ajami et al., 2007).

During development, microglia densely populate the embryonic VZ and SVZ amongst *Cxcl12* expressing $TBR2^+$ basal progenitors. As stated previously *Tbr2* conditional knockout results in a significant decrease in *Cxcl12* expression which negatively impacts the migration of interneurons into the cortex. In a similar fashion, Cxcl12 chemokine acts as a chemoattractant to microglia, driving the arrangement of

microglia in bands within the SVZ and VZ, which does not occur in its absence (Arnò et al., 2014). Once within the cerebral cortex, microglia have roles in regulating the number of neural progenitors within the cerebral cortex, capable of phagocytosing $TBR2^+$ IPCs and $Pax6^+$ RGCs to regulate their number (Cunningham et al., 2013). *In vitro,* culture of cortical embryonic precursors from $PU.1^{-/-}$ mice which lack microglia display decreased proliferation of precursors and astrogenesis, which were both rescued by introduction of microglia back into culture (Antony et al., 2011), thus outlining a complex cross talk between microglia and embryonic progenitors in neurogenesis. Strikingly a paediatric patient, carrying a mutation in levels of Colony Stimulating Factor 1 Receptor (CSFR1), completely lacked microglia in the brain. The patient displayed widespread debilitating congenital brain abnormalities as a result, further corroborating that the complex crosstalk of microglia and neural progenitors plays a vital role in embryonic brain development (Oosterhof et al., 2019).

2.2 Adult Neurogenesis and Neural Stem Cells

Traditionally it was believed by the neuroscience community that neurogenesis, the process of producing functionally integrated neurons from stem/progenitor cells, was a phenomenon restricted to embryonic development. As stated by Ramon Y Cajal in the early 19th century: “once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centres, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated” (Cajal, 1928)

In the 1960s Altman and Das published convincing evidence that new neurons are produced in the adult rat hippocampal dentate gyrus, by the utilization of new techniques involving [^{3}H]-thymidine autoradiography (Altman and Das, 1965). Altman also observed evidence of newly produced neurons within the rodent and cat neocortex, hypothalamus and olfactory bulbs (Altman, 1963). Despite this, the field of adult neurogenesis laid dormant, until Goldman and Nottebohm published their observations of adult neurogenesis in the canary, whereby new neurons are produced by ventricular zone cells within and surrounding the vocal control nucleus (hyperstriatum ventral, pars caudalis) (Goldman and Nottebohm, 1983).

Sophisticated techniques to detect dividing cells were later developed and utilized synthetic thymidine analogues such as 5-bromo-2'-deoxyuridine (BrdU), 5-ethynyl-2′-deoxyuridine (EdU) and [^{3}H]-thymidine. Incorporation of such analogues into cellular DNA during S-phase is indicative of active cell proliferation. As a result, two canonical niches of adult neurogenesis have been conclusively identified and accepted in most mammals, namely, the Subventricular Zone of the Lateral Ventricles (SVZ) and the Subgranular Zone of the Hippocampal Dentate Gyrus (SGZ). In addition to these established canonical neurogenic niches in the postnatal/adult mammalian brain, several new non-canonical niches are beginning to emerge, including, the rodent amygdala (Bernier et al., 2002; Fowler et al., 2008); hypothalamus (Haan et al., 2013; Kokoeva, 2005; Robins et al., 2013); and the cerebral cortex of species such as non-human primates (Gould et al., 1999) and rats (Dayer et al., 2005) to name but a few.

Progress in conclusively establishing these new non-canonical neurogenic niches in the postnatal/adult mammalian brain is slow. Several papers have observed neurogenesis in the adult primate cerebral cortex, by use of BrdU and neuronal nuclear antigen (NeuN) double labelling, detecting new-born neurons (Gould et al., 2001, 1999). Conversely some research groups could not replicate these findings despite use of the same techniques (Koketsu et al., 2003). In a 2007 review, Gould

postulated that difficulties in replicating data is likely due to subtle but crucial differences in protocol for BrdU pulsing and tissue processing, which may limit the detectability of low-level adult cortical neurogenesis (Gould, 2007).

2.2.1 Markers of Neural Stem/Progenitor Cells

Since the discovery of adult neural stem cells (NSCs), a large number of immunohistochemical markers have been identified in order interrogate neurogenesis, through detection of NSCs, more committed neural progenitor cells (NPCs) and immature neurons (**Table 2-1**). Many of the markers expressed by adult NSCs are also expressed within NSCs during embryonic development. For example, nestin is an intermediate filament protein that is expressed exclusively in NSCs within the embryonic CNS (Lendahl et al., 1990) and again within adult neural stem cells of the SVZ and SGZ (Lagace et al., 2007). Glial markers such as vimentin, glial fibrillary acidic protein (GFAP), Glutamate Aspartate Transporter (GLAST) and brain lipid binding protein (BLBP) are often used to analyse the degree of neuronal commitment of the stem/progenitors in question, in combination with proliferative markers (Anthony et al., 2004; DeCarolis et al., 2013; Doetsch et al., 1997).

Endogenous markers of proliferative cells include Ki67, present in active stages of the cell cycle (G_1, S, G_2 and mitosis) (Gerdes et al., 1983), and proliferating cell nuclear antigen (PCNA), a protein required alongside DNA Polymerase for DNA synthesis in replication, with its levels peaking in S phase (Celis and Celis, 1985; Juríková et al., 2016). Sex determining region Y-box2 (Sox2) is a transcription factor and functions to maintain characteristics of neural progenitors, by inhibiting differentiation (Graham et al., 2003). Additionally, markers have been identified in order to detect of new-born neurons in the CNS, for example doublecortin (DCX) which is expressed by neuroblasts/committed IPCs, and downregulated concomitantly with an increase in NeuN (mature neuronal marker) (Brown et al., 2003; Guselnikova and Korzhevskiy, 2015; Mullen et al., 1992; Sarnat et al., 1998).

Additional markers of new-born neurons or neuronally committed progenitors/neuroblasts, depending on combined expression of markers outlined above, include; the distal-less homebox transcription factors (DLX), Achaete-Scute Family BHLH Transcription Factor 1 (ASCL1 or MASH1), Polysialyated-neural adhesion molecule (PSA-NCAM), T-box transcription factor 2 (TBR2/Eomes), neural glial antigen 2 (NG2) and Neuron-specific Class III β-tubulin (TUJ1) (Belachew et al., 2003; Brill et al., 2008; Brown et al., 2003; Kim et al., 2011; Menezes and Luskin, 1994; Wang, 1996).

Table 2-1 Common markers utilised in adult neurogenesis research: The specific immune-markers used in adult neurogenesis research to detect cell proliferation and characterise neural stem/progenitor cells in both adult and embryonic neurogenesis.

Marker	Type	Cell type	Reference
Ki67	Protein	Proliferating cells (any)	Gerdes et al 1983
PCNA	DNA polymerase accessory protein	Proliferating cells (any)	Celis and Celis 1985
Nestin	Intermediate filament protein	Neural stem cells	Lendahl et al 1990
Vimentin	Intermediate filament protein	Glial (radial astrocytes/immature astrocytes)	Doetsch et al 1997
SOX2	Transcription factor	Neural stem/progenitor cells	Graham et al 2003
DLX	Transcription factor	Progenitor cell maintenance and neuronal differentiation	Brill et al 2008
DCX	Microtubule associated protein	Neural progenitor cells/ neuroblasts	Brown et al 2003
ASCL1	Transcription factor	Intermediate progenitors and neuroblasts(neural fate)	Kim et al 2011
PSA-NCAM	Neural cell adhesion molecule	Migrating neural cells (neuroblasts in combination with other markers)	Wang et al 1996
TBR2	Transcription factor	Intermediate progenitor cell (SGZ, Cerebellum and embryonic cortical neurogenesis)	Englund et al 2006, Englund et al 2005, Hodge et al 2008.
NG2	Chondroitin sulfate proteoglycan	Oligodendrocyte progenitor – neural potential	Belachew et al 2003
GFAP	Intermediate filament protein	Astrocytes (radial glia when in combination with other markers)	Doetsch et al 1997
GLAST	Astrocyte specific glutamate transporter	Astrocytes (radial glia when in combination with other markers)	Decarolis et al 2013
BLBP	Lipid binding protein	Astrocytes (radial glia when in combination with other markers)	Anthony et al 2004
TUJ1	Microtubule element	Young/differentiating neurons	Menezes et al 1994
NeuN	Nuclear/Perinuclear protein	Mature neurons	Sarnat et al 1998, Mullen et al 1992, Guselnikova 2015

2.2.2 Implications for Disease Treatment and Prevention

Adult neurogenesis has various potential applications in clinical practice. For example, adult neural stem cell research may, and indeed has, uncovered mechanisms through which we can potentially enhance the capacity of the human brain to recover following traumatic brain injury (TBI). Following TBI or focal ischemia injury, cell proliferation and neurogenesis within the SGZ and SVZ of adult mice increases (Kernie and Parent, 2010). Specifically within the hippocampal SGZ the neurogenic response was more robust in juvenile mice in comparison to adult and aged mice following TBI, which may underlie the age related differences concerning cognitive recovery (Sun et al., 2005).

Analysis of human brains after traumatic brain injury observed cells within the perilesional cerebral cortex that expressed markers of neural stem/progenitors such as DCX, Sox2 and PSA-NCAM, as well as upregulation of proliferative markers such as Ki67 in comparison to that of normal brain. This suggests that TBI in humans could induce neurogenesis, although it is unclear whether the observed NSC/NPCs were derived from canonical neurogenic niches, or locally within the cerebral cortex, nor whether they showed potential to mature into functionally integrated neurons (Zheng et al., 2013).

It is observed by some that the SVZ is capable of generating neural precursors (DCX^+) that migrate towards ischemic cortex via the rostral migratory stream and lateral cortical stream and may have a role in recovery following ischemic stroke (Jin et al., 2003). However, others show that following focal ischemia within the somatosensory cortex only a small minority of proliferative cells observed within the ischemic regions were neurogenic (3-6%), a vast majority of which expressed markers for glial, macrophage or endothelial cells (Gu et al., 2000). This was further corroborated by additional research into ischemic stroke injury, which showed that none of the proliferative cells within damaged cortical areas expressed neuronal markers 5 weeks following ischemic insult, whereas those within the striatum did (Parent et al., 2002). This has led to the hypothesis that the adult cerebral cortex lacks the instructive or survival cues necessary for integration of new-born neurons following injury. One such candidate that may act as a negative regulator of neuronal recruitment/neurogenesis within non-canonical regions are the ephrins-A2 and -A3. Within the CNS, ephrin-A2 and -A3 are expressed in high levels by astrocytes outside of canonical neurogenic niches and are thought to negatively regulate neural progenitor cell growth. Knockout of these ephrins results in substantial neurogenic proliferation within non-canonical CNS regions (Jiao et al., 2008)

Due to the limited capacity of canonical neurogenic niches to respond to injury, there is an accumulating need to determine conclusively the potential of non-canonical neurogenic regions, and mechanisms by which the endogenous neurogenic potential of non-canonical regions may be enhanced.

2.3 Canonical Neurogenic Niches

2.3.1 The Subgranular Zone of the Hippocampal Dentate Gyrus

2.3.1.1 Background

The hippocampus of the mammalian brain is a region that shows an impressive propensity for structural reorganisation, important in learning, memory, spatial navigation, emotional behaviour and in regulation of hypothalamic functions (Leuner and Gould, 2010). Structurally the hippocampus is comprised of the dentate gyrus and cornu ammonis (CA) fields (CA1-4), specifically the dentate gyrus receives input from the entorhinal cortex which acts as the major gateway between the hippocampus and cerebral cortex (Wible, 2013). Adult neurogenesis within the rat dentate gyrus was observed as early as 1965 (Altman and Das, 1965). However, this phenomenon was not studied in humans until 1998, through analysis of brain tissue from cancer patients treated with synthetic thymidine analogue BrdU therapeutically. The administered BrdU would be incorporated into any cell replicating DNA (S-phase) at the time of administration, and observation of any $BrdU^+$ cells post-mortem would be indicative of proliferation. Some $BrdU^+$ cells observed in the human hippocampus also expressed neuronal markers such as NeuN, providing evidence of new-born neurons in the adult human brain (Eriksson et al., 1998) Further elegant studies measured the concentration of ^{14}C isotope in genomic DNA and allowed for further elucidation of the dynamics of human SGZ neurogenesis. This was made possible by cold-war nuclear bomb testing between 1955 and 1963 which resulted in increased levels of atmospheric ^{14}C. The ^{14}C isotope was incorporated into plants via photosynthesis, which were in turn ingested by humans. As a result, ^{14}C was incorporated into any cell undergoing mitosis at the time and could be detected in human post-mortem tissue. This study determined that in adult humans, approximately 700 new neurons are produced within the SGZ each day, a rate comparable to that observed in mice (Spalding et al., 2013).

Despite such experimentally complex studies, conflicting papers are still emerging in regard to how long the SGZ retains its neurogenic capacity in humans. A study published in early 2018 reported that robust hippocampal neurogenesis is observed in children, which then falls to an undetectable level in adults. The study analysed pre-operative and post-mortem human samples, and macaque tissue, immunolabelling sections for markers of proliferation (Ki67), NSCs (Sox2 and Sox1) and young neurons (PSA-NCAM and DCX). However, the paper also acknowledges its limitations, in that samples obtained from humans were from patients with diseases such as epilepsy,

which may negatively impact levels of adult neurogenesis. Despite a broad range of causes of death, very little could be ascertained as to the health of post-mortem patients in this study (Sorrells et al., 2018).

In dispute, a second study published in the same month concluded that although the pool of quiescent NSCs within the dentate gyrus is smaller in aged humans ($GFAP^+$, $Sox2^+$ and $nestin^+$), the pool of intermediate progenitors ($Ki67^+$ and $nestin^+$) and immature neurons (DCX^+, $PSA\text{-}NCAM^+$) is stable across aging. In contrast to the aforementioned study no samples were obtained from patients suffering from neuropsychiatric disease or treatment and all had clear toxicology and neuropathology (Boldrini et al., 2018).

2.3.2 The Stem Cell Niche

Structurally the SGZ stem cell niche is sandwiched between the granule cell layer and the hillus of the dentate gyrus. The SGZ contributes new excitatory granule neurons to the dentate gyrus. Specifically, these new neurons incorporate into the mossy fibre tract, receiving input from the entorhinal cortex, and providing excitatory input to the hippocampal CA3 region, modulated by interneurons within the dentate gyrus and hillus regions (Kempermann et al., 2015; Kornack and Rakic, 1999; S. Liu et al., 2003).

The dentate gyrus comprises a specialised niche within which NSCs reside (Type 1 cells). Type 1 cells or 'radial astrocytes' (RAs) express glial fibrillary acidic protein (GFAP) and the neural stem/progenitor markers Nestin and Sox2 (Garcia et al., 2004; Lagace et al., 2007; Seri et al., 2001). Type 1 cells, project through the granule layer with a single process, terminating and branching within the inner molecular layer, and give rise to progenitor cells (type 2 cells), identifiable by varying marker expression (**Fig. 2-5**).

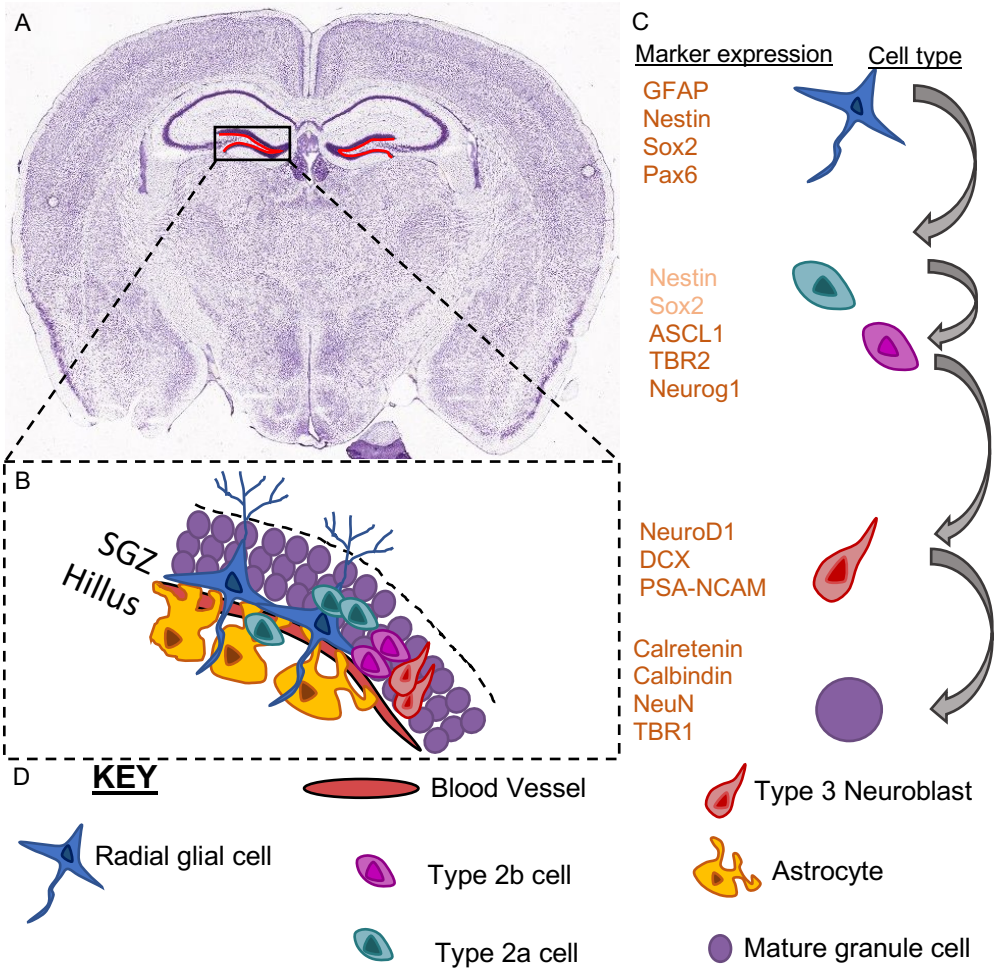


Figure 2-5 Cellular Organization of the Subgranular Zone in the Hippocampal Dentate Gyrus of the adult rodent brain: A coronal section of the rodent brain highlighting the proliferative subgranular zone (A) and a representation of the microstructure (B). Various cell types within the neurogenic niche may be identified by expression of specific markers (C) Cell types may be identified with the key (D). Adapted from Lin and Iacovitti 2015.

Type 2 progenitor cells, can be further subdivided into two categories, type 2a which in turn give rise to 2b. Broadly type 2 cells can be identified by expression of the transcription factor TBR2, which suppresses Sox2 and is essential for the differentiation of NSC (type 1 cell) to progenitor (type 2 cell) (Hodge et al., 2012). Type 2a and 2b cells can be differentiated by their degree of neuronal commitment. Type 2a cells express nestin and the glial marker Brain lipid binding protein (BLBP) but are negative for the immature neuron/neuroblast marker DCX. Type 2b cells are also nestin^{+}, but additionally express immature neuron marker DCX, and other neuronal markers such as the transcription factors Neuronal Differentiation 1 (NeuroD1) and Prospero Homeobox 1 (Prox1) thus committed to the neuronal lineage (Kronenberg et al., 2003; Lavado et al., 2010). The transcription factor Ascl1 (sometimes referred

to as Mash1) is essential for neural differentiation in embryonic neurogenesis and is present in low levels in type 1 cells and in high levels within type 2 cells, showing an increase in line with neural commitment (Kim et al., 2011).

The loss of nestin expression from type 2 cells results in transition into type 3 cells (neuroblasts), which still undergo low levels of proliferation, and express immature neuronal markers such as doublecortin and NeuroD1 until late stages of cell cycle exit (Kronenberg et al., 2003). The newly generated neurons express post-mitotic markers such as NeuN and integrate into dentate granule circuits (Hodge and Hevner, 2011).

The RA type 1 cells have end-feet that contact the vasculature within the SGZ. It is unknown whether the blood-brain barrier within the SGZ niche is 'leaky' and hence if the niche has direct contact with circulating factors (Palmer et al., 2000). It has been observed that mice housed in an enriched environment have increased levels of hippocampal VEGF expression. Forced gene over-expression of VEGF within the SGZ results in an two-fold increase in adult neurogenesis, with an associated improvement in cognition, linking environment, angiogenesis and neurogenesis (Cao et al., 2004; Jin et al., 2002).

Levels of neurogenesis within the hippocampal dentate gyrus can be influenced by external factors. Decreases in new neuron production within the SGZ have been related to aging (Kuhn et al., 1996) and serotonin depletion in rats (Brezun and Daszuta, 1999), prenatal stress in rhesus monkeys (Coe et al., 2003), and alcohol abuse in humans (Le Maître et al., 2017). Adult neurogenesis within the dentate gyrus may be enhanced in response to exercise in mouse studies (Van Praag et al., 1999). Furthermore, reduced levels of SGZ neurogenesis has been correlated with psychiatric disorders can be reversed by anti-psychotic/anti-depressant drugs such as fluoxetine, a specific serotonin reuptake inhibitor (Malberg et al., 2000; Santarelli et al., 2003).

In regions such as the adult SVZ and SGZ, high levels cell proliferation appear counterbalanced with apoptotic cell death, indicating that programmed cell death may have a regulatory role in eliminating excess cells not recruited into function (Ihunwo et al., 2016). In the dentate gyrus apoptosis is potent within the boarder of the hilus and granule cell layer, further suggesting an intrinsic link to neurogenesis (Biebl et al., 2000). It is thought that apoptosis of post-mitotic neurons within the dentate gyrus functions to modulate neuronal cell number as a mechanism of constant cell turnover. This is maintained throughout aging, as both cell proliferation and apoptosis within the SGZ slow (Heine et al., 2004). The level of apoptosis within the dentate gyrus is also

receptive to external stimuli, for example provision of environmental enrichment to mice has been shown to reduce the rate of apoptotic cell death in the rat hippocampus by 45% (Young et al., 1999).

In summary, the hippocampal NSCs add new granule neurons to the dentate gyrus throughout postnatal and adult life of rodents, non-human primates and likely within humans. Of particular interest is the strong effect of environment on hippocampal neurogenesis, and the potential link between this phenomena and various health conditions including those treatable by anti-depressants.

2.3.3 The Subventricular Zone of the Lateral Ventricles

2.3.3.1 Background

The neurogenic potential of the SVZ was observed by Altman in the 1960s in rats, using thymidine-H^3 analogues (Joseph Altman, 1969). The adult mammalian SVZ of the lateral ventricles generates new neurons which migrate along the rostral migratory stream into the olfactory bulbs (OB), where they differentiate into inhibitory interneurons.

Human adult neurogenesis in the SVZ, remains controversial. Marker analysis identified expression of proliferative and neuronal markers within the human SVZ such as nestin and PCNA (Bernier et al., 2000). However, by harnessing levels of the carbon isotope ^{14}C, in the genomic DNA it was determined that levels of SVZ neurogenesis in humans was low, if indeed existent at all. It should be noted, this was specifically in terms of contributing adult new-born neurons into the OBs, rather than focusing on proliferation within the SVZ itself (Bergmann et al., 2012). Conversely a study that cultured samples of human olfactory epithelium found that a subpopulation of the cells had an ability to differentiate into both neurons and glia. This suggests that some of the OB cells retain neurogenic potential (Roisen et al., 2001). These studies did not look directly at proliferation within the SVZ, only within regions where SVZ born neurons are thought to be recruited. It is hypothesised that new-born SVZ derived neurons in humans are destined for the striatum rather than olfactory bulbs, in part explaining the discrepancy (Ernst et al., 2014).

2.3.4 The Stem Cell Niche

The NSCs that inhabit the adult SVZ originate from embryonic development and remain largely dormant until they are reactivated postnatally (Fuentealba et al., 2015). The neurogenic cells of the adult SVZ are located next to the ependyma, the thin layer of cells that forms the lining of the lateral ventricles. The NSCs within the SVZ are

referred to as B1 cells (Radial Astrocytes), and are $GFAP^+$ and $BLBP^+$, a characteristic shared with the SGZ type 1 cells. In terms of orientation the SVZ niche is sandwiched between the ependymal cells, in contact with cerebrospinal fluid (CSF) and basal neural vasculature (Young et al., 2007).

The B1 cells of the SVZ have a long basal process that terminate onto endothelial cells that comprise the surrounding vascular plexus (Shen et al., 2008). The regions in which B1 cells contact blood vessels are often lacking astrocyte end feet, and pericyte coverage. This results in a 'leaky' blood brain barrier specifically within the SVZ, allowing circulating small molecules to penetrate the niche (Tavazoie et al., 2008). Apically B1 cells are able to penetrate between the ependymal cell layer and hence have access to signalling molecules present within the CSF (Mirzadeh et al., 2008). Evidence has been presented that shows signals from the choroid plexus, which produces CSF, can regulate adult neural stem cell proliferation within the rodent SVZ (Silva-Vargas et al., 2016).

It has been observed that type B cells exist as either active ($nestin^+$) or quiescent ($nestin^-$) cells (Codega et al., 2014; Mich et al., 2014). Once activated, slow cycling B1 cells give rise to rapidly dividing type C cells (transit amplifying cells), which stop expressing GFAP and begin to express distal-less homeobox-2 (Dlx2) and ASCL1 (Kim et al., 2011). Type C cells differentiate into neuroblasts (type A cells) which begin expressing DCX, PSA-NCAM.(Doetsch et al., 1997). Type A cells migrate in chains out of the SVZ niche along the rostral migratory stream, to their final destination within the olfactory bulbs in most mammals (Alvarez-Buylla and Garcıa-Verdugo, 2002; Lim and Alvarez-Buylla, 2016) (**Fig. 2-6**).

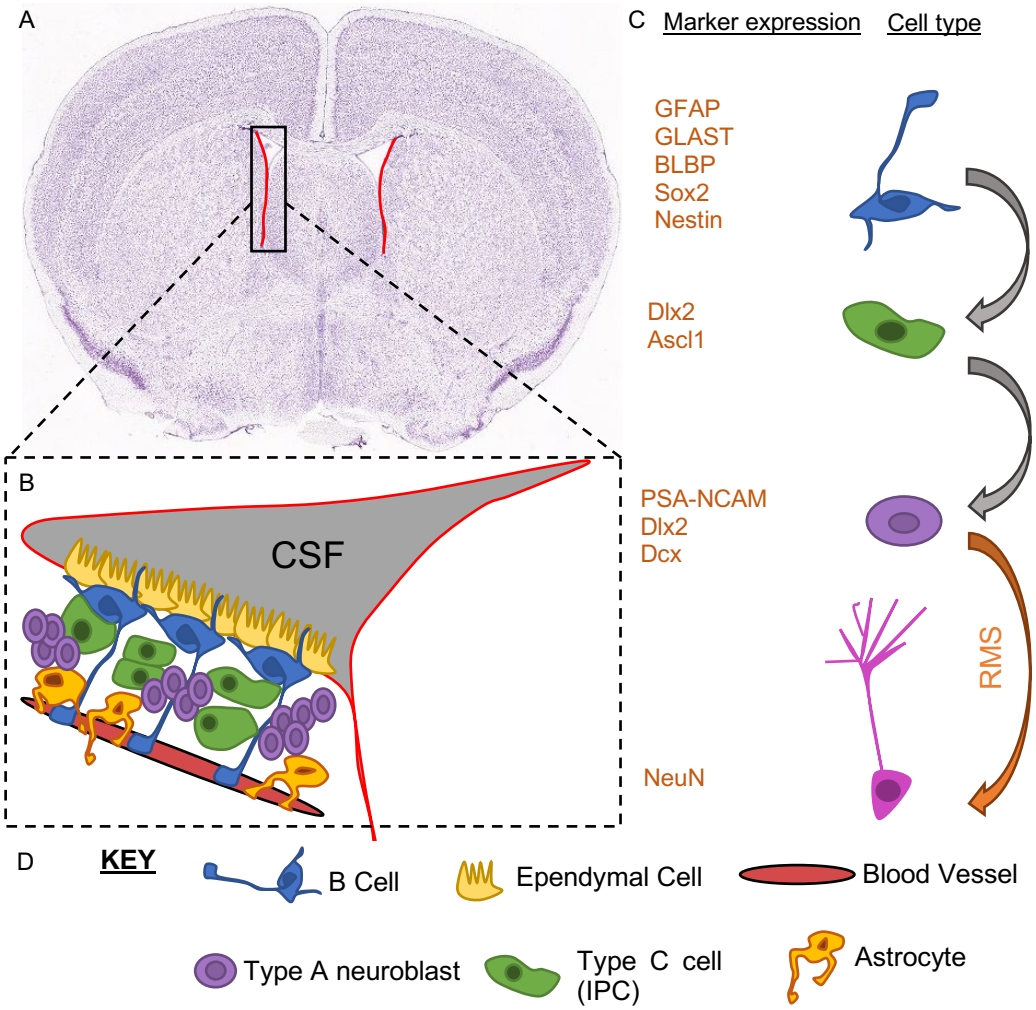


Figure 2-6 Cellular organization of the Subventricular Zone surrounding the lateral ventricles of the adult rodent brain: A coronal section of the rodent brain highlighting the proliferative subventricular zone of the lateral ventricles (A) and a representation of the microstructure (B). Various cell types within the neurogenic niche may be identified by expression of specific markers to determine commitment to neurogenic lineages (C). Cell types may be identified with the key (D). Adapted from Lin and Iacovitti 2015.

In rodents, approximately only half of the new-born SVZ derived neurons within the olfactory bulbs survive, as a result of apoptosis thought to be dependent on sensory input (Winner et al., 2002). Sensory deprivation results in an increase in apoptosis of newly generated olfactory bulb neurons, indicating the crucial role of such sensory input to neuron survival (Yamaguchi and Mori, 2005). The conclusive role of SVZ neurogenesis to olfactory bulb function in rodents remains largely elusive, although evidence indicates a potential role in odour discrimination and odour memory. Exposure of mice to an enriched odour environment results in an increase of SVZ derived neuron survival, and an improvement of odour memory (Rochefort et al.,

2002). Furthermore, mice deficient in neural cell adhesion molecule (NCAM) have deficits in migration of SVZ derived neurons into the olfactory bulb, resulting in a sizable reduction of this region. This decrease in the newly generated neuronal population results in a reduced ability of mice to discriminate between odours, whilst their ability to detect odours was unaltered (Gheusi et al., 2000).

In summary, accumulating evidence has confirmed the existence of the SVZ neurogenesis within mammals, and its contribution to granule cells within the olfactory bulb. However, much conflict remains as to evidence of SVZ neurogenesis within humans, specifically as to where the SVZ contributes new-born neurons and the resulting role of SVZ neurogenesis.

2.4 The Non-Canonical Neurogenic Niches

Since the discovery of the SVZ and SGZ as neurogenic niches, accumulating evidence has identified other mammalian 'non-canonical' neurogenic regions. Adult zebrafish are able of constitutive neurogenesis throughout their adult life, localised to regions of the CNS including the SVZ and rostral migratory stream as observed in mammals, and also regions such as the hypothalamus, dorsal telencephalon and cerebellum (Grandel et al., 2006; Zupanc et al., 2005) (**Fig. 2-7**). The generation of neurospheres from primary cultured cells from the rodent CNS has been used as an indicator of neurogenic potential in non-canonical regions such as the striatum, which observed that a population nestin^{+} cells, could proliferate in response to epidermal growth factor and differentiate into neurons (Reynolds and Weiss, 1992). Further studies utilising similar *ex vivo* techniques identified neurogenic populations isolated from the adult mouse spinal cord (Weiss et al., 1996); the postnatal non-human primate neocortex (Homman-Ludiye et al., 2012) and amygdala (Arsenijevic et al., 2001), that are capable of growth factor stimulated proliferation *in vitro*.

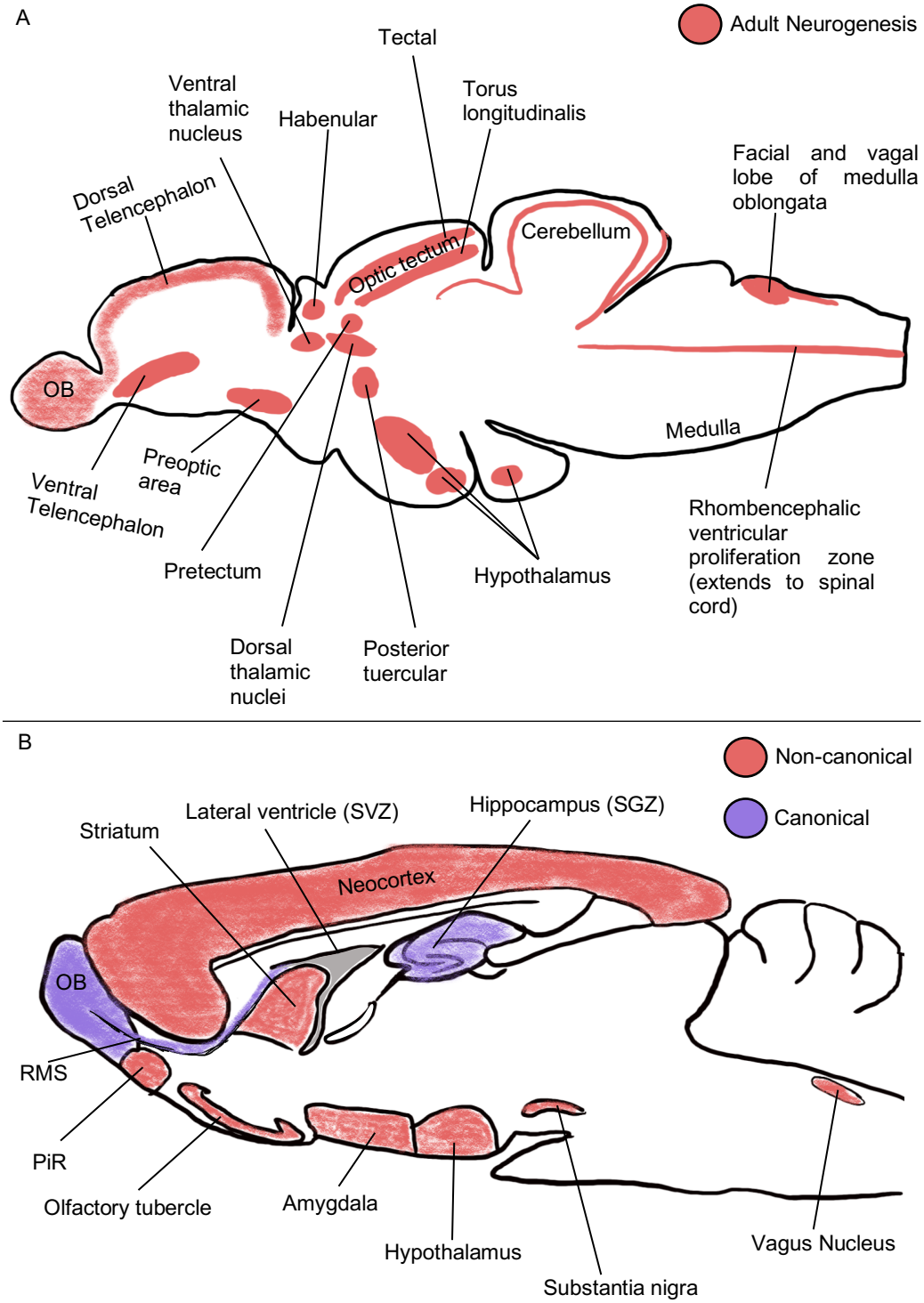


Figure 2-7 Regions capable of adult neurogenesis in zebrafish and rodent brain: Within the adult zebrafish brain numerous regions are capable of generating new-neurons (A). Comparison of adult neurogenic capability in zebrafish to the rodent brain highlights a number of common neurogenic regions of both canonical and non-canonical types such as the hypothalamus, cerebral cortex and olfactory bulbs (B). Adapted from Grandel and Brand 2006 (A) and Gould 2007 (B). OB, olfactory bulbs; RMS, rostral migratory stream; PiR, piriform cortex.

Within CNS of *Fgf10*^{nLacZ} mice, cells expressing *Fgf10* or derived from an *Fgf10* expressing lineage were identified within the cerebral cortex and regions such as the amygdala and cerebellum and hypothalamus, that are widely speculated as retaining neurogenic capacity into adulthood (Hajihosseini et al., 2008). Within this section, the postnatal/adult neurogenic potential of the aforementioned regions will be discussed (**Fig. 2-8**).

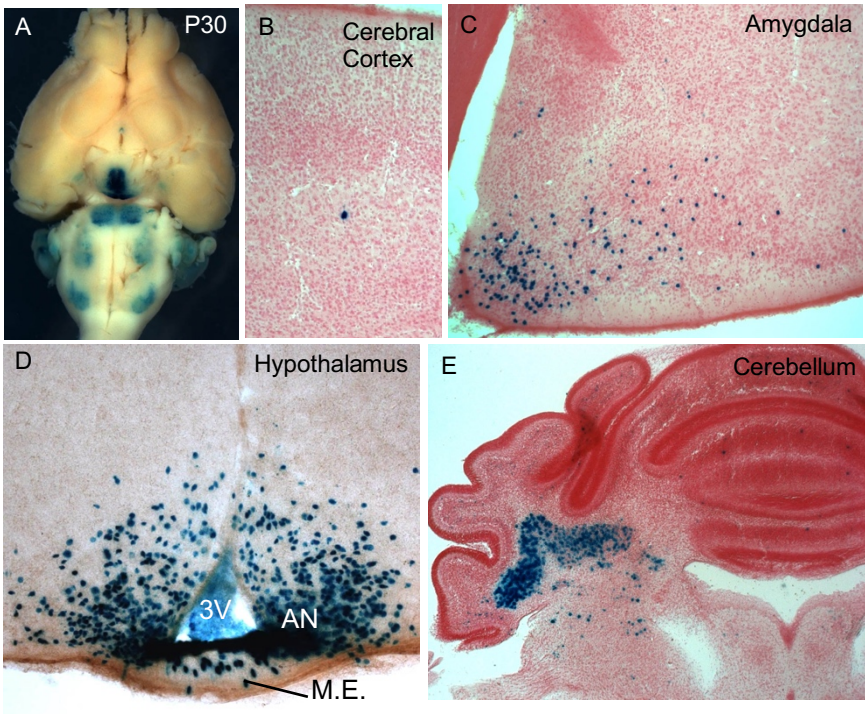


Figure 2-8 Distribution of LacZ expressing cells in the adult Fgf10^{nLacZ} mouse brain overlaps with non-canonical neurogenic regions: In 2008, use of the *Fgf10*^{nLacZ} mouse allowed for transient lineage tracing of cells expressing *Fgf10*. Cells expressing *Fgf10* and hence either derived from an *Fgf10* expressing lineage were observed within the brain (A), specifically within the cerebral cortex (B); amygdala (C); Hypothalamus (D); and cerebellum (E). Hajihosseini *et al*. 2008. AN, Arcuate nucleus; M.E., Median eminence; 3V, third ventricle.

2.4.1 The Amygdala

The amygdala is an almond shaped region of the brain responsible for emotional processing, threat assessment, aggression and addition. Studies in both humans and mice show that the amygdala has a pivotal role anxiety related disorders, specifically in relation to its hyper-connectivity as result of early life stress (Johnson et al., 2018).

Initially BrdU and NeuN double positive cells were observed within the amygdala of adult primates, speculated to originate from the rostral migratory stream (Bernier et al., 2002). However, subsequent studies within rodents were unable to detect any

proliferation within the amygdala without stimulation. One such study subject mice to a model of neuropathic pain for two months, and subsequently pulsed with BrdU for 3 days before sacrifice. Behavioural analysis indicated mice displayed a depressive behaviour in the absence of anxiety. On a molecular level there was a higher number of cells within the amygdala, contributed to by new-born neurons ($BrdU^+$ $NeuN^+$) within the neuropathic pain model only.

Additional studies studied the neurogenic potential of the amygdala in response to stimuli such as electroconvulsive seizures (Ehninger et al., 2011; Okuda et al., 2009; Wennström et al., 2004). However, were unable to observe adult new-born neurons. Instead, these studies showed that new-born $BrdU^+$ cells within the amygdala were oligodendrocytes, or proliferative cells expressing proteoglycan neuron-glia 2 (NG2) which primarily differentiate into oligodendrocytes. It should be noted that the comparison of these studies is incredibly difficult, due to large variation in terms of their BrdU dosage and duration of the pulse.

Recently, primary cell culture assays identified a population of cells within the rodent basolateral amygdala that are neural progenitors and have the capacity to generate new neurons *in vitro.* The study also used a comprehensive routine of *in vivo* experiments with transgenic mouse lines to identify new-born neurons ($BrdU^+$ and DCX^+) within the amygdala (Jhaveri et al., 2018).

In conclusion, it's likely that some cells in the amygdala retain proliferative capacity into adult mammalian life. However, conflict remains in terms of the degree of new neuron production, and whether it is a phenomenon only observable after stimulation.

2.4.2 The Cerebellum

The cerebellum is a region of hindbrain that is critical for motor control, specifically motor control and cognition (Armengol et al., 2013). Structurally, the adult cerebellum can be separated into 3 layers, the more superficial molecular layer, the Purkinje cell layer and the internal granule layer. During embryonic development, there are two transient germinal zones within the cerebellum; the ventricular zone and the external granule layer (EGL). Accumulating evidence shows that Purkinje neurons (GABAergic) and granule neurons (glutamatergic) are derived from the ventricular zone and EGL respectively.

The cerebellar ventricular zone is most active during embryonic development, whereas neurogenesis within the EGL persists into postnatal development, until approximately P14, generating new-born granule neurons destined for the internal

granule layer (Espinosa and Luo, 2008). It is thought that this neurogenesis contributes to the growth of the characteristic cerebellar folia, a process stimulated and regulated by sonic hedgehog (Shh) expressed by Purkinje neurons (Corrales, 2006). Postnatal born granule cell neurons leave the proliferative EGL and migrate inward, past the embryonic born Purkinje neurons and into the internal granule layer; a process aided by a subset of glial cells named Bergmann glia, which provide a scaffold for radial migration (Lewis et al., 2004).

Some groups have been able to isolate and culture populations of neural progenitors from the postnatal rodent cerebellum, of a separate lineage from the known protracted granule cell postnatal development. Rare cells expressing Prominin-1 (CD133) were isolated by FACS sorting, thus selecting for a population separate to the known granule cell precursors within the postnatal EGL. The group was able to isolate a distinct population of neural stem/progenitor cells from the postnatal day 7 cerebellum, able to differentiate into neurons, astrocytes and oligodendrocytes *in vitro*. However, whether this population persists into adulthood was beyond the scope of the study (Lee et al., 2005).

Recently, a population of $sox2^{+}/nestin^{+}/S100\beta^{-}$ undifferentiated Bergmann glia within the Purkinje cell layer of the adult mouse cerebellum have been identified. Cells from $Sox2^{+}$ lineage proliferated in the adult mouse cerebellum ($BrdU^{+}$) and differentiated into neurons. This phenomenon could be enhanced by physical exercise, and a similar population of *Sox2* expressing cells were identified in human cerebellum samples (Ahlfeld et al., 2017).

In studies using the rabbit as a model organism, the EGL is replaced by a second proliferative layer (coined the subpial layer). Within this layer neural precursors (DCX^{+} and PSA-$NCAM^{+}$) are observed into puberty, but are no longer detectable at 6 months old (Ponti et al., 2006). More in depth studies in ages after the subpial layer ceases to exist (1-3 years of age) detected neural progenitors (DCX^{+} PSA-$NCAM^{+}$) that persisted within cerebellar layers (Ponti et al., 2008).

It is known that development of cerebellar granule neurons is a protracted process extending into postnatal mouse development. However, separate populations of progenitors within the cerebellum have been identified in two separate models. Further research is required to confirm whether these are separate populations to those already identified, and whether neurogenesis continues into adult life.

2.4.3 The Hypothalamus

More recently it has been demonstrated that postnatal/adult neurogenesis also occurs within the rodent hypothalamus. The hypothalamus is situated below the thalamus and surrounds the lateral aspects of the third ventricle, in close contact with the median eminence, a circumventricular organ. Functionally, the hypothalamus regulates energy balance and behaviours of instinct and autonomic function such as temperature regulation, water balance and sexual activity (Yuan and Arias-Carrión, 2011).

Lining the third ventricle (3V) are tanycytes, which first arise during late embryonic development (Ahlfeld et al., 2017). In the postnatal/adult mouse brain tanycytes can be subdivided into alpha and beta subtypes, both of which possess a radial morphology, with long processes extending into the hypothalamic parenchyma (Goodman and Hajihosseini, 2015) (**Fig. 2-9**).

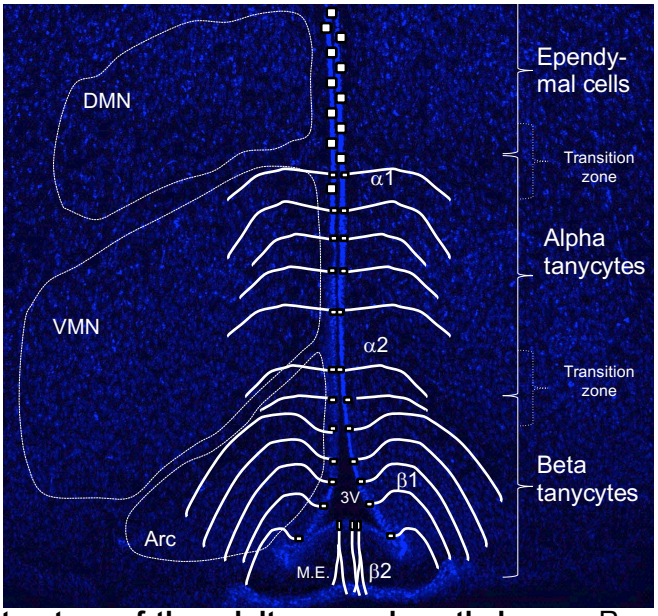


Figure 2-9 The structure of the adult mouse hypothalamus: Recently, cells named tanycytes have been implicated as having a role in adult neurogenesis within the hypothalamus. Based on their location within the ventricular lining, tanycytes may be classified into different groups denoted by alpha and beta. In a coronal section of mouse brain tissue Hoechst staining allows the delineation of hypothalamic nuclei. Populations of alpha and beta-tanycytes and ependymal cells are discernable, between each tanycyte/ependymal cells lie transition zones relaying difficulties in accurately discerning populations. Goodman and Hajihosseini 2015. Arc, arcuate nucleus; VMN, ventromedial nucleus; DMN, dorsomedial nucleus; 3V, third ventricle; M.E, Median eminence.

In 2002 a research group were able to culture mitotic cells from samples of rodent hypothalamus and brain stem, which also expressed markers of immature neurons (Evans et al., 2002). Further studies in the adult rodent CNS observed strong nestin

expression within cells lining the 3V. Many nestin^{+} tanyctes were also BrdU^{+} (Ernst and Christie, 2005; Xu et al., 2005). Cells within the adult rodent hypothalamus were found to form neurospheres in culture, capable of proliferating, and following withdrawal of growth factors, differentiating into astrocytes, oligodendrocytes, and neurons (Xu et al., 2005). *In vivo* it was discovered that BrdU^{+} cells within the median eminence and hypothalamic parenchyma give rise to new-born neurons within mouse hypothalamic nuclei, some displaying functional phenotypes in specific regard to energy balance (Kokoeva, 2005).

Research aimed at identifying which cells within the hypothalamus constitute NSCs is ongoing. Previous studies from our laboratory sought to elucidate the expression profile of Fibroblast growth factor-10 (Fgf10) in the embryonic, postnatal and adult CNS by use of a transgenic reporter mouse strain, namely *Fgf10*^{nLacZ}. It was observed that there is intense *Fgf10* expression in the embryonic, postnatal and adult hypothalamus (Hajihosseini et al., 2008).

It was hypothesised that cells derived from the *Fgf10*-expressing lineage may delineate the neural stem/progenitor cells in the hypothalamus, therefore further research aimed to elucidate this. In 2013 research by our laboratory determined that cells from the FGF10^{+} derived lineage were restricted to the Beta-tanycytes, and express neural stem/progenitor markers such as sox2 and nestin within the hypothalamic 3V. With subsequent BrdU and EdU (another thymidine analogue) identifying actively dividing *Fgf10*-expressing lineage derived cells within the hypothalamic parenchyma and β-tanycyte domain (**Fig. 2-10**) Furthermore, parenchymal FGF10^{+} lineage derived cells were also identified as neurons by the mature neuronal marker NeuN. It was determined that *Fgf10*-expressing lineage derived NSC/NPCs proliferate in the postnatal/adult mouse hypothalamus and add new neurons to energy balance regulating circuitry. It remains to be shown whether cells derived from the FGF10^{+} lineage identified within other brain regions, such as the cerebral cortex, also delineate a population of neural stem/progenitor cells (Haan et al., 2013).

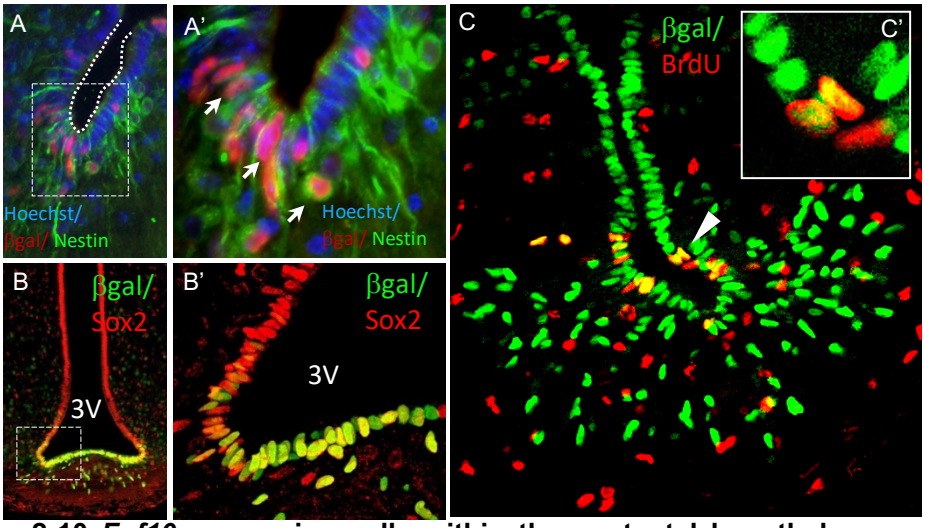


Figure 2-10 *Fgf10* expressing cells within the postnatal hypothalamus resemble neural stem/progenitor cells: Further studies using the *Fgf10*^{nLacZ} mouse focused on LacZ^{+} cells within the hypothalamus. It was shown that LacZ^{+} cells within the β-tanycyte domain expressed markers of neural stem/progenitor cells such as nestin (A), sox2 (B) and were capable of incorporating BrdU, hence were actively dividing at P28 (C). Haan et al. 2013.

Conversely, Robins et al concluded it was in fact the more dorsal α-tanycytes that are neural progenitors, and that these in turn give rise to more ventrally situated β-tanycytes, astrocytes and sparse neurons within the mouse hypothalamic parenchyma (Robins et al., 2013). Some research groups have observed proliferation of the cuboid ependymal cells situated ventrally in regard to both α- and β-tanyctes, by use of a continuous pump administering BrdU into the ventricles for 3 days (Latifa et al., 2003). However, this phenomenon was not replicated by a less intensive BrdU pulsing paradigm, which instead identified proliferation of tanycytes in response to insulin-like-growth-factor-1 infusion (Pérez-Martín et al., 2010).

The role of hypothalamic neurogenesis in rodents is being elucidated. Under physiological conditions hypothalamic neurogenesis is slow. Long-term high fat diet (HFD) impairs the survival and subsequent neurogenesis from hypothalamic NSCs within the medio-basal hypothalamus (Li et al., 2012). This is postulated to be attributed to vulnerability of hypothalamic NSCs to obesity related inflammation via IKKβ/NF-κB over-activation, and may contribute a neurodegenerative mechanism for obesity related diabetes. The median eminence has been shown as capable of generating new-born neurons at a 5-fold higher rate than other hypothalamic regions, derived from β2-tanycytes. Blocking neurogenesis in the ventro-basal hypothalamus (hence the median eminence) of mice fed on HFD results in higher weight gain than relative controls, additionally HFD is capable of activating median eminence-based

neurogenesis (D. A. Lee et al., 2012). Discrepancies between such HFD studies on hypothalamic neurogenesis may be as a result of differences in hypothalamic region studied, postnatal/adult ages analysed and the duration of HFD. Additionally, sex-related differences may have a role, as shown with HFD causing an increase in ME neurogenesis in female mice but not males (Blackshaw et al., 2016). None-the-less such studies demonstrate an important role of hypothalamic neurogenesis in energy balance and metabolism in rodent models.

Hypothalamic neurogenesis has yet to be extensively explored using non-human primates or human tissue. A paper focused on the SVZ noted expression of nestin within the human 3V as a side-note, but did not explore further (Bernier et al., 2000). Another in 2013 explored DCX expression within the hypothalamus and observed extensive DCX expressing cells within the hypothalamus of adult humans, mice and sheep. This may stand as evidence of adult new-born and immature hypothalamic neurons in these organisms. (Batailler et al., 2014).

2.4.4 The Cerebral Cortex

The cerebral cortex is a laminar structured brain tissue which covers a majority of the cerebrum. It is a complex multifunctional brain region responsible for higher order cognition, sensory and motor functions to name a few (Section 1.1). Broadly, the of compliment of neurons within the cerebral cortex may be subdivided into two heterogeneous populations, inhibitory GABAergic interneurons and excitatory glutamatergic pyramidal neurons. In embryonic development interneurons and pyramidal neurons are born in the ganglionic eminences and ventricular/subventricular zone respectively and migrate post-mitotically into the cortical plate in a process largely thought to be confined to embryonic development. In a strict temporal sequence cortical neurogenesis is superseded by gliogenesis which peaks during postnatal life.

During postnatal development the rat brain increases in mass by six times, in a process previously attributed to gliogenesis and increased neuronal size without neurogenesis. However, it has been shown that during the first postnatal week the neurons within the cerebral cortex double in number, followed by a second wave of an additional 6 million neurons added in the two weeks after birth (Bandeira et al., 2009). Studies in mice observed a similar contribution of new-born neurons to the cerebral cortex between birth and P16 of approximately 80-100%. This was coupled with a delay of NeuN marker expression in deep layer cortical neurons until P16, suggesting a retention of immature phenotypes and of neurogenic potential of these cells (Lyck et

al., 2007).

2.4.4.1 Parenchymal Progenitors

Research using adult macaques as a model observed BrdU positive cells in the neocortical regions concerned with behavioural plasticity, some of which expressed markers of mature neurons within layers I-V, but not VI (Gould et al., 1999). Subsequent research by the same group postulated that new-born neocortical neurons are transient, as the number of $BrdU^+$ cells decreases by 9 weeks after BrdU administration. This study also utilised adult rats, and observed $BrdU^+$ and $TUJ1/NeuN^+$ cells in the rostral neocortex (Gould et al., 2001).

Additional BrdU focused studies identified GABA-ergic $BrdU^+$ interneurons in the postnatal rat cerebral cortex. None of the new-born neurons expressed DCX, however minority of new-born neocortical interneurons expressed NG2, a marker or oligodendrocyte progenitor cells (OLPs). This suggests new-born neurons may derive from the NG2 expressing lineage present in the cortical parenchyma (Dayer et al., 2005). The $NG2^+$ cells that contribute to new neurons of the neocortex have been characterised as PLP-promoter-expressing NG2 progenitors (PPEPs). These cells were observed as multipotent *in vivo* giving rise to low levels of neurons, astrocytes and oligodendrocytes in postnatal development, between P8 and P15 (Guo et al., 2009).

Populations of immature neurons (DCX^+) have also been identified within the mouse cerebral cortex, at low levels that are retained into adulthood, potentially as a result of adult neurogenesis. Additionally, cells expressing Ki67, were observed migrating from the dorsal rostral migratory stream and into the neocortex through the corpus callosum, leading to the hypothesis that the SVZ may contribute new-born neurons to the adult neocortex (Nkomozepi et al., 2018). Chains of neuroblasts migrating towards the corpus callosum have also been observed in the rabbit (Luzzati et al., 2003), and again within the mouse contributing to neurons of both the amygdala and associated cerebral cortex (Bernier et al., 2002). However, DCX^+ cells may not necessarily be representative of postnatal/adult neurogenesis. Supposed 'immature' DCX^+ neurons in the sheep, rat and guinea pig cerebral cortex have been shown to be born embryonically, but remain in a prolonged state of immaturity into adulthood (Gómez-Climent et al., 2008; Piumatti et al., 2018; Yang et al., 2015).

2.4.4.2 The Meninges as a Neural Stem/Progenitor Cell Niche

The meninges have also been identified as a potential niche of neural precursors that contribute neurons to the cerebral cortex postnatally. The meninges are a three-

layered structure, consisting of the outer dura mater and the leptomeninges (arachnoid and pia mater), covering the entire brain and penetrating within it (Decimo et al., 2012). Capillaries extending from the pia mater invade the cerebral cortex embryonically, establishing an intracerebral microvascular component, whereby each perforating vessel is associated with extroflexions of the meninges, forming a perivascular space (Zhang et al., 1990). Initial evidence showed that nestin^{+} cells extracted from the rat leptomeninges postnatally (P15) could differentiate into neurons *in vitro,* which when injected into the adult brain were able to survive and differentiate (Bifari et al., 2009).

The rat postnatal/adult leptomeninges contain cells that express markers associated with neural stem/progenitor cells such as nestin, vimentin, DCX and sox2 (Bifari et al., 2015). More recently embryonic born cells within the meninges, were observed migrating into the caudal cerebral cortex, and differentiating into neurons within layers II/III and V (Bifari et al., 2017). A majority of the meningeal derived neurons were from the PDGFRβ^{+} lineage, of both projection and interneuron subtypes. The embryonic birth of these progenitors suggests the postnatal cerebral cortex may be capable of recruiting new-born neurons, but potentially in the absence of parenchymal cell divisions (Bifari et al., 2017).

2.4.4.3 Cortical Neurogenesis Following Injury

The cerebral cortex has been shown to have proliferative potential in response to insult. Studies utilising focal ischemia as a stroke model in rats observed proliferation of layer 1 neocortical progenitor cells and generation of integrated GABAergic neurons (Ohira, 2011). Others have observed sustained neurogenesis within the cortical ischemic lesion by cells of SVZ origin (Yang et al., 2007). Further studies determined that endogenous cortical progenitors produced new neurons (DCX^{+}/BrdU^{+}) within the lesioned cortex that did not mature. However, given that there is evidence that cortical neurons may persist in an extended immature state under normal conditions, such immature neurons produced following injury may still have a functional role (Li et al., 2015). Cortical neurogenesis can also be stimulated by targeted degeneration of layer VI corticothalamic neurons, originated from both the SVZ and endogenous cortical progenitors (Magavi et al., 2000).

The meninges have also been shown to have neurogenic potential in response to ischemia and stroke, in addition to the low-level neurogenesis observed under physiological conditions. In a mouse model of ischemic stroke nestin^{+} and PDGFRβ^{+}/NG2^{+} leptomeningeal cells were proliferative (Brdu^{+}/Ki67^{+}) and capable of generating neurons when cultured *in vitro* (Nakagomi et al., 2011). A subsequent study

observed DCX^+ in the post-stroke leptomeninges that were capable of migrating into the cerebral cortex and differentiating into neurons *in vitro* (Nakagomi et al., 2012).

2.4.4.4 Cortical Neurogenesis and Perinatal Hypoxia

In developed countries, systemic asphyxia occurs up to a rate of 20 per 1,000 births at full term, and the incidence is higher in babies that are premature or very-low-birth-weight (Vannucci, 2000). Syndromes such as apnoea and respiratory distress at birth result in cerebral hypoxemia, and in turn loss of cortical neurons and glia, as well as altered differentiation of neurons and synaptogenesis. Through adolescence the incidence of brain-damage related disability as a result of perinatal hypoxia decreases, however largely the mechanism for this plasticity is unknown (Ment et al., 2003).

New-born pups that were reared in sublethal hypoxic conditions between P4 and P11 displayed a 30% reduction of neurons within the cerebral cortex at P11. Following 4 weeks of recovery, normoxic and hypoxic reared mice were indistinguishable in terms of brain weight, cortical volume and NeuN immunolabelling. It was determined that following hypoxic injury there is enhanced proliferation within the SVZ, and aberrant migration of neuroblasts dorsally from the rostral migratory stream into the deep layers of the cerebral cortex. This phenomenon was two-fold higher in hypoxia treated pups in comparison to control, suggesting this SVZ contribution of neurons to the postnatal cerebral cortex may occur under normal conditions at low levels, which may be further stimulated following hypoxic insult (Fagel et al., 2006). In the rat, a model of neonatal hypoxia-ischemia (carotid artery ligation followed by systemic hypoxia in a chamber at P6) observed SVZ derived neocortical neurogenesis. This recruitment of new-born interneurons to cortical regions was sustained at least until 5 months following injury (Yang et al., 2007).

Further studies determined that cortical neurogenesis following perinatal hypoxia requires fibroblast growth factor receptor-1 (*Fgfr1),* by use of $Gfap^{Cr}::Fgfr1^{floxed/floxed}$ conditional knockout (cKO) mice, in which Fgfr1 was knocked out in all $GFAP^+$ astrocytes. It was noted that *Fgfr1* cKO mice failed to mount a cortical neurogenic response and recover following hypoxic injury, and interestingly showed a decrease in SVZ neurogenesis in comparison to an increase in hypoxic treated wild-type mice (Fagel et al., 2009).

This illustrates that the cerebral cortex may retain a limited ability to recruit new-born neurons from the canonical niches in response to injury. However, these studies did not comment on the existence of actively proliferating endogenous cortical progenitors and varied in regard to the observed ability of new-born neurons to integrate and

mature within the cerebral cortex.

2.4.4.5 Conflicting Evidence

Despite accumulating evidence for neocortical neurogenesis in the postnatal/adult brain, in a broad range of species, conflict still remains. Replicative BrdU pulsing experiments in the adult macaque did not identify any $BrdU^+$ $NeuN^+$ cells in the frontal cortex in compared to that identified by Gould *et al.* 1999 but did observe close association of $NeuN^+$ and $BrdU^+$ cells (denoted as glial satellite cells). Analysis of human tissue for integration of the carbon isotope ^{14}C, and integration of BrdU identified no adult born neuronal cell within the neocortex. However, the paper acknowledges the limits of the techniques used in terms of their sensitivity (<1%). Thus, both transient new-born neurons and those produced in low quantity will not have been detectable (Bhardwaj et al., 2006).

This is of particular significance considering that the neocortex is postulated to only be capable of low levels of neurogenesis under physiological conditions, below the sensitivity parameters of many current methodologies. Furthermore, research within the field vastly varies in terms of BrdU dosage, method and length of administration period, and time (days) of the subsequent 'chase' following. As mentioned previously, a review in 2007 highlighted this and postulated variable experimental paradigms are the root cause of the discrepancies in the field (Gould, 2007).

2.4.4.6 Conclusion

In conclusion the field of postnatal/adult neurogenesis within the cerebral cortex is a conflicting one. There appears to be some consensus on the potential of meningeal derived neural stem/progenitors, and of NG2/PDGFRβ/α origins of parenchymal cells with neurogenic potential. However, there is yet to be agreement on the origin of these progenitors, the niche in which they occupy, and whether they remain quiescent until stimulated by insult or proliferate at low-levels.

2.5 The Fibroblast Growth Factor Family

The fibroblast growth factors (FGFs) are a family of signalling molecules responsible for a broad range of processes in embryonic development and adult tissue homeostasis. Fibroblast growth factor activity was initially identified in an extract of the bovine pituitary, able to stimulate proliferation of 3T3 cultured fibroblasts. This would later be purified and referred to as basic FGF or FGF-2 (Armelin, 1973; Lemmon and Bradshaw, 1983). There are currently twenty-two known members of the fibroblast growth factor family, eighteen of which function canonically, in a paracrine/autocrine/endocrine manner and four that signal via intracellular mechanisms. The FGF family can be further divided into seven subfamilies dependant on biochemical function, sequence homology and structural properties (**Fig. 2-11**).

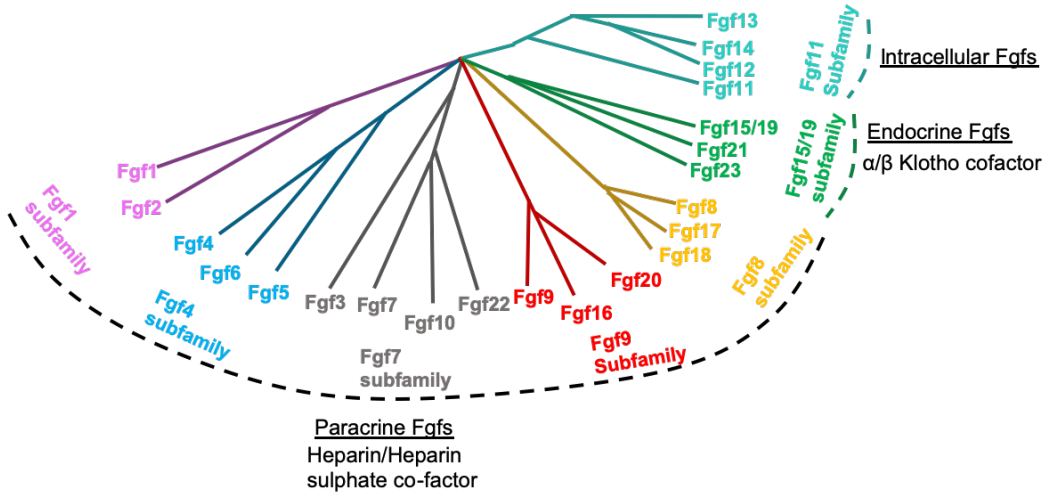


Figure 2-11 The Fibroblast Growth Factors: The fibroblast growth factor subfamilies as determined by phylogenetic analysis, whereby branch lengths are approximately proportional to the evolutionary distance between each gene. Adapted from Ornitz and Itoh 2015.

2.5.1 FGF Receptors

The canonical FGFs signal via FGF tyrosine kinase transmembrane receptors (FGFR), of which there are five identified thus far (FGFRs 1-5) (Zhang et al., 2006). Broadly, FGFRs consist of three extracellular immunoglobulin-like domains (DI-DIII), a transmembrane domain and two intracellular tyrosine kinase domains (split tyrosine kinase). Ligand specificity and binding is determined by the DII and DIII domains. Interestingly, the DI domain and the acid-box motif that links it to DII have an autoinhibitory role on ligand binding. This has shown with an alternative splicing event removing the DI domain and DI-DII linker having an increased affinity for FGF ligands (Olsen et al., 2004; Plotnikov et al., 1999).

Further ligand binding specificity is introduced to FGFR1-3 by alternative splicing of the DIII domain, coded for by exons IIIb and IIIc, resulting in either IIIb or IIIc isoforms of these receptors, which are generally restricted to epithelial or mesenchymal tissues, respectively (**Fig. 2-12**) (Chellaiah et al., 1994; Miki et al., 1992; Ornitz and Itoh, 2015; Orr-Urtreger et al., 1993). The FGF ligands have robust selective affinity for the specific FGFRs, as investigated by mitogenic assay utilising canonically low FGFR expressing BaF3 cell lines, genetically engineered to express only a specific FGFR isoform (Ornitz et al., 1996; Zhang et al., 2006) (**Table 2-2**).

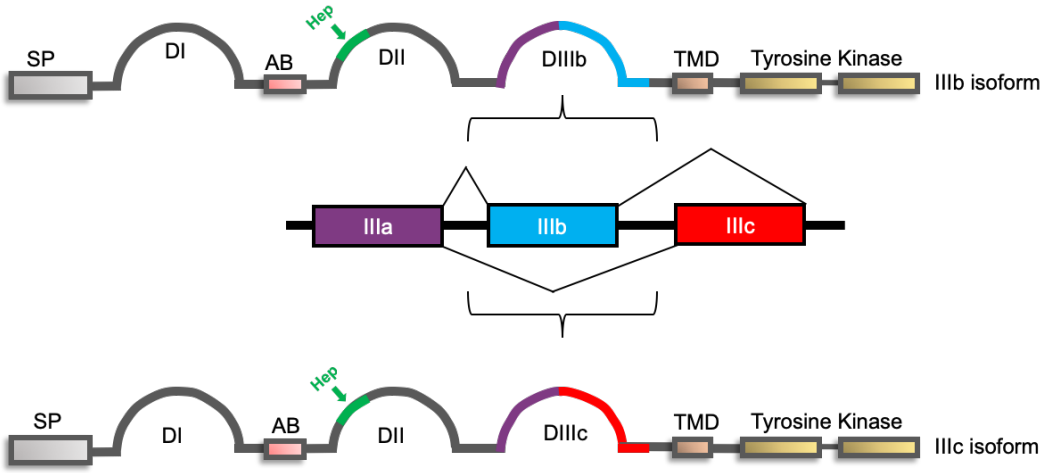


Figure 2-12 Alternative splicing of FGFR1-3: By alternative splicing to include either the IIIb or IIIc exon of the FGFR gene, additional ligand binding specificity is conferred resulting in either IIIb or IIIc splice isoforms. Sp, cleavable secreted signal sequence; AB, acid box; TM, transmembrane domain.

Table 2-2 Receptor binding specificity of the FGF ligands and respective FGFRs: As determined by alternative splicing of FGFR1-3 in various cell culture studies. FGFR4Δ denotes at two immunoglobulin-like domain form. Adapted from Ornitz and Itoh 2015.

FGF Ligand	Receptor Specificity	Cofactor
FGF1 FGF2	ALL FGFR 1c, 3c >2c, 4Δ	Heparan Sulphate
FGF4 FGF5 FGF6	FGFR 1c, 2c, > 3c, 4Δ	
FGF3 FGF7 FGF10 FGF22	FGFR 2b > 1b	
FGF8 FGF17 FGF18	FGFR3c > 4Δ > 2c >1c >>3b	
FGF9 FGF16 FGF20	FGFR3c >2c >1c,3b >>4Δ	
FGF15/19 FGF21 FGF23	FGFR 1c, 2c > 3c, 4Δ FGFR 1c, 3c FGFR 1c, 3c, 4	β-Klotho α-Klotho

Several variants of the FGFRs exist, such as FGFR5 (also known as FGFRL1) which does not possess a tyrosine kinase domain and binds FGF2 (but not members of the Fgf-7 subfamily) with low affinity (Sleeman et al., 2001). The lack of tyrosine kinase domain lead to the assumption that FGFR5 functions as a decoy receptor to modulate FGF signalling. For example, in *Xenopus* development FGFRL1 is shed from the membrane and directly antagonises FGF signalling (Steinberg et al., 2010). However, in other models such as in pancreatic β-cell cytokine-induced inflammation, FGFRL1 acts as a co-receptor for FGFR1, forming 2:1 heterocomplexes (Regeenes et al., 2018). A second is a secreted variant of FGFR1, which encodes only Ig like domains II and III and functions to inhibit FGF-FGFR signalling (Duan et al., 1992). The secreted sFGFR1 binds FGF2 and FGF1, but potentially also FGF-4, -5 and -6 and has been shown promise in combination with sVEGFR in anti-tumour angiogenesis

(Ogawa et al., 2002).

2.5.2 FGF Signaling

In canonical paracrine FGF signalling, FGF-FGFR binding forms a 1:1 complex. Activation of the signalling pathway requires formation of FGF:FGFR dimers and in turn requires heparan sulphate as a co-factor (Spivak-Kroizman et al., 1994), and activates tyrosine kinase domains by transphosphorylation. This results in recruitment of the adaptor protein FGFR substrate 2α (FRS2) which in turn phosphorylates and activates downstream substrates for four major signalling pathways; PLCɣ, and the RAS-MAPK and PI3K-AKT (dependent on binding of CRKL). The activated FGFR complex also activates STAT-1, -3, -5 and in turn regulates STAT target genes (**Fig. 2-13**) (Ornitz and Itoh, 2015).

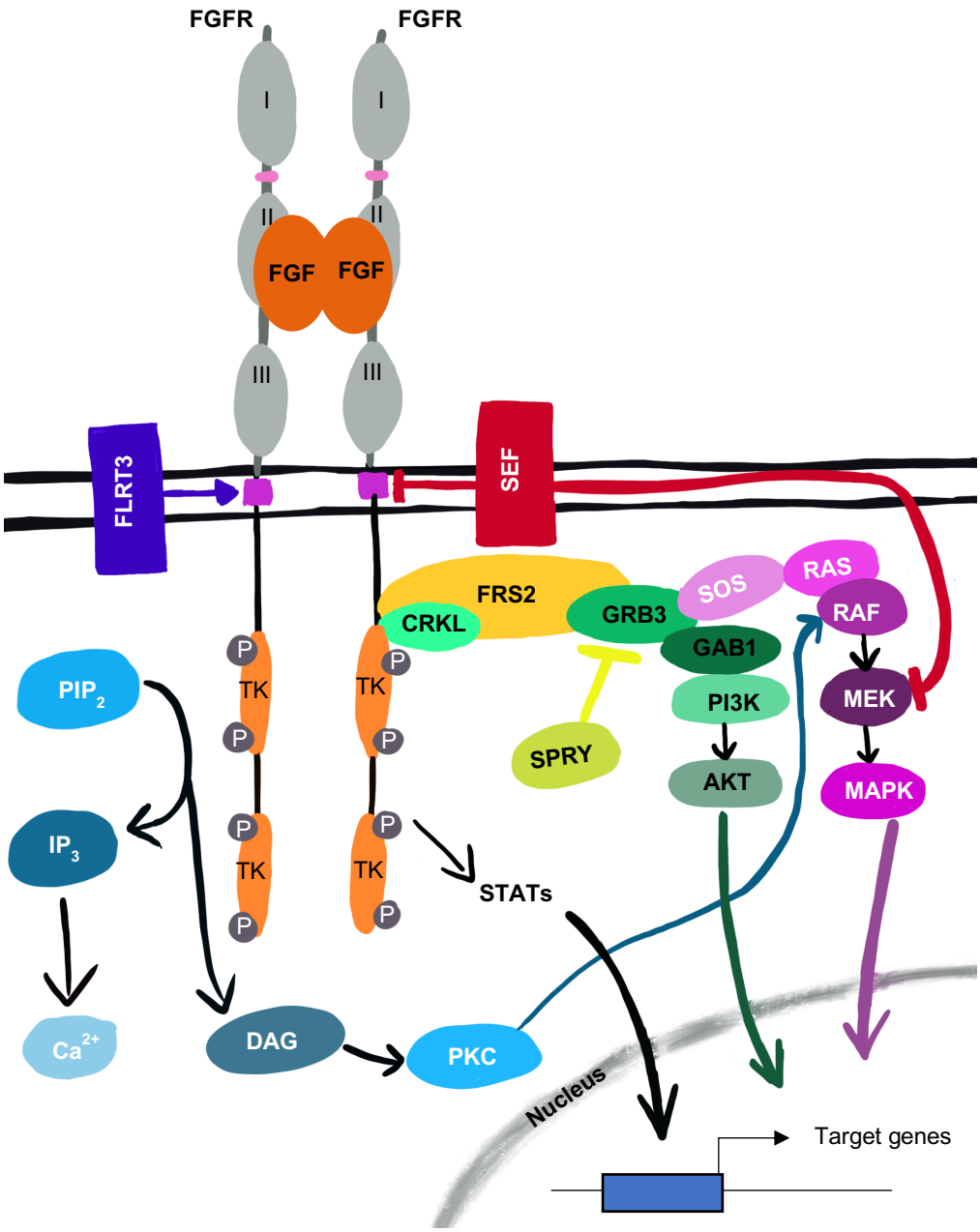


Figure 2-13 The FGF-signaling pathway: A schematic of the transduction of signaling induced by FGF-FGFR binding. Upon binding the FGF ligand and receptors form a dimer, resulting in mutual transphosphorylation of intracellular tyrosine kinase domains. This in turn activates Ras/MAPK and PI3K-AKT downstream signaling, as well as STAT and phospholipase-Cγ. Examples of negative regulators are highlighted such as SPRY and SEF. Adapted from Ornitz et al 2015.

2.5.3 Modulators of FGF Signaling

A plethora of molecules exert an influence to modulate FGF signalling, in order to maintain tight control on levels of signalling and the broad range of complex processes that FGF signalling has a role in. Canonical signalling of endocrine and paracrine FGFs is dependent on the co-factors α/β-klotho and heparan sulphate respectively.

Heparan sulphate proteoglycans (HSPGs) are comprised of a core protein linked to heparan sulphate glycosaminoglycan chains. They are able to interact with both FGFs and FGFRs to increase binding affinity and stabilise the FGF-FGFR dimer. Heparan sulphate also acts as a component of the extracellular matrix and is therefore able to trap FGF ligands and modulate their diffusion to regulate signalling gradients. This is a necessary component in regulating branching morphogenesis of glands in response to gradients of FGF10 and FGF7 that form as a result of differential HSPG binding affinities, which when altered perturb branching morphogenesis (Makarenkova et al., 2009; Matsuo and Kimura-Yoshida, 2013; Ornitz and Itoh, 2015).

The Klotho family of transmembrane glycoproteins act as cofactors in endocrine FGF signalling and consist of α-klotho, β-klotho and KLPH (Ornitz and Itoh, 2015). During endocrine signalling of FGF-23, it was found that Klotho increases the affinity of it to bind to its FGFR, and enhanced phosphorylation of FRS2 and ERK signalling (Kurosu et al., 2006).

Sprouty is a regulator of signalling transduced via intracellular tyrosine kinases; including but not limited to FGF and vascular endothelial growth factor (VEGF) signalling. The sprouty (Spry) family consists of four members Sprys 1-4, which interact with Grb2 adaptor protein to inhibit and modulate the Ras-MAPK and PI3K-AKT pathways. As part of a negative feedback loop, FGF signalling can activate *Spry* expression, in patterning of the mid-hindbrain boundary by FGF-8, -17 and -18 and kidney development induced by FGF7 (Chi et al., 2004; A. Liu et al., 2003).

A transmembrane protein called Fibronectin leucine-rich transmembrane protein 3 (FLRT3) is expressed following activation of FGF signalling pathways and downregulated following pathway inhibition. The *Xenopus* homologue, XFLRT3, facilitates the FGF-induced MAP-kinase downstream signal transduction of FGF8 by interaction with FGFRs (Böttcher et al., 2004).

A second transmembrane protein that influences FGF signalling is Sef, which acts intracellularly as an antagonist of the Ras-MAPK pathway by binding to MEK and

inhibiting the nuclear translocation of MAPK. Extracellularly Sef protein has been speculated as capable of binding to FGFRs and inhibit the phosphorylation of intracellular tyrosine kinase domains (Fürthauer et al., 2002; Kovalenko et al., 2006).

2.5.4 FGF Signaling within the Embryonic and Adult Brain

Several members of the FGF signalling family are implicated in embryonic development of the central nervous system and within the post-natal/adult brain under physiological and pathological conditions, some of which are discussed below.

2.5.4.1 The Fgf1 Subfamily (Fgf-1 and -2)

Of all the FGF ligands, FGF1 is the only one capable of signalling via all FGFRs and their isoforms, whereas FGF2 is only capable of signalling the FGFR1-3 IIIc isoforms and FGFR4 (**Table 2-2**). Both *Fgf1* and *Fgf2* are both highly expressed within the embryonic CNS, *Fgf1* exclusively within neuronal cells and *Fgf2* by both neuronal and non-neuronal cells (Reuss and von Bohlen und Halbach, 2003). In studies utilising embryonic cell lines, application of FGF1 and FGF2 was able to enhance neurogenesis of embryonic stem cell lines into primitive neuroblasts (Chen et al., 2010).

During embryonic development, studies indicate that FGF2 has roles in proliferation and neuronal differentiation of progenitors. Mice that are deficient in *Fgf2* are viable but have 45% reductions in cortical neuronal number by the end of neurogenesis, and lack deep cortical layers, with no change in apoptosis. Further analysis determined that *Fgf2* is expressed by neuroepithelial cells in cortical development, and increases the proliferative capacity of embryonic cortical progenitors to give rise to both neurons and glia (Raballo et al., 2000; Vaccarino et al., 1999). Expression of FGF1 is also observed in a similar pattern to FGF2 in embryonic neurogenesis, however the two do not show any compensatory roles, with the brain of $FGF1^{-/-}$ mice exhibiting a normal structure (Miller et al., 2000).

In adult animal models of injury, both FGF1 and FGF2 are reported to have protective roles involved in a range of different processes. Both FGF1 and FGF2 *in vivo* have a role in maintenance of blood brain barrier integrity to prevent secondary injury following intracerebral haemorrhage (Huang et al., 2012). Specifically, FGF1 appears to prevent apoptosis and necrotic cell death following hypoxic-ischemic perinatal brain injury (Russell et al., 2006). Following insult, FGF2 levels increase, and may function to increase cell proliferation within the adult hippocampus in response (Yoshimura et al., 2001). Additionally driven long-lasting expression of FGF2 following ischemic

stroke enhances the neurogenic response and results in an improved functional recovery (Leker et al., 2007), further contributing to the 'neuroprotective' function of these FGFs.

2.5.4.2 The Fgf4 Subfamily (Fgf-4, -5 and -6)

The Fgf4 subfamily signals specifically via IIIc isoforms of the FGFRs 1-3 (**Table 2-2**). In the CNS both *Fgf4* and *Fgf5* are expressed, whilst *Fgf6* expression is restricted to embryonic skeletal muscle (Armand et al., 2005; deLapeyrière et al., 1993). Specifically *Fgf5* is expressed in most adult brain regions including the cerebral cortex and hippocampus, located within neurons and thought to influence astrocyte populations by paracrine signalling (Haub et al., 1990). In combination with FGF2, FGF5 *in vivo* influences astrocyte differentiation, which in turn has an impact on blood brain barrier integrity either directly or indirectly (Reuss et al., 2003). *In vivo* studies of glioblastoma tumour samples and astrocytic tumour cell culture also implicate FGF5 as an oncogenic growth factor and malignant progression of such cancers by autocrine and paracrine signalling (Allerstorfer et al., 2008).

The *in vitro* study of FGF4 on embryonic stem cell culture implicate it along with FGF1 and FGF2 (as referred to previously) in neurogenesis (Chen et al., 2010). In has been observed that astrocytes display a potential to de-differentiate into multipotent progenitors, capable of further proliferation and multipotent differentiation into neurons in response to *in vivo* spinal cord injury and *in vitro* injury models (Feng et al., 2014; Lang et al., 2004; Yang et al., 2009).

2.5.4.3 The Fgf7 Subfamily (Fgf-3, -7, -10 and -22)

All members of the Fgf7 subfamily can bind to and signal via IIIb splice isoform of FGFR2, and additionally FGF3 and FGF10 are capable of activating FGFR1-IIIb (**Table 2-2**). The inclusion of FGF3 within this subfamily is a subject of debate, based on chromosomal localisation it is argued FGF3 best fits in the Fgf4 subfamily, or alternatively in a subfamily of its own (Ornitz and Itoh, 2015). During Zebrafish embryonic development, *Fgf3* is expressed alongside *Fgf8* and influences telencephalic gene expression in the forebrain (Walshe and Mason, 2003). In further zebrafish studies, it was shown that *Fgf8* and *Fgf3* expression at mid-hindbrain boundary (MHB) occurs at an earlier time-point then *Fgf22,* and genetic ablation of *Fgf3/8* resulted in complete loss of posterior *Fgf22* expression. This led to the hypothesis that FGF22 is genetically downstream of FGF3/8 signalling at the MHB. Specific analysis of FGF22 action in zebrafish midbrain development showed that it was required for cell proliferation and formation of the roof plate and tectum (Miyake

and Itoh, 2013). However, analysis of *Fgf3*$^{-/-}$ and *Fgf3/Fgf8* double knockout mice indicates that expression of *Fgf3* is not required in rodent forebrain development, therefore this phenomenon is likely restricted to zebrafish (Theil et al., 2008).

Initially, Fgf7 subfamily members Fgf-7, -10, and -22 were implicated as having a role in synaptogenesis (Umemori et al., 2004). However subsequent studies focused on FGF7 and FGF22 only, as *Fgf10*-null mice do not survive past birth. In the developing hippocampus, FGF7 functions in the formation of inhibitory synapses, and as a result *Fgf7*$^{-/-}$ mice are susceptible to epileptic seizures. Similarly, FGF22 has been shown to have a role in the establishment of excitatory synapses, and mice that are *Fgf22*$^{-/-}$ are resistant to epileptic seizures (Terauchi et al., 2010). Further studies on the effects of *Fgf7* deficiency determined that the impaired inhibitory synapse formation enhanced embryonic neurogenesis and abnormal sprouting of mossy fibres which contributes to epilepsy susceptibility (C. H. Lee et al., 2012).

In embryonic development, FGF10 has a crucial role in the formation of the pituitary gland, which functions as directed by the hypothalamus and secreting neuroendocrine factors. In *Fgf10*-knockout mice the pituitary gland is entirely absent (Ohuchi et al., 2000). In embryonic and postnatal development *Fgf10* is highly expressed within the pituitary and hypothalamus of the mouse, and was hypothesised to have a role in both embryonic and postnatal/adult neurogenesis (Hajihosseini et al., 2008). In the chick embryo, *Fgf10* expression demarcates a population of embryonic progenitors that give rise to the infundibulum (also known as the pituitary stalk) (Pearson et al., 2011). In 2017 further research identified that these *Fgf10*$^{+}$ progenitors within the chick hypothalamus are not only retained within the tuberal hypothalamus as non-proliferative cells of the infundibulum but expand along the rostro-caudal axis giving rise to the presumptive hypothalamus (Fu et al., 2017). It is speculated that these embryonic *Fgf10*$^{+}$ progenitors may be retained within the hypothalamus into adulthood, demarcating the population of *Fgf10* expressing neural progenitors within the postnatal β-tanycyte domain (Haan et al., 2013). During early embryonic development of the mouse cerebral cortex transient expression of *Fgf10* has been implicated in regulating the timely differentiation of neuroepithelial cells to radial glia cell, and as a result regulation of the number of basal progenitors and neurons within the cerebral cortex (Sahara and O'Leary, 2009).

2.5.4.4 The Fgf8 Subfamily (Fgf-8, -17 and -18)

The Fgf8 family of canonical paracrine FGFs, specifically activating the IIIc splice isoforms of FGFR1-3 and FGFR4 (**Table 2-2**). Initially expression of FGF8 is observed

within the anterior neuronal ridge (ANR) early in embryonic brain development and is positively upstream of *Fgf17* and *Fgf18* expression. In the embryonic mouse, modification of FGF8 signalling by electroporation mediated gene transfer showed that expansion of anterior *Fgf8* expression into posterior regions resulted in posterior cortical regions taking on a more anterior fate (Fukuchi-Shimogori, 2001a). Similarly, attenuation of FGF8 signalling by the use of hypomorphic mouse mutants results in a shift of transcription factor expression and expansion of posterior neocortex (Garel, 2003). Thus, FGF8 has a role in specification of anterior telencephalon fates, and repression of caudal telencephalic fates. Genetically downstream of *Fgf8*, subfamily member FGF17 has roles in local patterning within the anterior cortex, specifically dorsal frontal cortex (Cholfin and Rubenstein, 2007). Additionally, FGF8 has been shown to have roles alongside FGF17 and FGF18 in patterning and proliferation of the developing cerebellum, displaying some functional redundancy (Xu et al., 2000). During development, expression of *Fgf8* is also essential for cell survival within the mouse embryonic mid-brain and the cerebellum (Chi, 2003) indicating a dual role in addition to patterning.

In humans and rodents the first exon of *Fgf8* is further subdivided into four sub-exons which are alternatively spliced, producing eight potential splice isoforms in mice (FGF8 a-h) (Blunt et al., 1997). Isoform FGF8b has been shown have a role in cell survival. Pre-treatment, co-treatment and even treatment two hours post insult of primary cultured E18 rat hippocampal neurons with FGF8b has been shown to be neuroprotective against oxidative stress (Mark et al., 1999). In studies of cultured E15 cortical cells FGF8b has been shown to promote survival and direct differentiation of cortical precursors into astrocytes. Whilst FGF8a appeared to share the survival effect of FGF8b, the FGF8c splice isoform did not display either (Hajihosseini and Dickson, 1999). Analysis of *Fgf18* knockout mice demonstrate that its signalling is involved in expression of Pea3 subfamily members of the Ets transcription factors in embryonic cortical progenitors, which in turn confers radial migration of cortical neurons in mid-late neurogenesis (Hasegawa et al., 2004).

2.5.4.5 The Fgf9 Subfamily (Fgf-9, -16 and -20)

The Fgf9 subfamily are canonical paracrine Fgfs and all signal via FGFR3-IIIb and the IIIc splice isoforms of FGFR1-3 (**Table 2-2**). Members of the Fgf9 subfamily are widely expressed throughout the CNS. Produced predominantly by neurons, FGF9 is a potent mitogenic and survival enhancing factor in cultures of embryonic motor neurons from the spinal cord (Garces et al., 2000; Todo et al., 1998). Exogenous application of FGF9 to slice cultures of embryonic mouse cortical tissue resulted in premature and

enhanced gliogenesis of oligodendrocytes, and hence may have a role in the neurogenic-gliogenic switch in development (Seuntjens et al., 2009). The knockout of *Fgf9* specifically within the neural tube also showed that FGF9 has a key role in the postnatal migration of granule neurons within the cerebellum, secreted by mature neurons and having a role in the formation of the Bergmann glia scaffold that newborn glial neurons migrate along (Lin et al., 2009).

In the adult mouse CNS, FGF16 has been shown to be expressed in the olfactory bulbs. However, little is known about its function in this region (Fon Tacer et al., 2010). During Zebrafish embryonic development, the knockdown of *Fgf16* resulted in decreased cell proliferation in the forebrain and midbrain, and specifically in the specification of both oligodendrocytes and GABAergic interneurons (Miyake et al., 2014).

In the adult, paracrine signaling by FGF20 has been identified as a potential route to ensure survival of dopaminergic (DA) neurons in Parkinson's. Parkinson's disease is associated with loss of DA neurons within the substantia nigra pars compacta, which in turn play a role in the control of voluntary movement. Within this brain region FGF20 is expressed, and signals in a paracrine manner with FGFR1-IIIc, enhancing the survival of DA neurons *in vivo* and *in vitro* (Murase and McKay, 2006; Ohmachi et al., 2003, 2000).

2.5.4.6 The Endocrine Fgf15/19 Subfamily (Fgf15/19, -21 and -23)

The endocrine FGFs activate FGFRs using members of the Klotho family as a co-factor. They bind HSPGs with a low affinity facilitating release from the confines of the extracellular matrix (Goetz et al., 2007). In the presence of their co-factors FGF15/19 activates the IIIc isoforms of FGFR1-3 specifically, as well as FGFR4. Both FGF21 and FGF23 are able to signal via the IIIc isoforms of FGFR1 and FGFR3, additionally FGF23 can also signal via FGFR4 (**Table 2-2**). The rodent *Fgf15* is considered an ortholog of human *Fgf19*, hence determined as *Fgf15/19* (Ornitz and Itoh, 2015). Studies using *Fgf15*$^{-/-}$ mice have shown that FGF15 has a role in patterning of the embryonic cerebral cortex, and is secreted anteriorly, opposing the gradient of FGF8 signalling and suppressing the specification of anterior telencephalic fates. Furthermore, FGF15 suppresses proliferation and promotes neural differentiation and caudoventral telencephalic fates (Borello et al., 2008).

Under physiological conditions members of the Fgf15/19 subfamily are not expressed within the adult mouse CNS, however due to long-distance endocrine roles may still have functions within the brain and spinal cord (Fon Tacer et al., 2010). Following

demyelination injury of the mouse spinal cord, leakage of circulating FGF21 derived from the pancreas into the CNS by a disrupted blood brain barrier is capable of promoting remyelination and driving proliferation of oligodendrocyte precursor cells in CNS regeneration (Kuroda et al., 2017). A study of humans with varied cardiovascular (CV) risk factors sought to investigate the link between FGF23 and brain integrity. It was shown that elevated levels of FGF23 in high CV risk patients is associated with axonal loss in the frontal lobe (Marebwa et al., 2018). In the gut-brain axis, FGF15/19 produced by the small intestine and released into circulation after a meal can have a direct effect on the central nervous system and hypothalamus, in particular interacting with FGFR1/FGFR4 to illicit its effects in regulation of glucose and energy expenditure (Ryan et al., 2013).

2.5.4.7 The Intracellular Fgf11 Subfamily (Fgf-11, -12, -13 and -14)

The intracellular FGF (iFGF) subfamily are a family of Fgfs that are not secreted, and function independently of the FGFRs. At their N-terminus instead of the classical secretory signal, the iFGFs possess a nuclear localisation motif, and are expressed in both the adult and developing nervous system (Smallwood et al., 1996). It is known that iFGFs are important in the activity of sodium voltage gated channels, and may help regulate the localisation of these channels during development and modulate the initiation/propagation of action potentials (Zhang et al., 2012). In the embryonic nervous system, *Fgf13* is expressed extensively and when applied to embryonic rat neuronal primary culture it specifically increases the GABAergic differentiation of neurons (Greene et al., 1998). Additional studies indicate that FGF13 acts to stabilise microtubules as required for migration and polarisation of cortical neurons into the cortical plate. This was confirmed by knockout of *Fgf13* in mice, and the neuronal migration defects observed in the neocortex and hippocampus resulting in impairments in learning and memory (Wu et al., 2012).

Accumulating research demonstrates FGF14 mutations are a risk factor for complex brain disorders. In addition to having a role in sodium voltage gated channels. Mutations in FGF14 have been attributed to the disorder spinocerebellar ataxia type 27 (SCA27), $Fgf14^{-/-}$ mice show decreased excitatory transmission in the cerebellum (Tempia et al., 2015). In the hippocampus, there is a reduction in the ready-releasable store of glutamate in presynaptic terminals of excitatory neurons, and a reduced expression of GAD67 in interneurons following genetic deletion of *Fgf14*, implicating it in the cognitive impairments associated with schizophrenia (Alshammari et al., 2016). In general, $Fgf14^{-/-}$ mice present with severe cognitive impairment, attributed to a variety of roles for FGF14 in the CNS, not limited to those discussed above.

2.5.5 Summary

In summary, various FGFs have a broad range of roles within both the developing and adult central nervous system, under pathological and physiological conditions. Often in a manner that is dependent on brain region, timepoint of analysis (in development and otherwise) experimental paradigm (*in vitro* or *in vivo*) and model organism. This pleiotropic nature of FGF ligands leads to difficulty in determining the multitude of roles they are likely to function in within the CNS.

2.6 Aims and Hypothesis

In current research there is accumulating evidence that the phenomenon of adult neurogenesis is not restricted to the SVZ and SGZ. Regions such as the amygdala, hypothalamus and cerebral cortex to name but a few have since been shown to possess postnatal/adult neurogenic potential, either under physiological conditions or stimulated by insult. The expression of *Fgf10* in the postnatal/adult CNS as determined by Hajihosseini *et al* 2008 observed that regions where $Fgf10^{+}$ cells were identified overlapped with regions that were speculated as neurogenic, such as the aforementioned. Further research showed that postnatal/adult hypothalamic $Fgf10^{+}$ cells that lined the third ventricle were proliferative, and contributed new-born neurons to the postnatal/adult hypothalamus (Haan et al., 2013).

It is the hypothesis of this project that the *Fgf10* expressing cells observed within the postnatal cerebral cortex also demarcate a population of neural stem/progenitor cells as had been shown in the hypothalamus. This study aims to explore the dynamic of *Fgf10* expressing cells within the cerebral cortex, to:

- Determine when *Fgf10* is first expressed and by which cortical cell types
- Elucidate the function of *Fgf10,* whether in migration, proliferation, differentiation or survival of cells within the cerebral cortex.
- Investigate whether $Fgf10^{+}$ cells within the cerebral cortex represent a population of NSCs/NPCs as observed within the hypothalamus.

3 MATERIALS AND METHODS

3.1 Transgenic Mouse Lines

Unless specified, all mouse lines were bred and maintained as heterozygotes on a mixed C57BL6/129Ola background. Mice were housed under a 12-hour light/dark cycle and raised on a chow diet, according to local regulation for use of transgenic animals, and within terms of a Home Office licence possessed by Dr Mohammad Hajihosseini.

3.1.1 $Fgf10^{CreERT2}$

To generate this transgenic lineage tracing mouse line a tamoxifen-inducible Cre recombinase was inserted into the first exon of the *Fgf10* locus (Cre-ERT2-IRES-YFP) (**Fig 3-1A**) (El Agha et al., 2012). The transgene element renders a copy of *Fgf10* non-functional, thus homozygotes have near absent levels of FGF10 and do not survive past birth. Mice were bred as heterozygotes which were viable and fertile. The CreERT2 construct is only transcribed in *Fgf10* expressing cells. When bred with mice that carry 'floxed' constructs, administration of tamoxifen results in Cre mediated recombination specifically within *Fgf10* expressing cells at the time of administration.

3.1.2 $Fgf10^{flox}$

This mouse line possesses LoxP sites flanking exon 2 of the *Fgf10* allele. Cre-mediated deletion of exon 2 results in an Fgf10-null allele (Abler et al., 2009) (**Fig. 3-1B**).These mice were used to generate triple transgenic $Fgf10^{CreERT2/floxed::Rosa26\text{-}tdTomato}$ lineage tracing mice.

3.1.3 $Rosa26^{tdTomato}$

The Rosa26 locus is widely used as a site for the knock-in of transgenes to achieve constitutive gene expression within all somatic cells of the mouse (Soriano, 1999). In this case, the ubiquitous Rosa26 locus contains a floxed-Stop-casette controlled fluorescent marker gene (tdTomato) (Madisen et al., 2010) (**Fig. 3-1C**). Crossing mice carrying the $Rosa26^{tdTomato}$ mice with $Fgf10^{CreERT2}$ allows for lineage tracing of *Fgf10* expressing cells by inducing constitutive expression of tdTomato following tamoxifen administration as a double transgenic model.

3.1.4 $Fgf10^{nLacZ}$

The Fgf10-LacZ reporter mouse strain was generated by insertion of the nuclear targeted LacZ transgene (*Mlc1v-nlacZ-24*) 114kb upstream of the Fgf10 transcriptional start site (Kelly et al., 2001)(**Fig. 3-1D**). The Fgf10-LacZ mouse is a slight hypomorph of Fgf10 despite this insertion not disrupting translation of the Fgf10

gene, for this reason mice were bred and maintained as heterozygote, despite homozygotes also being viable (Mailleux et al., 2005). Expression of the β-Galactosidase, the product of the LacZ gene closely matches *Fgf10* expression patterns and can be detected by immunohistochemistry or staining using 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal).

3.1.5 NPY^{rGFP}

Neurons that contain neuropeptide Y (NPY) are found throughout the brain. Within the hypothalamus NPY expressing cells delineate a population of orexigenic neurons, which release NPY as an anabolic signalling molecule to increase food intake (Gehlert, 1999; Glenn Stanley et al., 1986). Within the cerebral cortex NPY is expressed within a population of GABAergic interneurons, found widely distributed throughout the cerebral cortex but more concentrated within layers II/III and VI (Hendry et al., 1984; Karagiannis et al., 2009). The NPY^{rGFP} mice express humanized *Renilla* GFP under the control of the neuropeptide Y (NPY) promoter, deleting the first 41bp of the NPY coding sequence. Patterns of GFP fluorescence are consistent with *NPY* gene expression. Obtained from JAX stock #006417 (van den Pol et al., 2009)(**Fig. 3-1E**).

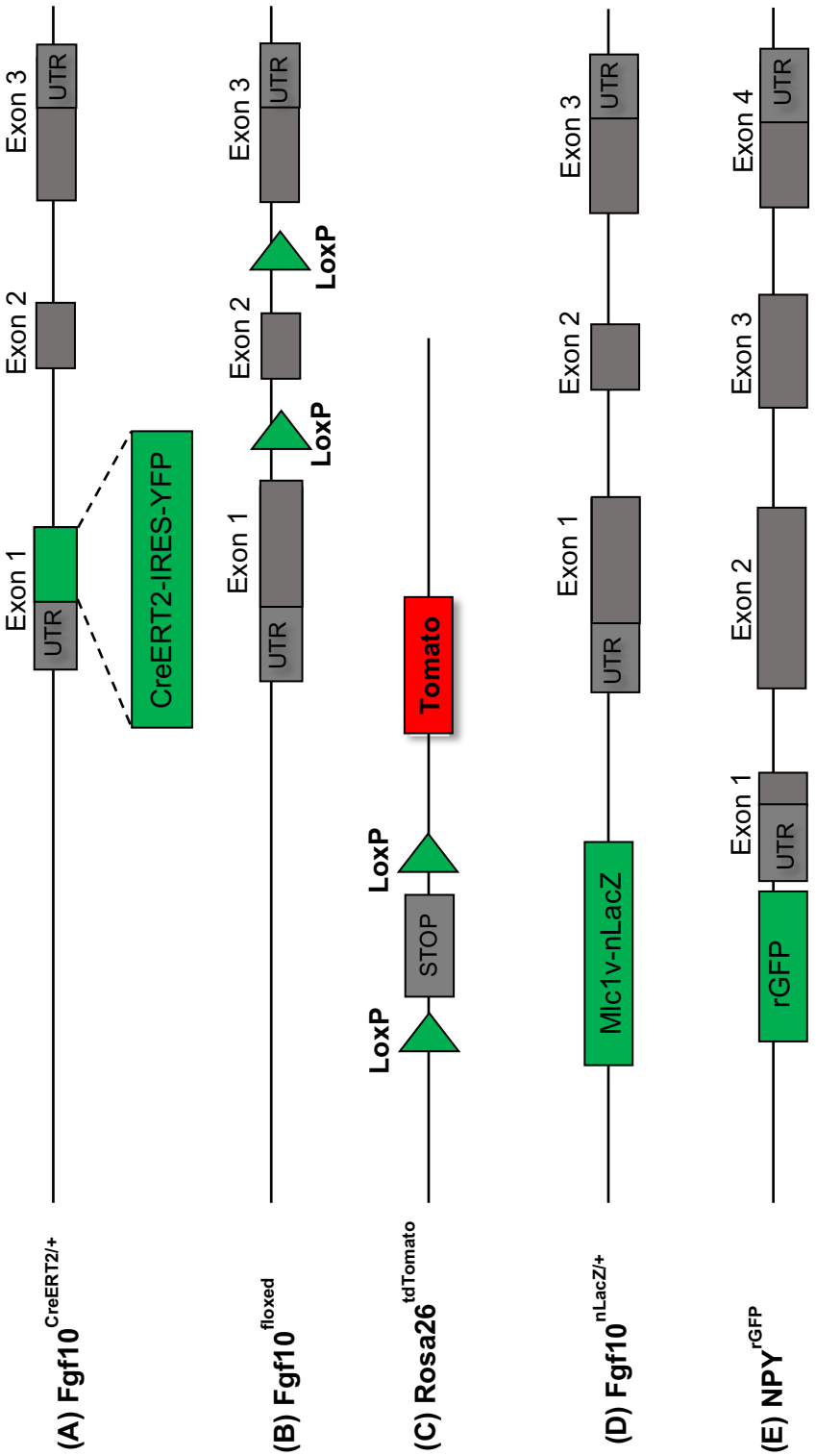


Figure 3-1 Transgenic Mouse Alleles: Schematic representations of the transgenic mouse alleles used in single, double and triple transgenic models for lineage tracing experiments highlighting the transgenic elements inserted into the alleles.

3.2 Genotyping

For DNA genotyping, ear biopsies were harvested and digested overnight at 55°C in 100µl of Tail Digestion buffer (0.1M Tris Base, 5mM EDTA, 0.2M NaCl, 0.2% w/v SDS in ddH_2O, pH 8) containing 7µg/ml Proteinase K (Sigma). Genomic DNA was then precipitated using an equal volume of isopropanol. The pellet of DNA was re-suspended in a 30% solution of TE buffer made up in ddH_20 and incubated overnight at 37°C to allow the pellet to return into solution.

The DNA samples were subject to Expand Long Template PCR (Roche) using gene specific primers (**Table 3-1**), subject to conditions specified in **Table 3-2**.

Table 3-1 Primers for genotyping of transgenic mouse lines: The allele being tested, corresponding primers and product size in Kb visualised in subsequent agarose gel electrophoresis of genotyping PCR product.

Allele	Primer Sequences (5'-3')	Product size (Kb)
Fgf10$^{CreERT/+}$	AGC AGG TCT TAC CCT TCC AGT ATG TTC C	CreERT2: 0.5
	TCC ATG AGT GAA CGA ACC TGG TCG	
	AGC AGG TCT TAC CCT TCC AGT ATG TTC C	WT: 0.3
	CTC CTT GGA GGT GAT TGT AGC TCC G	
Fgf10^{Floxed}	GAG GCA GGA TAA CCA GTA TCT GG	WT: 1.8 Floxed: 1.2
	GAA ATT GCA GAG ATT GCA AAG GAA GC	
Rosa26^{tomato}	CTG TTC CTG TAC GGC ATG G	Tomato: 0.2
	GGC ATT AAA GCA GCG TAT CC	
	AAG GGA GCT GCA GTG GAG TA	WT: 0.3
	CCG AAA ATC TGT GGG AAG TC	
NPY-GFP	TATGTGGACGGGGCAGAAGATCCAGG	GFP: 0.4 WT: 0.5
	CCCAGCTCACATATTTATCTAGAG	
	GGTGCGGTTGCCGTACTGGA	
Fgf10$^{nLacZ/+}$	GCATCGAGCTGGGTAATAAGCGTTGGCAAT	LacZ: 0.8
	GACACCGACACAACTGGTAATGGTAGCGAC	
	CGAGTGGAGCATGTACTTCCGTGTCCTGAA	WT: 0.5
	TCCCTACCCAGTCACAGTCACAGCTGCATA	

Table 3-2 PCR conditions for transgenic mouse genotyping: The PCR cycles utilised in genotyping PCR reactions to detect transgenic alleles.

Allele	Temperature (°C)	Time (min:sec)	Number of cycles
$Fgf10^{nLacZ/+}$	94	2	x1
	94	0:30	x15
	62	0:45	
	68	1	
	94	0:30	x17
	62	0:30	
	68	1(+0:40/cycle)	
	68	7	x1
	6	15<	x1
$Fgf10^{CreERT2/+}$ and NPY-GFP	94	2	x1
	94	0:30	x9
	61	0:30	
	68	2:40	
	94	0:30	x17
	61	0:30	
	68	2:40 (+0:40/cycle)	
	68	7	x1
	6	15<	x1
$Fgf10^{Floxed}$	94	2	x1
	94	0:30	x10
	61	0:30	
	68	3:10	
	94	0:30	x16
	61	0:30	
	68	3:10(+0:30/cycle)	
	68	7	x1
	6	15	x1
$Rosa26^{Tomato}$	94	2	x1
	94	0:30	x15
	60	0:30	
	68	1:10	
	94	0:30	x17
	60	0:30	
	68	1:10(+0:20/cycle)	
	68	7:0	1
	6	15	x1

3.3 Animal Treatments

3.3.1 Tamoxifen Administration

To make up a 20mg/ml stock solution 0.3g of Tamoxifen (Sigma) was diluted into 1.5ml of ethanol, made up to 15ml with corn oil (Mazola). The resulting solution was vortexed for 5 minutes and incubated with shaking for 5 hours at 55°C until tamoxifen went into solution. Aliquots of tamoxifen solution were stored at -20°C and protected from light. For P4 and P5 tamoxifen pulsing pups were administered 100μg of tamoxifen stock solution by suckling.

For P27-28 pulsing young adult mice received intraperitoneal (IP) injections of tamoxifen at 200mg/kg body weight once daily for two days. Adult 12-month old mice were administered tamoxifen by oral gavage once daily for 4 days at a dose of 100mg/kg, followed by a 7-day chase before tissue harvest. Pregnant dams were tamoxifen pulsed at approximate days of gestation by gavage, receiving a daily dose of 100mg/ml tamoxifen over the course of one or two days.

3.3.2 Bromodeoxyuridine (BrdU) Administration

Bromodeoxyuridine (BrdU) is a synthetic thymidine analogue which is incorporated into the DNA of dividing cells during S-phase of the cell cycle. BrdU (sigma) was dissolved in alkaline PBS (0.7ml of 0.1M NaOH per 10ml of PBS). Pups received one dose of 50mg/kg twice daily at postnatal ages P8, P9 and P10 by intraperitoneal injection.

3.4 Tissue Processing

For immunohistochemical analysis, animals were schedule 1 culled and perfused transcardially with a solution containing 4% Paraformaldehyde (Sigma) in 1X PBS (pH Adjusted to 7.4 with NaOH pellets). The brain was dissected from the skull and fixed overnight at 4°C in 4% Paraformaldehyde (PFA) or 2% PFA solution depending on the primary antibody to be used.

For cryostat sections, post-fixed brains were washed in PBS and transferred into a solution of 30% sucrose for cryoprotection for 2 days at 4°C. Tissue was then embedded and orientated in optimal cutting temperature compound (OCT) (Tissue Tek) on dry ice and stored at -80°C. For tissue sectioning using the vibratome, brains were removed from PFA post-fix and dehydrated in a graded series into absolute ethanol (30%, 50%, 70% 100% EtOH), allowing 1 hour for each wash, and being stored at 4°C until rehydrated for sectioning, with tissue destined for Gad67

immunolabelling being the exception, and not EtOH dehydrated prior to sectioning.

For X-Gal staining ($Fgf10^{nLacZ}$), embryos and pups were decapitated, brains dissected and fixed for 2 hours in 4% PFA at 4°C. A X-Gal stock solution consisted of 20mg/ml X-Gal (Melford) in dimethylformamide. From this stock a 0.5ug/ml X-Gal working solution was made up in X-Gal buffer (2mM $MgCl_2$, 5mM $K_4Fe(CN)_6$, 5mM $K_3Fe(CN)_6$ in PBS). Tissue was incubated in X-Gal working solution whilst rotating at 37°C for 5 hours minimum or over overnight. Following this, tissue was post-fixed in 4% PFA for 3 hours at 4°C and processed for either cryostat or vibratome sectioning.

3.5 Tissue Sectioning and Orientation

For vibratome sectioning brains were rehydrated in a graded series from ethanol into PBS (100%, 70%, 50%,30% EtOH). Subsequently tissue was incubated in 3% agar dissolved in ddH_2O for 30 minutes and set in the desired orientation for coronal sectioning. Using a vibrating microtome (Leica), 60µm serial sections were collected and stored at 4°C in 48 well plates filled with PBS until processing. Cryostat sections were obtained using a freezing microtome (Microm HM560). Sections of 60µm were collected and stored free floating in 48 well plates containing PBS at 4°C. For 20µm sections, brain slices were collected 6 per slide and stored at -80°C. In was important for each individual section to be collected serially, so that the regions of cerebral cortex could be determined. The coordinates of each section in mm from bregma was evaluated by comparing morphological characteristics to a stereotaxic atlas. Bregma point 0 being a reproducible point on the skull where the coronal and sagittal sutures meet.

3.6 Immunohistochemistry

The details of primary and secondary antibodies used in this study can be found in **Table 3-3** and **Table 3-4.**

3.6.1 Pre-treatments and antigen retrieval

3.6.1.1 Hydrochloric acid treatment

Prior to BrdU immunolabeling, sections were incubated with 1M HCl at 47°C, or 2M HCl at 37°C. Different incubation times were trialled to determine the optimal for BrdU detection, which was determined as 30 minutes in 1M HCl at 47°C. After incubation sections were washed twice in ddH_2O to remove excess HCl and returned to PBS.

3.6.1.2 Citrate antigen retrieval

Sections were incubated in a citrate retrieval buffer (10mM tri-sodium citrate, 0.05% Tween 20, pH 6.0) for 15 minutes at room temperature. Following this, pre-warmed 70°C citrate buffer was applied, and sections further incubated for 15minutes at 70°C. Once allowed to cool, sections were washed 3 times in PBS for 5 minutes.

3.6.1.3 Acid alcohol treatment

Sections were incubated on slides in a solution of ethanol with 5% glacial acetic acid pre-cooled to -20°C for 20 minutes, covered in parafilm to ensure sections did not dry out. Following the treatment, slides were washed 4 times in PBS, each wash being 10 minutes.

3.6.1.4 Blocking of endogenous biotin and avidin

For experiments where Biotin-Streptavidin signal amplification was required, it was necessary to block any endogenous biotin/avidin binding sites to reduce background signal. This was achieved by use of the Endogenous Avidin/Biotin Blocking Kit (Abcam), immediately after the serum blocking step (two-hour incubation in 20% normal goat serum and 1% Triton X-100). Briefly, sections were incubated at room temperature (RT) for 15 minutes with the avidin blocking solution and washed three times in PBS with each wash being 2 minutes. Sections were then incubated for 15 minutes at RT in the biotin blocking solution, washed 3 times in PBS for 2 minutes, after which the sections were incubated in primary antibody.

3.6.2 Immunohistochemistry of cryostat sections.

For immunolabeling, unless otherwise stated, all solutions were made up in 1X PBS. Following any required pre-treatments specified in **Table 3-3**, non-specific binding sites were blocked in a solution comprised of 5% normal goat serum (NGS) 2.5% Bovine serum albumin (BSA) and 1% Triton X-100 to permeabilise the section at room temperature for 2 hours. Primary antibodies were applied overnight in 5% NGS, 2.5% BSA and 0.1% Triton X-100 at 4°C. The following day, sections were washed 5 times, each wash being an hour in block solution. The relevant secondary antibodies were applied overnight at 4°C in a solution made up of 5% NGS, 2.5% BSA and 0.5% NP-40.

3.6.3 Immunohistochemistry of vibratome sections

Non-specific binding was blocked by treatment with 20% NGS and 1% Triton for 2 hours at room temperature. Sections were incubated in primary antibody overnight at 4°C diluted in a solution of 0.2% NGS and 0.1% Triton X-100. Any excess primary

antibody was washed off by five 1-hour washes in 0.2% NGS and 0.1% Triton X-100 before incubating the sections in secondary antibody overnight at 4°C in a 0.5% NP-40 and 0.2% NGS.

3.6.4 Fluorescent conjugated secondary antibody

Where a fluorescence conjugated antibody was used, sections were washed 6 times in PBS with each wash being 30 minutes. Nuclei were stained using 5µg/ml Hoechst (Invitrogen) for 10 minutes, and sections mounted for imaging in Vectashield (Vector Laboratories).

3.6.5 Biotinylated secondary antibody

Where biotinylated secondary antibodies were used, after overnight incubation with secondary antibody sections were washed 5 times in 0.1% Triton X-100, 2.5% BSA and 5% NGS, with each wash being an hour. Sections were incubated in wash solution containing Streptavidin conjugated to AMCA or Cy2 overnight at 4°C. To remove any excess streptavidin, the sections were subject to six 30 minutes washes in PBS and counterstained in Hoechst. Due to incompatibles of Cy2 with phenylenediamine (contained within Vectashield) any Strep-Cy2 treated sections were mounted in Fluoroshield (Sigma). Any Strep-AMCA treated sections were mounted in Vectashield as previously.

Table 3-3 Primary Antibodies. Primary antibodies utilised in varying combinations for immunohistochemistry reactions on both cryostat and vibratome sections. Optimal concentrations determined by optimisation studies.

Antibody	Host	Manufacturer	Catalogue Number	Tissue Processing	Pre-treatment	Citrate retrieval	Concentration	Biotin/Strep
Immunolabelling of cryostat sections								
Tomato-dsred	Rabbit	Clontech	632496	Perfused	N/A	-	1:1000	--
TBR1	Rabbit	Abcam	AB18465	Perfused	N/A	Yes	1:300	-
CTIP2	Rat	Abcam	AB18465	Perfused	N/A	Yes	1:300	Biotin
PCNA	Mouse	Millipore	MAB424R	Perfused	Acid/EtOH	-	1:200	Biotin
Ascl1	Mouse	Santa Cruz	Sc-374104	Perfused	-	-	1:100	-
Beta-Galactosidase	Chick	Abcam	Ab-9361	Perfused	-	-	1:1000	-
TBR2	Rabbit	Abcam	ab23345	Perfused	-	Yes	1:300	-
Immunolabelling of vibratome sections								
Tomato-dsred	Rabbit	Clontech	632496	Perfused	N/A	-	1:1000	-
GFAP	Mouse	Millipore	MAB360	Perfused	N/A	-	1:1000	-
NeuN	Mouse	Millipore	MAB377	Perfused	N/A	-	1:1000	-
BrdU	Mouse	Sigma	B8434	Perfused	HCl		1:500	-
Gad67	Mouse	Millipore	MAB5406	Perfused	Acid/EtOH	-	1:300	-

Table 3-4 Secondary antibodies and conjugate: Details of the secondary antibodies and conjugates utilised in dilutions as determined by optimisation studies.

Host	Target	Manufacturer	Conjugate	Catalogue Number	Working dilution
Goat	Rabbit	Abcam	Alexa 568	AB175471	1:1000
Goat	Rabbit	Invitrogen	Alexa 488	A11008	1:1000
Goat	Mouse	Invitrogen	Alexa 488	A11001	1:1000
Goat	Mouse	Stratech	IgG2a-Biotin	115-065-206	1:1000
Goat	Mouse	Sigma	HRP	A4416	1:1000
Goat	Rat	Thermofisher Scientific	Biotin	31830	1:600
-	-	Dianova	Streptavadin-Cy2	016-070-084	1:500
-	-	Dianova	Streptavadin-AMCA	016-150-084	1:1000
-	-	Invitrogen	Hoechst 33342	H3570	1:1000

3.7 Microscopy

Fluorescently labelled sections were imaged on a Zeiss Axioimager M2 with apotome attachment which allowed for optical sectioning, three-dimensional reconstruction, and interrogation of double-labelling with cut view analysis. When taking Z-stack slices the thickness of optical sections was dependent on the objective used but ranged between 0.5µm and 2µm, imaging the entire 60µm of the section. For the purpose of imaging X-Gal treated sections an upright light microscope (Zeiss) was used.

All images were acquired using Axiovision 4.8 software and post-processed using FIJI (ImageJ). Z-stack images were projected into 2D, adjusted in terms of brightness, contrast and scale bars added. Double-labelling of immunohistochemical markers was interrogated within single optical sections with split channels.

3.8 Quantification and Statistical analysis

For purpose of lineage tracing Tomato$^+$ cells were counted along the rostro-caudal axis of the cerebral cortex, with the exception of the paleocortex and archicortex. Every section containing cortex was analysed amounting to approximately 80-100 sections per brain. Tomato$^+$ cells were categorised according to their approximate laminar distribution, bregma region, and any double labelling with additional markers. Compiling of Z-stacks and maintaining the rostro-caudal order of sections ensured no Tomato$^+$ cells were double counted. Given the sparse distribution of Tomato+ cells

within the cerebral cortex any cells within 70μM of each other were determined part of a pair/cluster.

All graphs were generated using excel and statistical analysis using SPSS (IBM). When comparing two means, unpaired t-tests were used, where comparisons between multiple means were required one-way ANOVA was used and suitable post-hoc analysis determined by whether equal variances could be assumed.

3.9 *Ex vivo* slice culture

The outlined slice culture technique was optimised by Stuart Nayar in our laboratory, and subsequently adapted for the cerebral cortex. Pups used for slice culture were *Fgf10*$^{CreERT2/floxed}$*::* *Rosa26*$^{LoxP\text{-}STOP\text{-}LoxP\text{-}Tomato\text{-}dsred}$ (Fgf10$^{CreRT2/floxed}$), as confirmed by genotyping and identification of tdTomato fluorescent marker expression in the hair follicles. As previously, animals used in slice culture studies were pulsed with tamoxifen P4-P5, and schedule 1 culled for analysis at P7 based on optimum survival of sections as determined by Stuart Nayar.

3.9.1 Buffers and culture media

3.9.1.1 Dissection buffer (500ml)

The dissection buffer consisted of; 120mM NaCl; 5mM KCl; 2mM $CaCl_2 2H_2O$; 1mM $MgCl_2.6H_2O$; 1mM NaH_2PO_4; 1mM $NaHCO_3$; 26mM HEPES. The HEPES was allowed to dissolve and pH adjusted to 7.31 with NaOH. The buffer was then supplemented with; 11mM Glucose; 2.5mM myo-Inositol (Sigma); 10ml BME Amino acids (Sigma); 5ml Glutamax-100 (Gibco); 0.5g BSA (Fract. V, cell culture tested) (Fisher); 1ml Penicillin/Streptomycin (Gibco) and made up to 500ml with ddH_2O. The final solution was then filter sterilised using a Corning filter system.

3.9.1.2 Cell Culture media (filter sterilised)

The cell culture media comprised of; 50ml of MEM with HEPES and Glutamine (Gibco) supplemented with glucose (5.6mM)**;** 1ml of Penicillin/Streptomycin (Gibco)**;** 25mL of heat inactivated horse serum (Gibco)**;** 25mL of Hanks Balanced Salt Solution (HBSS) (Sigma). The resulting media was filter sterilised before use.

3.9.1.3 Low melting point agarose

The slice culture tissue was set and orientated within LMP agarose, made up of 1.5g LMP agarose (Thermofisher) dissolved in 50ml dissection medium. Aliquots were stored at 4°C until needed.

3.9.2 Slice culture of cortical sections

3.9.2.1 Dissection and tissue sectioning

Prior to dissection aliquots of LMP agarose were heated to 70°C. Animals were sacrificed by cervical dislocation, brains removed from the skull and placed into ice cold dissection medium. Hair follicles were examined for evidence of tdTomato expression, and ear clips obtained to confirm the genotype. The olfactory bulbs and cerebellum were removed, and meninges dissected. The resulting tissue was oriented and set LMP agarose on ice. Once set, the live brain tissue was sectioned using the vibrating microtome (Lecia), into 180µm coronal sections, stored for 1 hour on ice cold dissection medium.

3.9.2.2 Culturing of slices

Sections were orientated in an 0.04µm pore cell culture insert (Millicell), then placed into a 6 well plate filled with pre-warmed culture medium. Sections were incubated at 37°C and 5% CO_2 overnight, with media replaced every two days.

3.9.3 Microscopy- Imaging of slices

Live Tomato$^+$ cells within the cerebral cortex were imaged using a Zeiss Axiovert microscope using a x10 0.45NA objective. Inserts containing slices were removed from culture media and transferred into wells containing pre-warmed dissection media (37°C). Five images were then taken at three-hour intervals daily between 9am and 9pm for three days. Images were edited using deconvolution software on Axiovision.

3.10 Reverse Transcriptase PCR

3.10.1 Tissue dissection

Wild-type C5Bl6 mice were sacrificed by cervical dislocation, the brains were removed from the skull and cerebral cortex dissected. Care was taken to remove as much meningeal tissue as possible to avoid contamination of the sample. Tissue was snap-frozen in liquid nitrogen into RNAse free cryotubes. Tissue was stored at -80°C until required for RNA extraction.

3.10.2 RNA extraction

Frozen cerebral cortex tissue was thawed and homogenized in 1ml of Trizol by trituration with a P1000 pipette, and incubated for 5 minutes at RT. To this solution, 200µl of chloroform was added, and the sample shaken vigorously for 20 seconds, before incubating for a further 3 minutes at RT.

In order to separate the upper aqueous phase, the sample was centrifuged for 15minutes at 12000rpm at 4°C. The upper aqueous phase was removed, and RNA precipitated by adding 500ul of isopropanol (per 1ml of Trizol used) and incubating at RT for 10 minutes.

The sample was then centrifuged again to pellet the RNA for 15 minutes at 12000rpm at 4°C. The RNA pellet was washed in 1ml of 75% ethanol, by vortexing at 4°C for 5 minutes. To re-pellet the RNA the sample was centrifuged for 10 minutes at 9000rpm. Once the pellet is reformed the supernatant is removed and the pellet re-suspended in DPEC-treated water. To ensure RNA was fully dissolved the sample was further incubated for 10 minutes at 55°C. Finally, to assess the quantity and quality of RNA obtained the sample was determined using a Nanodrop spectrophotometer.

3.10.3 Reverse transcriptase PCR reaction

For RT PCR analysis Illustra Ready-to-go RT-PCR beads (GE Healthsciences) were used according to manufacturer's instructions. This was a two-step RT-PCR process and used 1µg of template RNA. Respective primers used are detailed in **Table 3 -5,** designed across exons, and not considering intronic regions. Gene specific primers were added to the reaction mixture following the reverse transcriptase cycle (first strand synthesis). Cycle information is detailed in **Table 3-6**. The PCR product was resolved on a 1% w/v agarose gel and visualised with ethidium bromide. The gene Beta-actin was used as a positive control.

Table 3-5 Gene specific primers for RT-PCR. The primers and corresponding product sizes for detection of *Fgf*-receptors and ligand mRNA expression.

Fgf receptor/ligand	Primers (5'-3')	Product size (bp)
FgfR1-IIIb	GCA GGG CTG CCT GCC AAC GAG ACA GTG	300
	GGT CTG GTG CAG TGA GCC ACG CAG ACT G	
FgfR1-IIIc	GCA GGG CTG CCT GCC AAC GAG ACA GTG	306
	GAA CGG TCA ACC ATG CAG AGT GAT GG	
FgfR2-IIIb	CCC ATC CTC CAA GCT GGA CTG CCT	317
	CTG TTT GGG CAG GAC AGT GAG CCA	
FgfR2-IIIc	CCC ATC CTC CAA GCT GGA CTG CCT	310
	CAG AAC TGT CAA CAA TGC AGA GTG	
FgfR3-IIIb	GAC AGA CAC ACG GAT GTG CTG GA	350
	GTG AAC ACG CAG CAA AAG GCT TT	
FgfR3-IIIc	GAC AGA CAC ACG GAT GTG CTG GA	348
	AGC ACC ACC AGC CAC GCA GAG	
FgfR4	TAC AGC TAT CTC CTG GAT GTG CTG	195
	GAA ACC GTC GGC GCC GAA GCT GCT	
Fgf-3	GAT GGG CCT GAT CTG GCT TCT GCT	573
	CAC CAT CTC ATG GTC CTT GTG GCC	
Fgf-7	ATC CTG CCG ACT CCG CTC TA	487
	CCT TTT GAT TTA AGG CCA CGA ACA	
Fgf-10	CTC TTT TTG GTG TCT TCG TTC CC	228
	CGC TGA CCT TGC CGT TCT TCT C	
Fgf-22	GTG GGC GTC GTG GTC ATC AAA GCA G	256 and 233
	GCA GGA AGT GGG CGG ACA GGT GG	
Beta-Actin	GAA ATC GTG CGT GAC ATT AAG GAG	450
	ATA CTC CTG CTT GCT GAT CCA CAT	

Table 3-6 RT PCR cycle information: The cycles utilised in the two-step reverse-transcriptase PCR detection of specific Fgf and Fgfr mRNAs.

Stage	Temperature (°C)	Time (min:sec)	Repeat
First strand synthesis (+1ug RNA and Oligo $(dT)_{12-18}$ primers (Invitrogen))	95	5.0	x1
	42	15:0	
	6	5:0	
RT-PCR + 1.5pmol of forward and reverse primers (gene specific)	95	0:30	x1
	60	0.30	x34
	68	1:0	
	6	5	x1

4 DISTRIBUTION AND IDENTITY OF *FGF10* EXPRESSING CELLS IN THE POSTNATAL CEREBRAL CORTEX

4.1 Introduction

Increasing evidence points to the existence of non-canonical neurogenic niches within the mammalian CNS, such as within the cerebral cortex. However, much conflict remains. In a recent review variation in experimental paradigms and sensitivity of the techniques used to identify neural stem/progenitor cells were identified as a potential reason for this discrepancy (Gould, 2007). Two studies using rats as a model organism identified new-born neurons within the adult neocortex, both utilising either BrdU or H^3-thymidine as methods to detect proliferation and NeuN immunolabelling or electron microscopy to identify new-born cortical neurons. Research determined that the level of adult neurogenesis in the rodent neocortex to be extremely low, amounting 0.011% of total neurons (Dayer et al., 2005; Kaplan, 1981). Although it cannot be directly extrapolated to human adult neurogenesis, when considering ^{14}C incorporation from nuclear bomb testing it is not so surprising that there was no evidence of adult human neurogenesis within the neocortex, due to the low sensitivity of this method (approximately 1%). Analysis of macaques using BrdU pulsing methods showed that new-born cortical neurons may be transient in their existence, and therefore only detectable within small window, further contributing to the difficulties in establishing the phenomena of adult cortical neurogenesis (Bhardwaj et al., 2006).

Previous research from our laboratory indicated that *Fgf10* expressing cells can be found in discrete regions within the adult rodent CNS, overlapping with those proposed to be non-canonical neural stem cell niches. Such regions included the hypothalamus, the amygdala, cerebellum and the cerebral cortex to name but a few (Hajihosseini et al., 2008). It was hypothesised that *Fgf10* expression may delineate populations of neural stem/progenitor cells within the postnatal mouse CNS.

In subsequent research, focused on the *Fgf10* expressing population within the hypothalamus, evidence was gathered that *Fgf10* expressing cells generate neurons postnatally, that contribute to the hypothalamic parenchyma (Haan et al., 2013). By use of the *Fgf10*^{nLacZ} mouse model, it was discovered that cells derived from the *Fgf10* expressing lineage are largely restricted to the β-tanycyte domain of the 3rd ventricle and the hypothalamic parenchyma. Specifically, the nLacZ$^+$ β-tanycytes expressed markers of neural stem/progenitor cells such as nestin and sox2, a proportion of which also incorporated BrdU and were hence actively dividing within the postnatal mouse hypothalamus. Postnatally, cells derived from an *Fgf10* expressing lineage gave rise to neurons that incorporated into energy balance and appetite regulating circuitry. In conclusion it was determined that cells derived from a *Fgf10* expressing population of

β-tanycytes demarcate a lineage with neurogenic capacity in the postnatal/adult mouse hypothalamus.

Within the adult CNS FGF10 has been implicated in roles such as in synaptogenesis and dendritogenesis and (Huang et al., 2017; Umemori et al., 2004). The latter of which, within the somatosensory cortex attributes the action of FGF10 to paracrine interactions with its receptors FGFR1-IIIb and FGFR2-IIIb. The hypothesised potential of FGF10 to function in postnatal neurogenesis within the cerebral cortex has yet to be explored. Given the previous research into the *Fgf10*-expressing population within the postnatal/adult hypothalamus, this study seeks to determine whether cells derived from a *Fgf10* expressing lineage also demarcate a population of postnatal cortical neural stem/progenitors.

4.2 Aims

To elucidate the expression pattern of *Fgf10* within the postnatal cerebral cortex by use of the double transgenic *Fgf10*$^{CreERT2/+::Rosa26tdTomato}$ mouse. Determine the distribution, abundance and identity of *Fgf10* expressing cells and their descendants by immunohistochemistry. Gathering evidence as to the potential function of *Fgf10* is in this postnatal lineage, and whether its expression demarcates a population of neural stem/progenitor cells as had been observed in the hypothalamus.

4.3 Results

4.3.1 *Fgf10* Is expressed in the postnatal cerebral cortex in the absence of its specific paracrine receptors

RT-PCR was conducted on RNA isolated from the P4 mouse cerebral cortex, in order to determine expression of the FGF-7 subfamily and FGF-receptors (FGFRs) at this age. The FGFRs 1-3 come in two splice variants of an immunoglobulin like domain III, resulting in IIIb and IIIc isoforms of the receptors, determining ligand binding specificity. Fibroblast growth factor-10 is only able to signal via FGFR2-IIIb and to a lesser extent FGFR1-IIIb, as determined by structured modelling using a range of cell lines such as BaF3 (Zhang et al., 2006) and direct binding studies (Ornitz and Itoh, 2015). It was found that the specific FGFRs that FGF10 exclusively signals via are not expressed within the cerebral cortex at P4 (**Fig. 4-1A**).

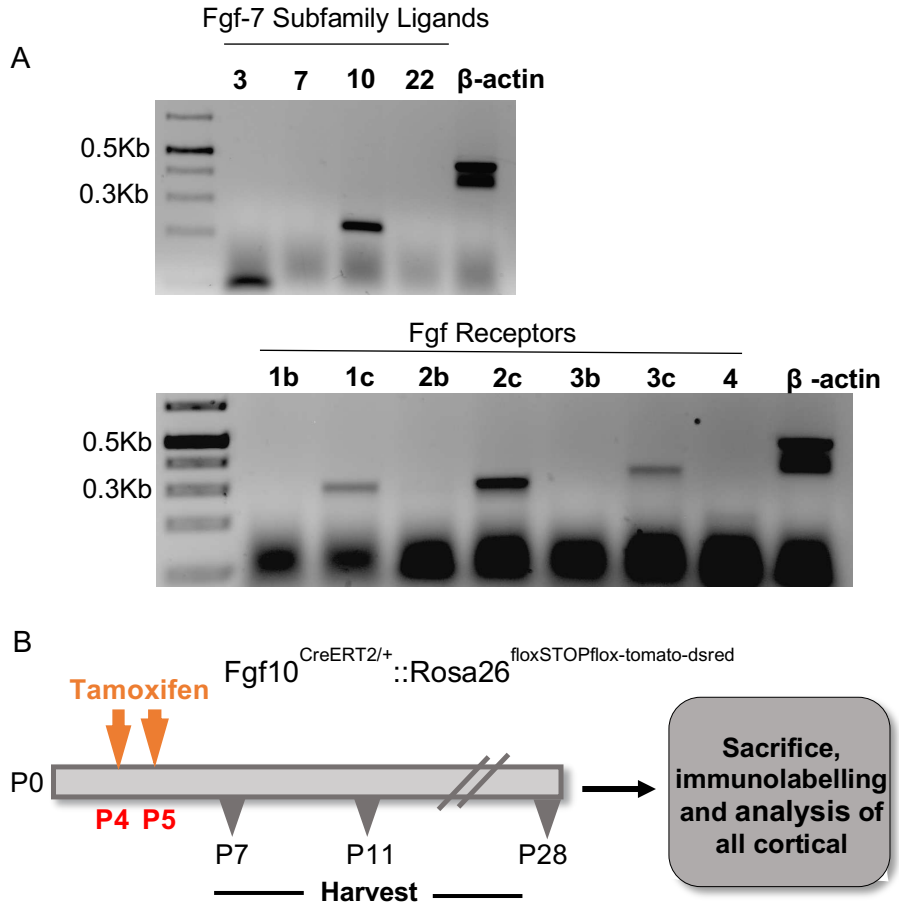


Figure 4-1 RT-PCR analysis of P4 wild-type cerebral cortex and tamoxifen regime: The P4 cerebral cortex exhibits expression of *Fgf10* at P4, and no expression of the *Fgfr1/2-IIIb* isoforms through which FGF10 functions in a paracrine manner (A). The subsequent tamoxifen pulsing regime for lineage tracing of P4-5 cortical *Fgf10*-expressing cells, as determined through analysis of hypothalamus tissue in parallel study (B).

4.3.2 Lineage tracing of P4-5 *Fgf10* expressing cells using Fgf10$^{CreERT2/+::Rosa26LoxP-STOP-LoxP-Tomato-dsred}$ mice

In order to understand where and by what cells within the cerebral cortex *Fgf10* is expressed, the CreERT2/tamoxifen system was utilised to trace the fate of this population. Double transgenic *Fgf10*$^{CreERT2/+::Rosa26LoxP-STOP-LoxP-tdTomato}$ (herein referred to as Fgf10$^{CreERT2/+)}$ were pulsed with tamoxifen at P4 and P5 by suckling. The CreERT2 construct is knocked-into exon 1 of the *Fgf10* locus, rendering one copy of *Fgf10* non-functional. Tamoxifen enables the CreERT2 protein access to the nucleus of *Fgf10* expressing cells, wherein it excises the floxed stop codon on the *Rosa26* allele, resulting in constitutive *tdTomato* (Tom) expression. The fluorescent Tom protein is expressed by all *Fgf10*$^{+}$ cells at the time of tamoxifen pulsing, and descendants thereafter. Brains were fixed and harvested at P7, P11 and P28, and immunolabelled for markers of neurons (NeuN) and astrocytes (GFAP) to lineage trace and characterise any descendants of a P4-5 *Fgf10* expressing population (**Figure 4-1B**).

Total cortical Tom^{+} cells were counted across all sections containing cerebral cortex, resulting in 87-100 60µm sections to analyse per brain. Using the mouse brain atlas and anatomical characteristics the rostro-caudal sequence of sections was maintained. Cortical Tom^{+} cells were quantified along the rostro-caudal axis, in terms of the approximate laminar distribution and NeuN double-labelling, with morphological observations to determine differentiation potential of the speculative *Fgf10* expressing progenitors.

4.3.2.1 Morphological observations of Tom^{+} cells in the cerebral cortex

At P7, Tom^{+} cells appear immature but neuronal in morphology, with poorly defined and sparse processes lacking the complexity observed in mature neurons (Rotheneichner et al., 2018) (**Fig 4-2A-B**). At P11 Tom^{+} cells acquire a more mature neuronal morphology, with clear axonal and dendritic projections; Tom^{+} cells with a glial morphology are also observed with blood-vessel association (**Fig 4-2C-D**). At P28 Tom^{+} cells with an immature morphology were still observed, often as closely associated pairs, potentially reminiscent of recent cell division or clustering of neurons by function. As with P11 analysis, glial Tom^{+} cells are observed at P28 with blood vessel association, as well as large pyramidal neurons with cell bodies in deep cortical layers, and cells with interneuron morphology (**Fig4-2E-H**). In a single P28 brain, a large cluster composed of Tom^{+} cells with neuronal morphologies and GFAP^{+} astrocytes with blood vessel association was observed, which may indicate multipotent potential of Tom^{+} cells in the postnatal cerebral cortex (**Fig 4-3**).

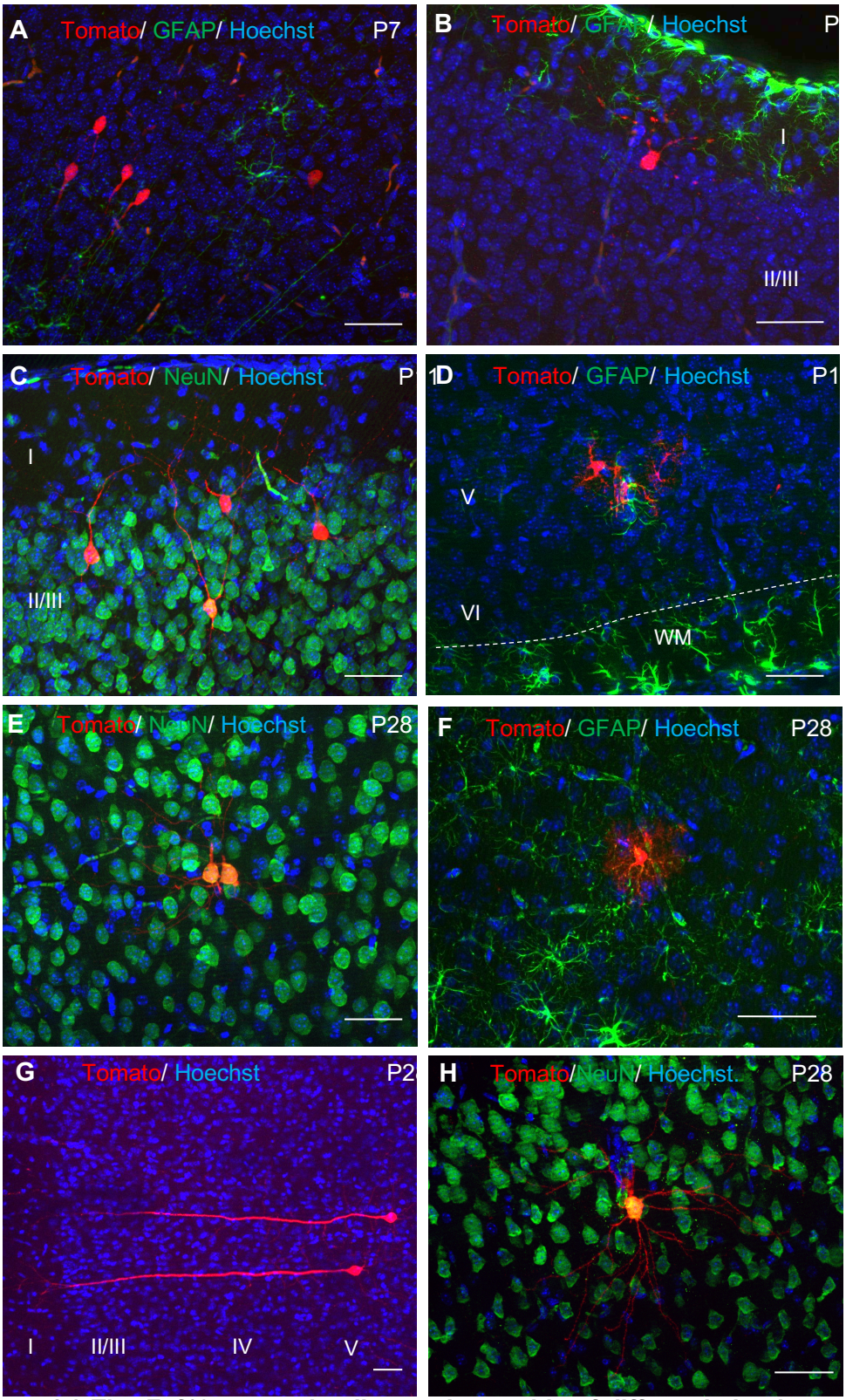


Figure 4-2 The *Fgf10*-expressing lineage is capable of differentiating into neurons and glia: Morphology of Tom^{+} cells at P7 (A-B), P11 (C-D) and P28 (E-H) following tamoxifen pulsing at P4-5. Scale 50μm.

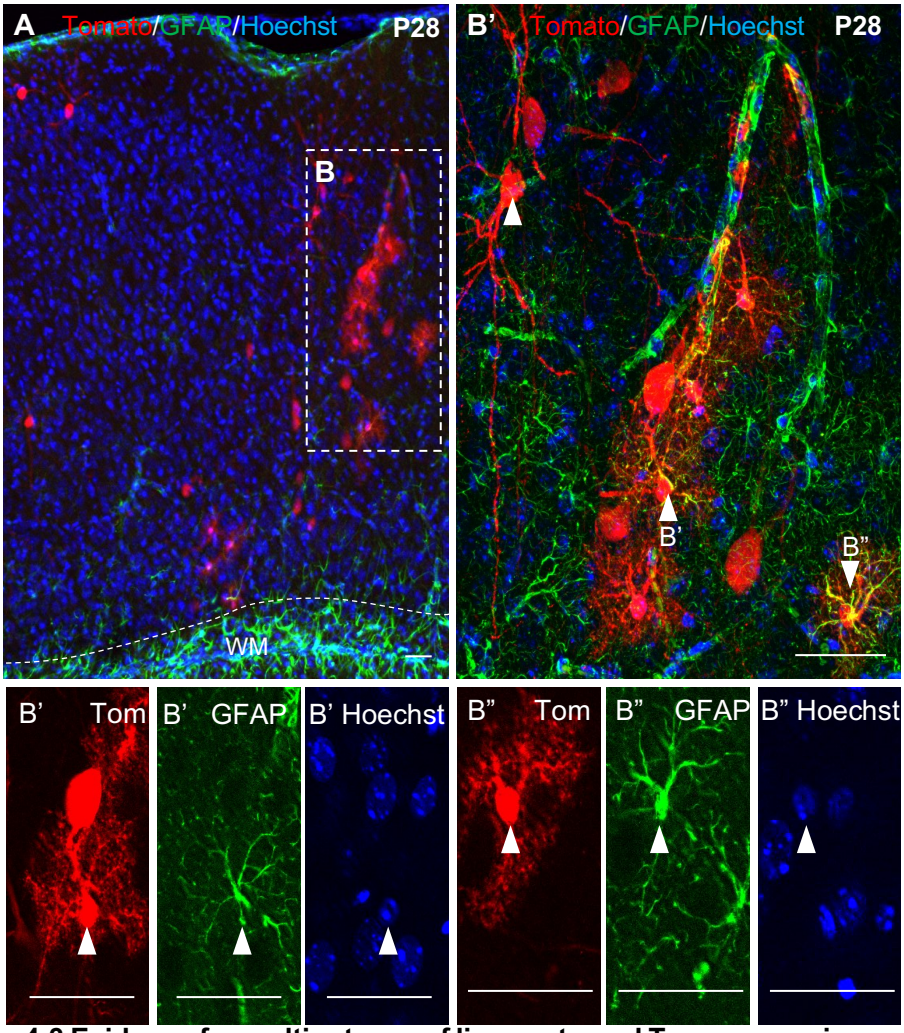


Figure 4-3 Evidence for multipotency of lineage traced Tom-expressing progenitors: In n=1 P28 brain a large Tom$^+$ cluster was observed (A) consisting of GFAP$^+$ astrocytes with blood vessel association and tom$^+$ cells with a neuronal morphology (B). Scale 50μm.

4.3.2.2 Distribution and amplification of Tom$^+$ cells in the postnatal cerebral cortex

Between P7 and P11 the total number of cortical Tom$^+$ cells increase significantly ($p<0.05$) to a level sustained into P28, suggestive of proliferation of cells derived from an *Fgf10* expressing lineage in the postnatal cerebral cortex (**Fig 4-4A**). This amplification was strikingly clear within the retrosplenial cortex, within which Tom$^+$ cells are observed more frequently (**Fig 4-4B-D**).

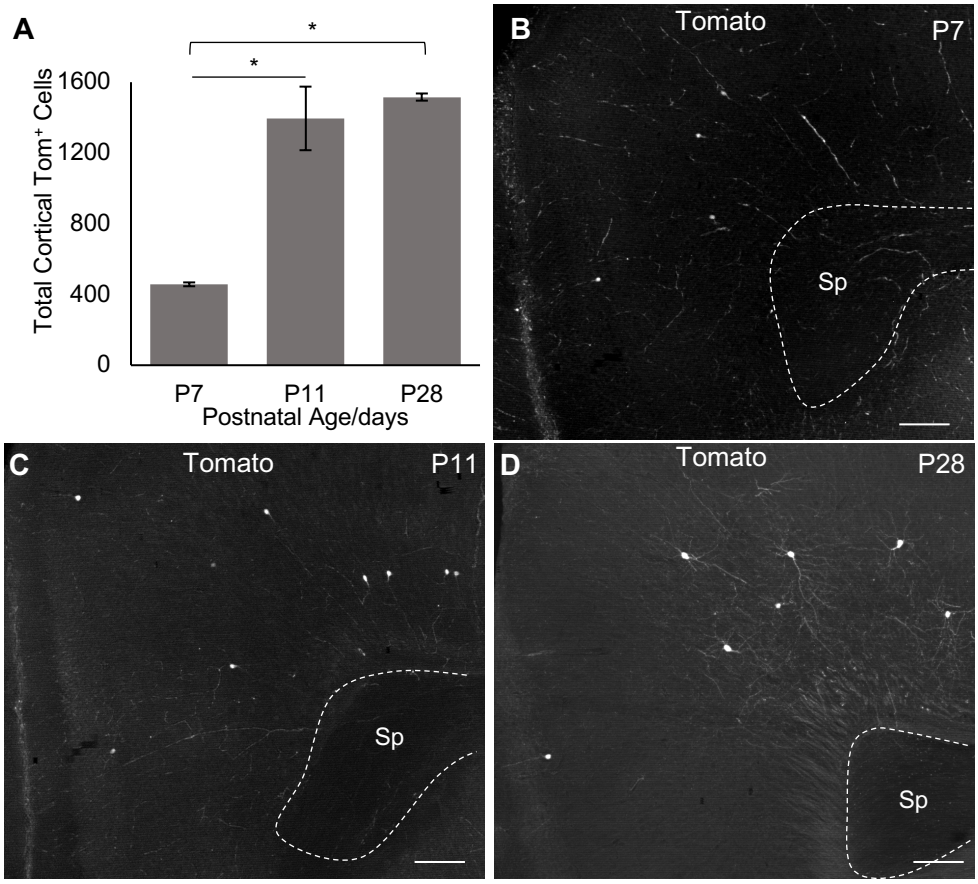


Figure 4-4 Cortical tdTomato-expressing cells amplify in early postnatal life: Analysis of n=3 brains at P7, P11 and P28 shows that between P7 and P11 cortical Tom$^+$ cells significantly increase in number (P= 0.03) to a level sustained at P28, where no significant difference is observed P11-P28 (P=0.788). By P28 there are significantly more Tom$^+$ cells than observed at P7 (P=0.02) (A). This amplification is easily identified within the retrosplenial cortex at P7 (B), P11 (C) and at P28 (D). Scale 50μm; Sp, Splenium. * = p<0.05 .

The neocortex is composed of 6 distinct layers and for ease of analysis the cortex was separated into three categories; layers I-II/III, layer IV and layer V-VI. Based on this paradigm Tom$^+$ cells are observed and amplify significantly in number within all neocortical laminae between P7 and P28 (I-II/III P=0.001; IV P=0.001; V-VI P=0.001). This increase in Tom$^+$ cell number seems broadest in the deeper laminae (V-VI, which could be speculated as a result of Tom$^+$ cell migration or amplification of Tom$^+$ cells being favoured within the deeper cortical layers (**Fig 4-5A**).

Brain sections analysed were also ordered according to anatomical landmarks associated with Bregma from the Allen Brain Atlas to determine the rostro-caudal distribution of cortical Tom$^+$ cells. At P7, P11 and P28 there was a distinct trend for Tom$^+$ cells to occupy the caudal cortical regions between -1.70mm and -3.80mm. At all Bregma points within the cerebral cortex there was no significant difference

between Tom$^+$ cell number P11-P28, validating that the retention of total cortical Tom$^+$ cells between P11-P28 has no regional differences. From Bregma point 1.34mm to -4.40mm there is a statistically significant difference between Tom$^+$ cell number at P7 and P28. However, only between Bregma -3.80mm and -4.40mm is there a significant difference between Tom$^+$ cell number between P7 and P11, likely a result of the large degree of variability in the P11 quantifications, perhaps as a result of final cell divisions of Tom$^+$ cells which stabilise in number by P28 (**Fig4-5B**).

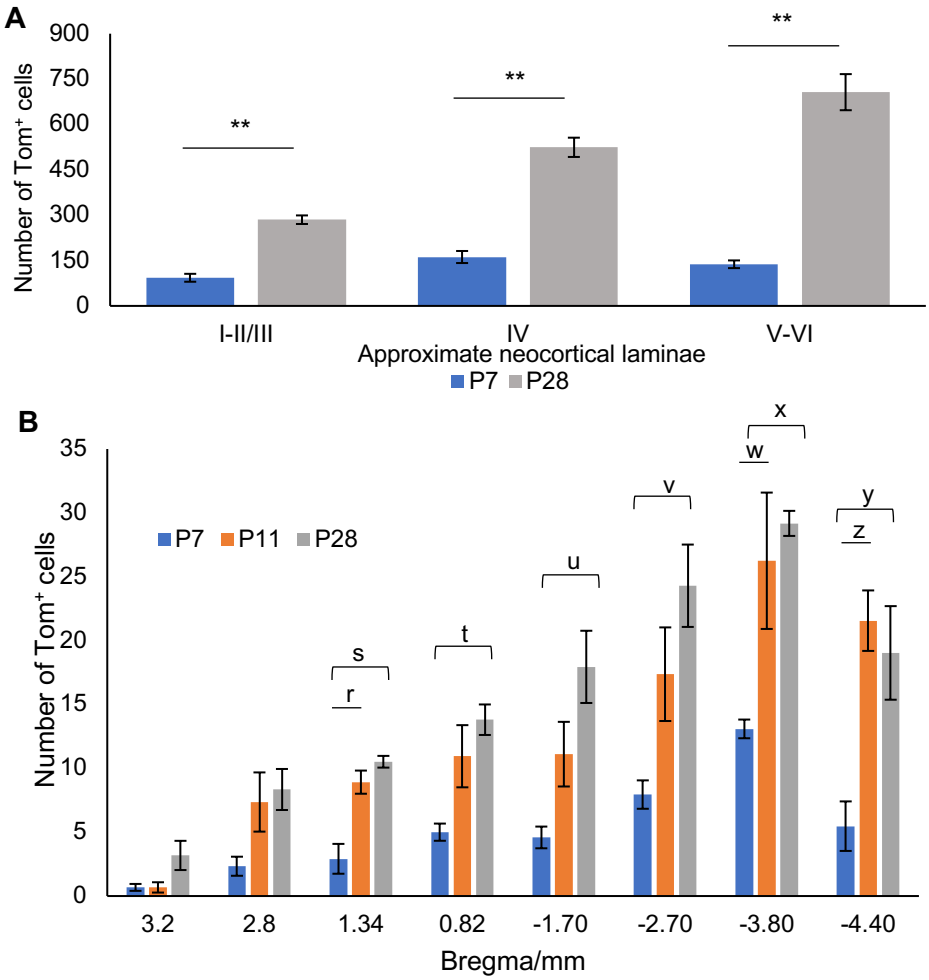


Figure 4-5 Laminar and rostro-caudal distribution of Tom$^+$ cells within the cerebral cortex: Tom$^+$ cells are distributed throughout all neocortical laminae, broadly separated into categories by Hoechst staining. Analysis of n=3 P7 and P28 brains shows that there is a significant increase in Tom$^+$ cells within all categories between P7 and P28 ($p<0.01$) (A). Tom$^+$ cells are found throughout the rostro-caudal axis of the cerebral cortex, most concentrated in caudal cortical regions. The number of Tom$^+$ cells between P11 and P28 are not significantly different at any point along the rostro-caudal axis, extending caudally significant differences between P7 and P11/P28 emerge (B). P values; r=0.012; s=0.002; t=0.019; u=0.013; v=0.017; w=0.045; x=0.027; y=0.018; z=0.037. ** = p <0.001.

4.3.2.3 Lineage traced cells display neurogenic potential

By immunolabelling alternating sections with GFAP and NeuN antibodies it was possible to identify astrocytes and mature neurons respectively, and hence determine

the differentiation potential of the cortical *Fgf10* expressing lineage. For each brain analysed, half of the sections were immunolabelled for NeuN, and any cortical Tom^+ cells interrogated for double-labelling.

At P7 43% (±0.41%) of Tom^+ cells were negative for NeuN, a marker of mature neurons, suggestive that Tom^+ cells with immature neuronal morphology observed at this age may not be post-mitotic and are potentially neuronal progenitors or neuroblasts (**Fig. 4-6B**). At P7 already 57% of Tom^+ cells are $NeuN^+$ and hence differentiated neurons, suggesting terminal division of $Fgf10^+$ stem/progenitors earlier than P7 (**Fig.4-6A**). Additionally, FGF10 may be pleiotropic in its nature within the cerebral cortex and already expressed by some mature neurons at P4/5. It is plausible that Tom^+ cells representing a 'salt and pepper' mix of progenitors and cells where *Fgf10* expression demarcates a separate population of differentiated cells. At P28 96% of Tom^+ cells are $NeuN^+$ (±0.47%) in a number that far exceeds that of P7, suggesting proliferation and neurogenic differentiation of Tom^+ cells postnatally (**Fig4-6D**). A small population of Tom^+ cells that were $NeuN^-$ persisted at P28 suggesting there is potential retention of immature Tom^+ neurons or quiescent Tom^+ progenitors at this age (**Fig 4-6C**).

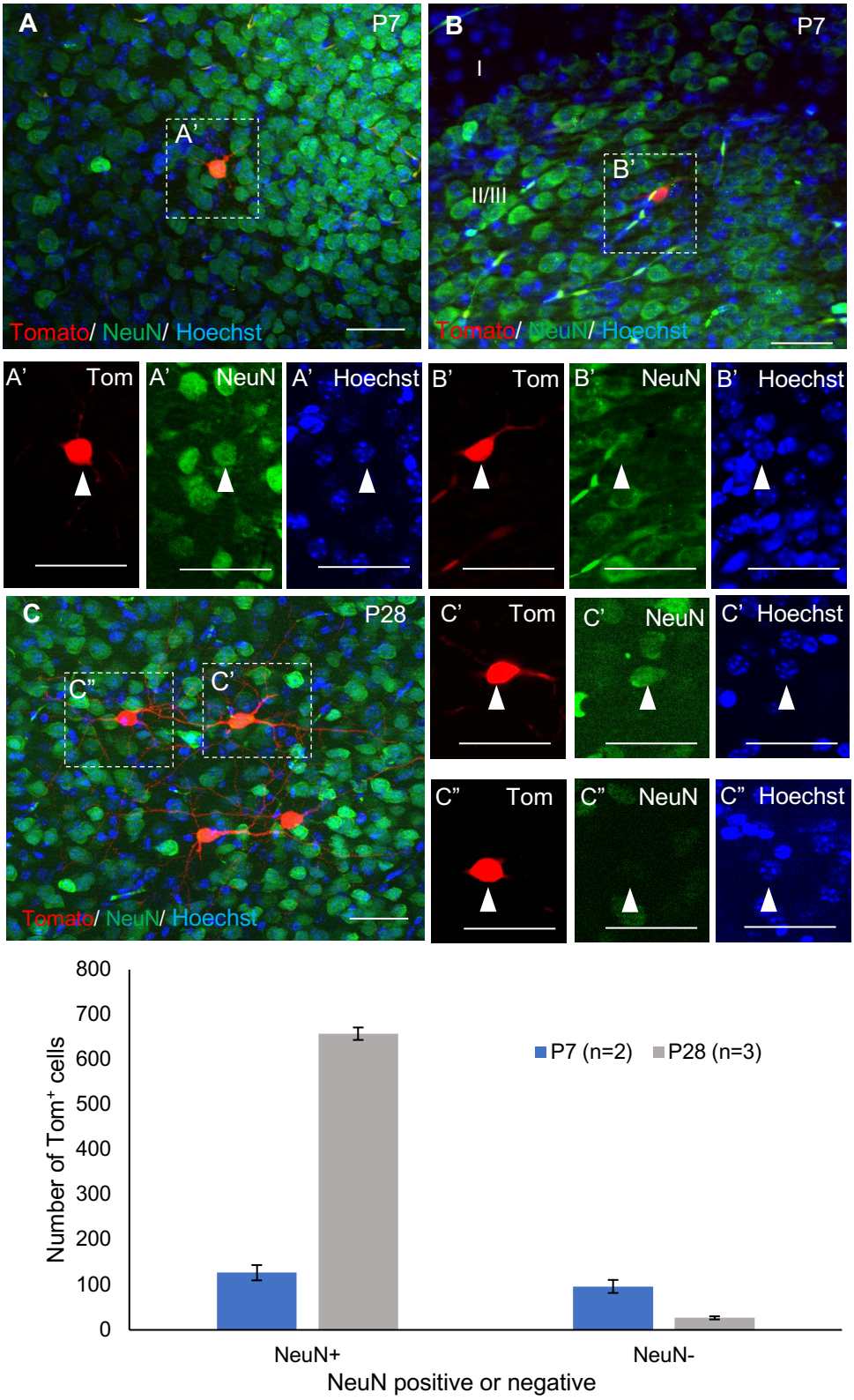


Figure 4-6 A proportion of Tom$^+$ cells at P7 and at P28 are immature neurons/progenitors: At P7 (A-B) and P28 (C) Tom$^+$ cells with an immature neuronal morphology and negative for the pan-neuronal marker NeuN can be observed. Quantification reveals that between P7 and P28 there is a large increase in Tom$^+$/NeuN$^+$ cells within the cerebral cortex and decrease in Tom$^+$/NeuN$^-$ cells. However due to the size of the dataset statistical analysis was not viable (D). Scale 50µm.

4.3.2.4 Tom$^+$ cells can differentiate into both GABAergic and glutamatergic neurons in the cerebral cortex

In order to determine the sub-types of neurons that *Fgf10* expressing cells give rise to postnatally, the *Fgf10$^{NPY\text{-}rGFP}$* mouse was utilised to create a triple transgenic mouse line, *Fgf10$^{CreERT2/+::Rosa26LoxP\text{-}STOP\text{-}LoxP\text{-}Tomato\text{-}dsred::NPY\text{-}rGFP}$*. In the cerebral cortex neuropeptide Y (NPY) is expressed in a subset of GABAergic neurons (Hendry et al., 1984) and may be used as a marker of interneurons (Markram et al., 2004). For the purposes of additional studies on the P28 *Fgf10$^{CreERT2/+}$* cortex only caudal sections (-2.80mm to -3.80mm) were analysed to maintain focus on the regions a majority of Tom$^+$ cells inhabited (n=3). It was observed that a small minority of Tom$^+$ cells (0.6% ±0.15%) at P28 were also NPY$^+$ indicating that Tom$^+$ cells are capable of differentiating into cortical interneurons (**Fig. 4-7**).

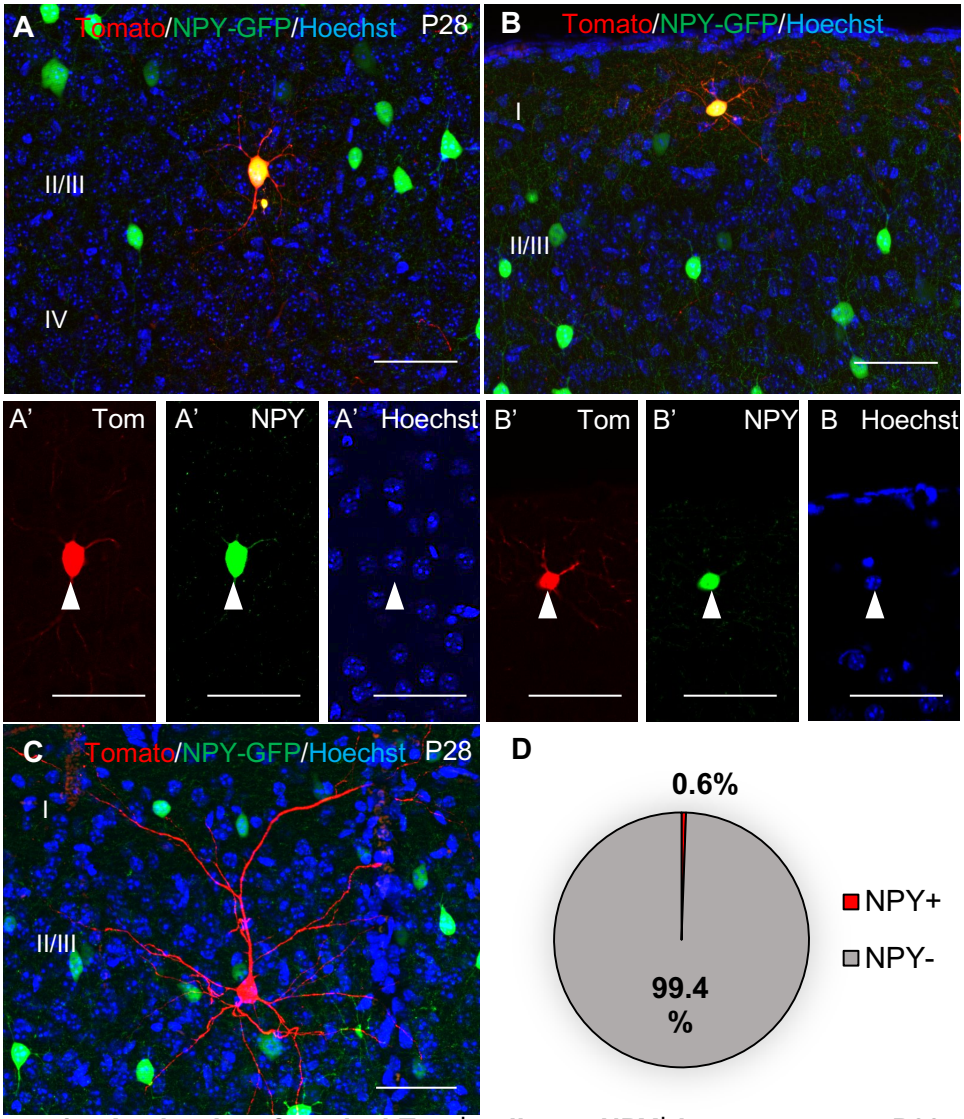


Figure 4-7 A minority of cortical Tom$^+$ cells are NPY$^+$ interneurons at P28: Analysis of the caudal cerebral cortex shows that cortical Tom$^+$ cells are capable of differentiating into NPY$^+$ neurons (A-B). NPY is not expressed in Tom$^+$ cells with a pyramidal morphology (C). NPY$^+$Tom$^+$ cells constitute a minority of Tom$^+$ cells within the caudal cerebral cortex (D). Scale 50μm.

To identify pyramidal neurons within the cerebral cortex the marker T-box brain 1 (TBR1) was used. This transcription factor is expressed by a majority but not all cortical glutamatergic neurons, (Hevner et al., 2001). Of the caudal Tom$^+$ cells analysed at P28, 45% (±4.27%) were TBR1$^+$ and are therefore glutamatergic neurons. However, it does not suffice to say that the remaining 55% (±4.27%) of Tom$^+$ cells are interneurons, immature neuroblasts or glial cells, as neither NPY nor TBR1 are expressed by all GABAergic and glutamatergic neurons respectively (**Fig.4-8**).

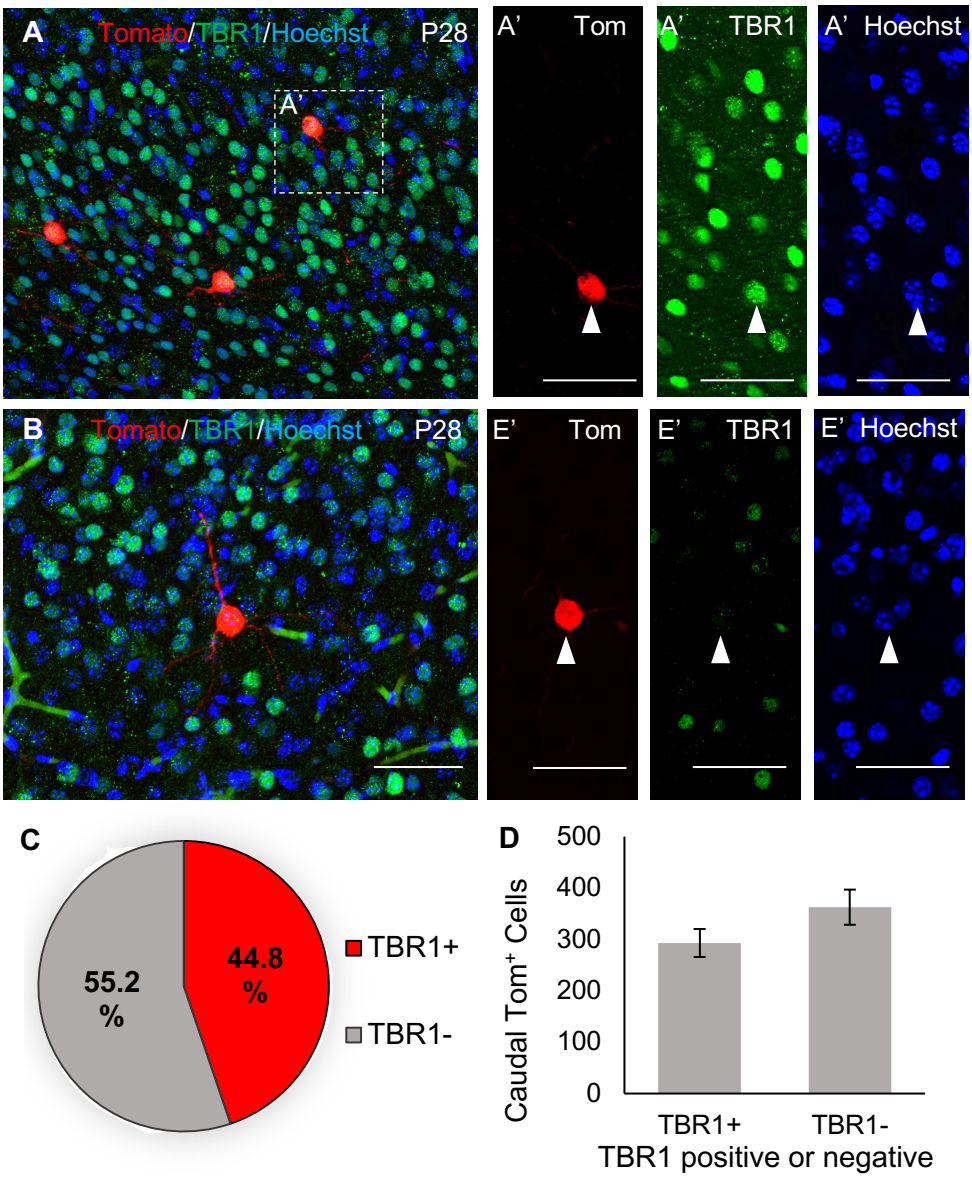


Figure 4-8 Evidence for differentiation of Tom$^+$ cells into glutamatergic TBR1$^+$ neurons: Analysis of n=3 caudal cerebral cortices shows that Tom$^+$ cells are capable of differentiating into TBR1$^+$ and TBR1$^-$ neurons with pyramidal (glutamatergic) morphologies (A-B). A small majority of Tom$^+$ cells are not TBR1$^+$ (C) but not to a level that reaches significance ($p<0.05$, $p=0.186$) (D). Scale 50µm.

4.3.2.5 Descendants of the P4-5 Fgf10 expressing lineage are retained into early adulthood

In order to determine how long descendants of the early postnatal *Fgf10* expressing lineage persist within the caudal cerebral cortex, a single P65 $Fgf10^{CreERT2/+}$ (P4-5 tamoxifen pulsed) mouse was analysed (**Fig. 4-9**). In terms of total caudal Tom^+ cells, it appears that the number of Tom^+ cells at P28 is retained into P65, in a near identical laminar distribution, with a similar peak between Bregma -2.80mm and -3.80mm. This suggests that there is neither laminar, nor rostro-caudal migration of Tom^+ cells between P28 and P65, nor any further amplification of the lineage. However, additional replicates are necessary to draw statistically meaningful conclusion.

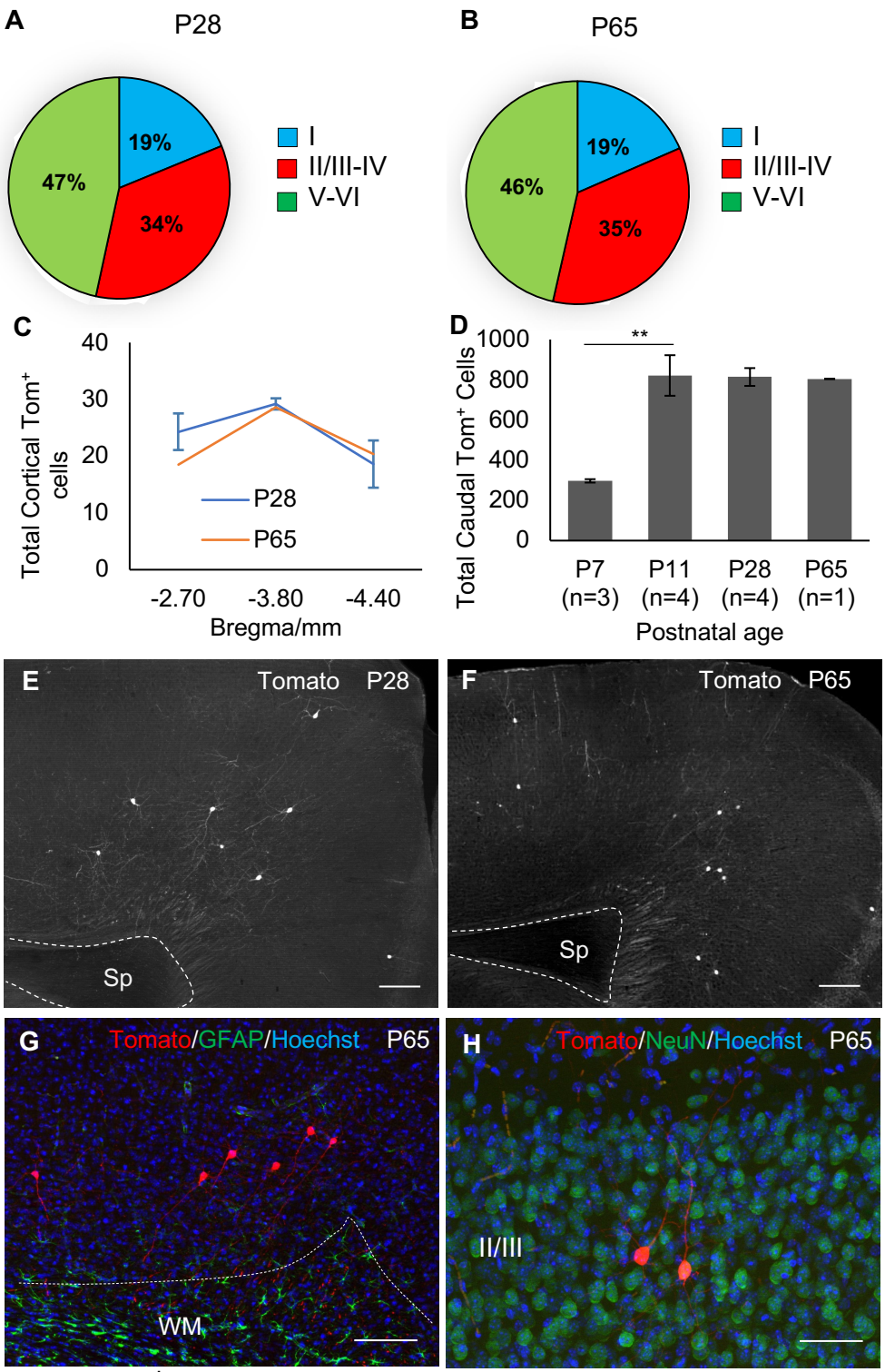


Figure 4-9 Tom$^+$ cells activated at P4-5 persist in the adult cerebral cortex: Analysis of the P4-5 tamoxifen treated caudal cerebral cortex of a P65 mouse (n=1) shows that Tom$^+$ cells remain in a similar laminar (A-B) and rostro-caudal (C) distribution throughout the cerebral cortex, and are retained likely in a similar quantity in caudal regions, despite the significant amplification in early postnatal life P7-P11 (p=0.001)(D). Tom$^+$ cells are still observed within the retrosplenial cortex (E-F) and as clusters and pairs within deep and more superficial laminae (G-H). Scale E-F, 100μm; Scale G-H, 50μm. **= P<0.01.

4.3.3 Interrogation of cortical Tom$^+$ cells for evidence of proliferation

In order to determine if the postnatal amplification of Tom$^+$ cells is a result of cell proliferation, Fgf10$^{CreERT2/+}$ pups were pulsed with tamoxifen P4-5 and received doses of 5-bromo-2'-deoxyuridine (BrdU) at P8, P9 and P10 before they were culled at P11. The synthetic thymidine analogue BrdU is incorporated into any dividing cells during S-phase and is used to detect actively proliferating cells. To detect BrdU by immunohistochemistry, sections of brain tissue must be incubated in hydrochloric acid to denature DNA and expose the BrdU antigen. Other methods of detecting proliferation include the use of endogenous markers such as PCNA. During DNA synthesis, PCNA (cyclin) acts as a sliding clamp, increasing the efficiency of polymerases during DNA replication (Bravo et al., 1987). Any cells in active cell cycle at the time of sacrifice will show PCNA localisation within the nucleus. For the purposes of this investigation, only caudal cerebral cortex sections were isolated for analysis.

4.3.3.1 Immunolabelling of BrdU is incompatible with tdTomato protein

The process of HCl treatment to denature DNA can have a negative effect on antigenicity of the sections in some cases resulting in a complete elimination of immunostaining for some proteins (Boulanger et al., 2016). To determine the optimum incubation for sections in hydrochloric acid two paradigms were used; one using a higher hydrochloric acid concentration at a lower temperature (2M at 37°C), and one of a lower molarity HCl but a higher temperature (1M at 47°C) based on established laboratory protocol. Incubation times were varied for 15, 20, or 30 minutes to determine the optimal incubation time. As previously, only the caudal cerebral cortex was analysed, narrowing the focus to regions where a majority of Tom$^+$ cells are observed. In both conditions, HCl treatment quenched Tom fluorescence beyond detection, and negatively impacted ability of the anti-dsred antibody to recognise its antigen. The quality of the BrdU signal and the quality of the Tomato signal were inversely correlated with the time of incubation. For BrdU immunolabelling 47°C in 1M HCl for 30 minutes produced the clearest signal, however under these conditions' Tom$^+$ cells were not detectable (**Fig. 4-10**). As a result of the experimental paradigms explored it was not possible to obtain visualisation of both Tom and BrdU signal, thus alternative techniques were utilised to analyse proliferation of Tom$^+$ cells.

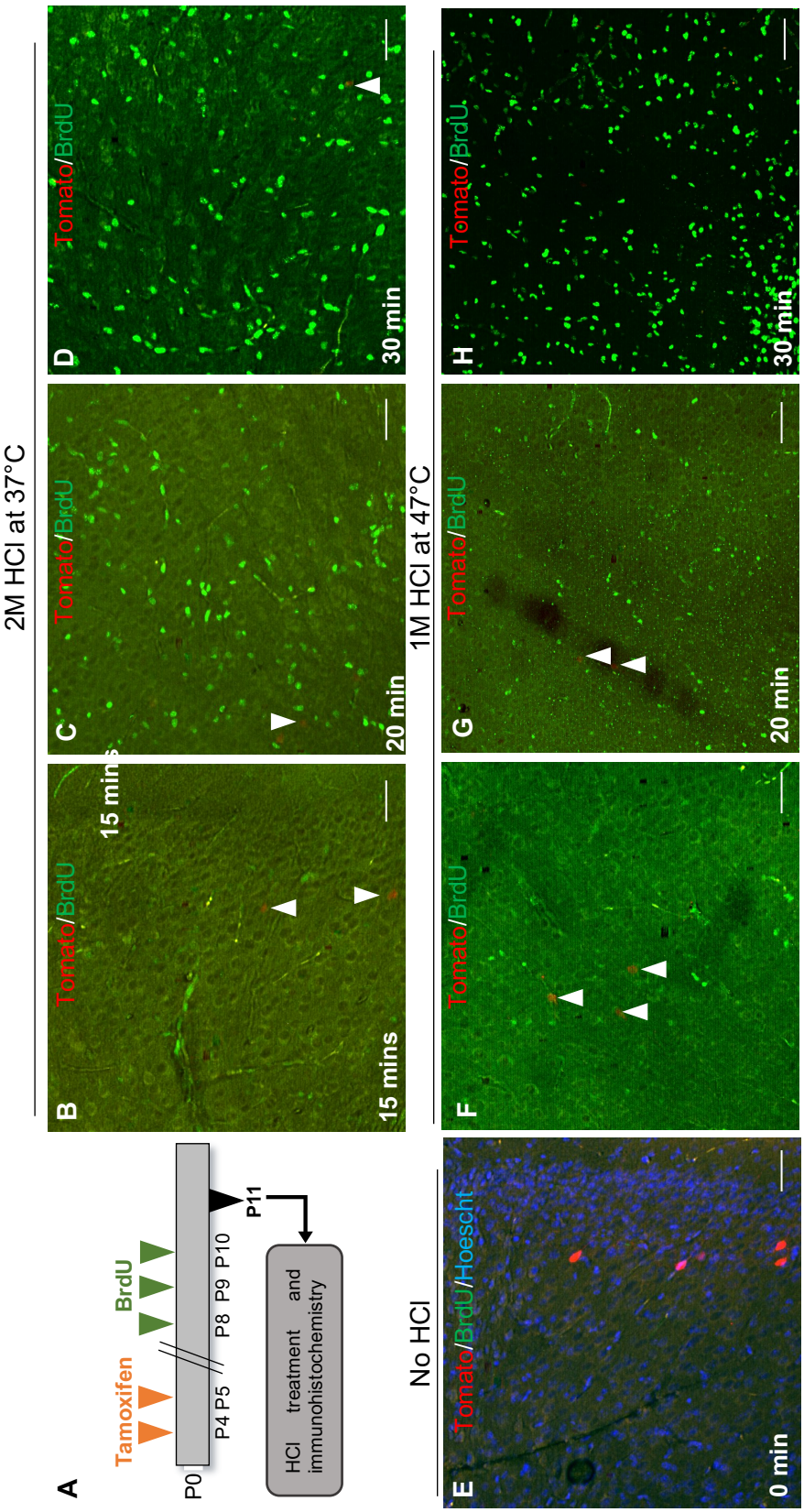


Figure 4-10 BrdU immunolabelling is incompatible with tdTomato: The tamoxifen and BrdU pulsing paradigm utilized for interrogation of P7-P11 amplification of Tom^+ cells (A) Tomato signal greatly diminishes with incubation in HCl at both 2M 37 °C (B-D) and 1M 47°C (F-H). For reference, tdTomato signal is shown in the absence of any HCl treatment (E). Arrow-heads indicate partially visible Tom^+ cells. Scale 50µm.

4.3.3.2 Detection of Tom⁺ cell division by PCNA immunolabelling

In the cell cycle PCNA is highly expressed in S-phase, and is required as an accessory protein to DNA polymerase in DNA replication where it is localised to the nucleus, and may be used as an indicator of cell proliferation (Celis and Celis, 1985). As a positive control the SGZ of the dentate gyrus was selected as a region where PCNA expression is extensive due to the high levels of postnatal neurogenesis ongoing (He et al., 2005). To interrogate the increase of Tom⁺ cells between P7 and P11 (**Fig. 4-4**) pups aged P8 (pulsed P4-5) were selected for analysis of caudal cerebral cortex for PCNA expression.

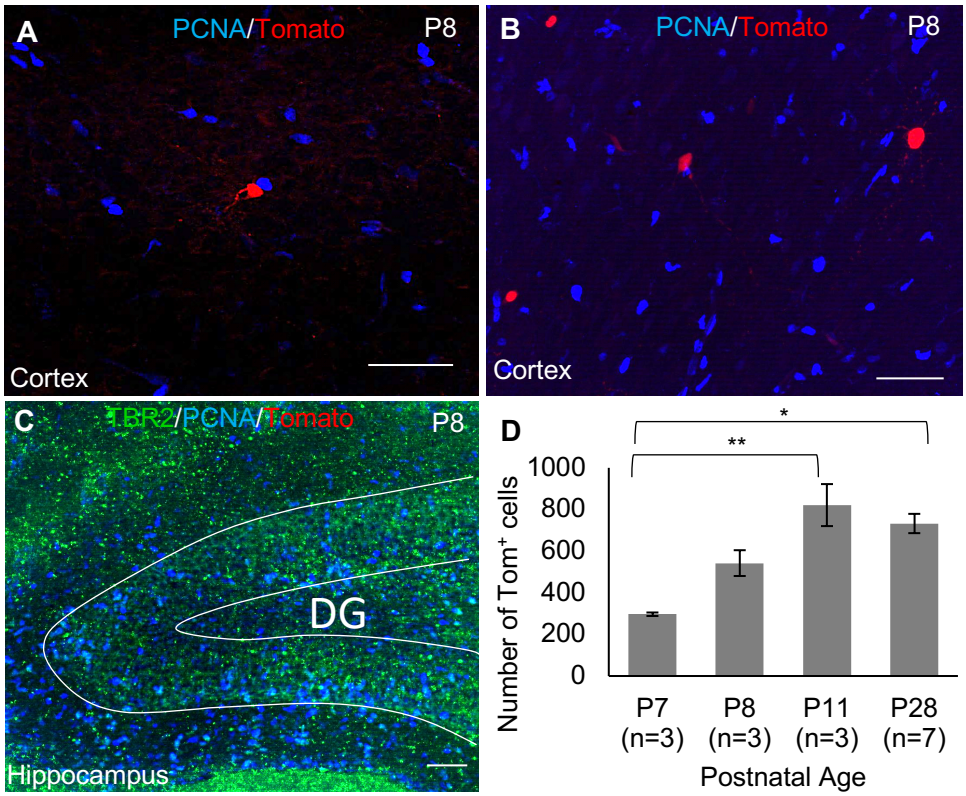


Figure 4-11 PCNA analysis shows evidence of recent cell division in lineage traced Tom⁺ cells: At P8 PCNA⁺ cells are observed in the caudal cerebral cortex often in close association with Tom⁺ cells (A-B). The hippocampus was utilized as a control where PCNA⁺ cells constitute a population of neural stem/progenitor cells shown in conjunction with TBR2 immunolabelling (C). Based on caudal Tom⁺ counts and ANOVA analysis there is no significant increase in Tom⁺ cells between P7 and P8 ($p=0.165$). No significant differences were observed between P8-P11 ($p=0.074$), and P11-P28 ($p=0.725$). However, there was still a significant increase in Tom⁺ cells observed between P7 and P11 ($p=0.001$), and P7-P28 ($p=0.002$) (D). Scale 50μm. DG, Dentate gyrus. *= $p<0.05$; **= $p<0.01$.

However, no PCNA⁺/Tom⁺ double-labelled cells were observed within the P8 cerebral cortex. Many Tom⁺ cells were identified closely associated with PCNA⁺ nuclei which may be an artefact of a recent cell division, giving rise to a Fgf10⁺ progeny at the time

of tamoxifen induction (**Fig 4-11A-B**). Based on total caudal cortical Tom$^+$ cell counts it appears there is some amplification of the lineage between P7 and P8, which does not reach significance, indicating that amplification is ongoing at this timepoint (**Fig. 4-11C**).

4.3.3.3 No expression of intermediate progenitor marker TBR2 in the P8 cerebral cortex

In embryonic development TBR1$^+$ post-mitotic glutamatergic neurons are derived from a sequential lineage progression from Pax6$^+$ radial glia to TBR2$^+$ intermediate progenitors which differentiate into TBR1$^+$ early born post-mitotic neurons (Englund et al., 2005). Due to the existence of TBR1$^+$/Tom$^+$ cells within the P28 cerebral cortex, and the amplification of tom$^+$ cells between P7 and P11, it was hypothesised that at P8 a minority of Tom$^+$ cells were TBR2$^+$ intermediate progenitors. Upon analysis, it was found that there is no TBR2 expression in the caudal cerebral cortex. However, TBR2 was strongly detected within the cerebellum within the internal granule cell layer confirming the success of reactions (**Fig. 4-12**).

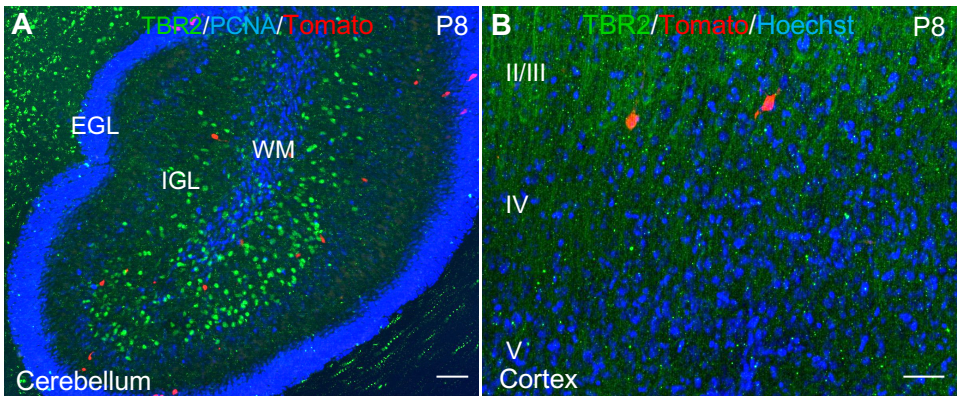


Figure 4-12 TBR2 is not expressed within the caudal postnatal cerebral cortex at P8: Within the cerebellum Tom$^+$ cells are observed, as well as strong TBR2 signal within the internal granule layer (IGL) and white matter (WM), with PCNA$^+$ proliferative cells within the external granule layer (EGL) (A). Within the P8 caudal cerebral cortex no TBR2+ cells are observed (B). Scale 50μm.

4.3.4 *Fgf10* expression persists in the adolescent cerebral cortex

To determine whether *Fgf10* expression persists within the adolescent cerebral cortex, *Fgf10*$^{CrerERT2/+::Rosa26\text{-}LoxP\text{-}STOP\text{-}LoxP\text{-}tdTomato}$ mice were pulsed twice with tamoxifen via intraperitoneal injection at P27 and P28, and sacrificed after a 12 day chase at P40. Expression of *Fgf10* was still observable in the cerebral cortex, however with this lineage tracing paradigm the population of Tom$^+$ cells observed were entirely neuronal in morphology. In terms of their distribution, Tom$^+$ cells were localised almost entirely within deep neocortical laminae (**Fig. 4-13**). The cortical Tom$^+$ cells observed were found to be organised in clusters of a similar morphology, which may be reminiscent

of recent cell division, or a form of functional clustering. Interestingly, no Tom$^+$ glial cells were identified in adolescence, indicating that expression of Fgf10 in glial populations may be restricted to early postnatal life.

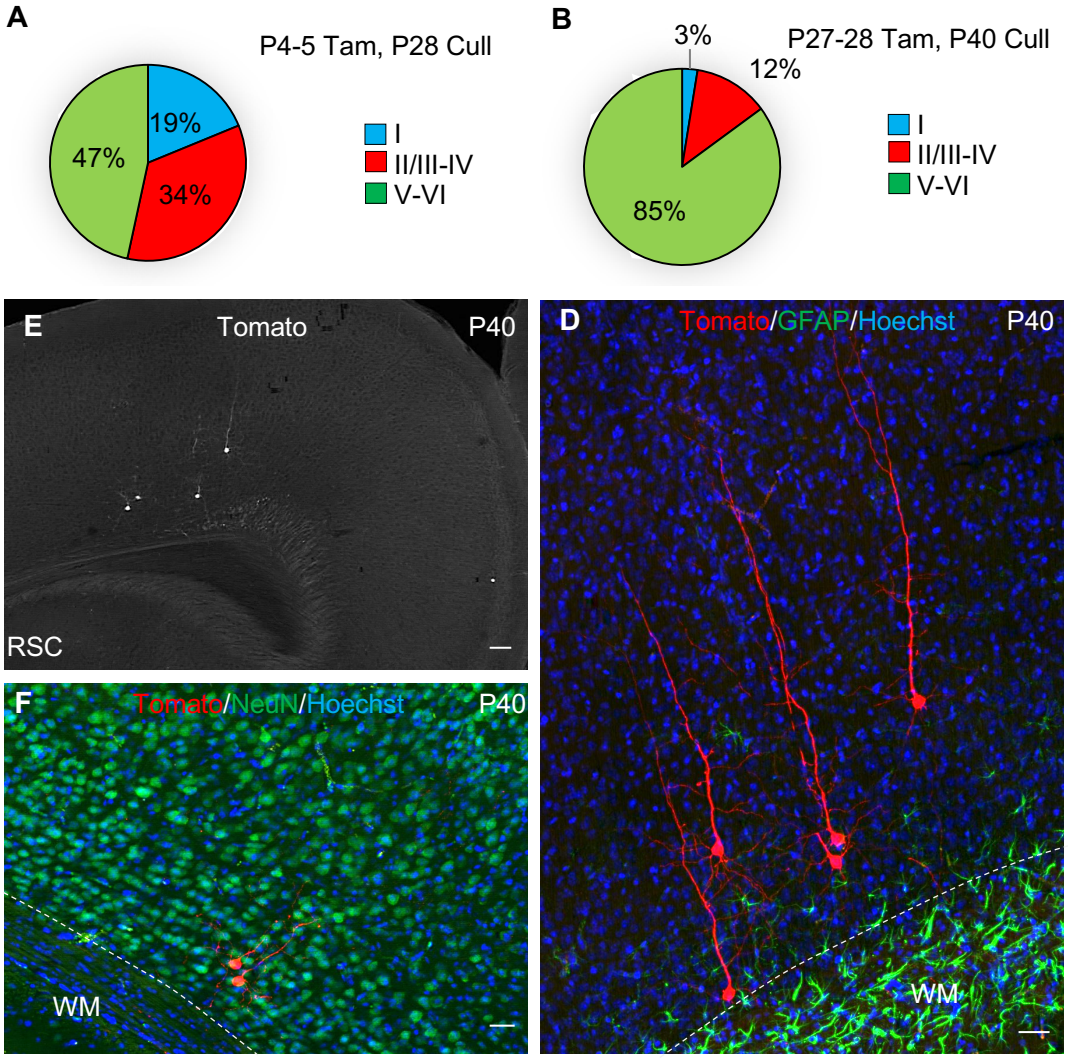


Figure 4-13 Caudal cortical *Fgf10* expression persists into early adolescence: *Fgf10*$^{CreERT2/+::Rosa26LoxP\text{-}STOP\text{-}LoxP\text{-}tdTomato}$ mice were pulsed at P27 and P28 with tamoxifen and culled at P40 (n=2). The expression of *Fgf10* persists at P27-28, however with a strikingly different laminar distribution to P4-5(P28) pups, with a majority of Tom$^+$ cells inhabiting the deep cortical layers (A-B). Tom$^+$ cells are still found within the retrosplenial cortex (D) and display morphological characteristics of glutamatergic pyramidal neurons (E-F). Scale 50μm.

In collaboration with work ongoing within the hypothalamus some aged mouse brains were obtained to determine whether expression of *Fgf10* extends within the adult mouse cerebral cortex. In this paradigm, 12month old *Fgf10*$^{CrerERT2/+::Rosa26\text{-}LoxP\text{-}STOP\text{-}LoxP\text{-}tdTomato}$ mice were pulsed with tamoxifen by oral gavage once a day for four days, and sacrificed after a week-long chase period. The caudal cerebral cortex, as previously, was interrogated for distribution of Tom$^+$ cells. As with mice pulsed with tamoxifen in

adolescence (P27-P28), a majority of Tom$^+$ cells were neuronal in morphology and within the deep cortical layers (V-VI) in striking contrast to early postnatal tamoxifen pulsed mice (P4-5). As in the adolescent mouse brain clusters of Tom$^+$ cells were observable, specifically within separate functional units of the cerebral cortex such as the visual and somatosensory cortex, further suggestive of a clustering by function like phenomena of cortical Tom$^+$ neurons (**Fig. 3-14**).

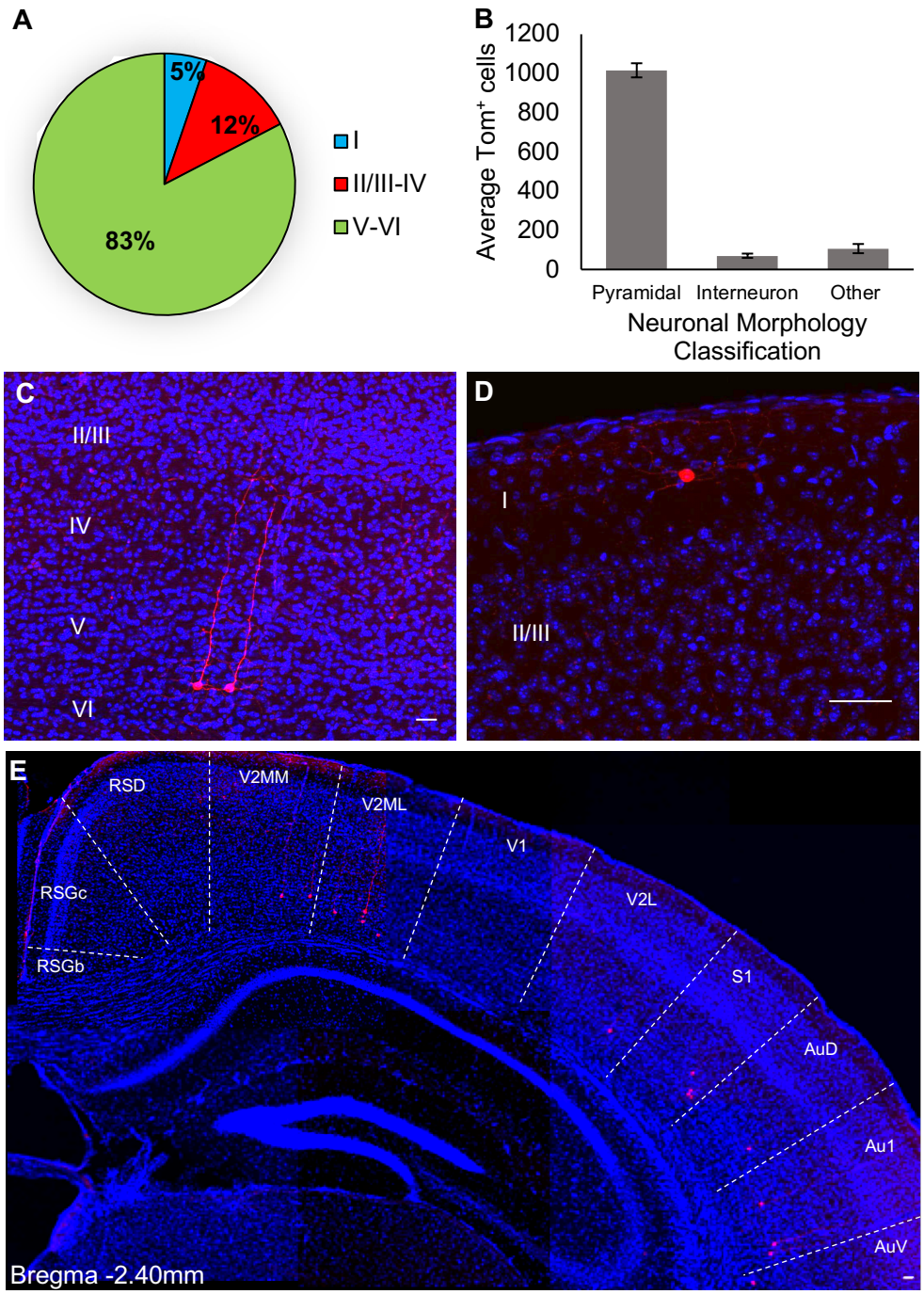


Figure 4-14 Caudal cortical *Fgf10* expression persists into adulthood: *Fgf10*$^{CreERT2/+::Rosa26LoxP\text{-}STOP\text{-}LoxP\text{-}tdTomato}$ mice were pulsed once a day for 4 days with tamoxifen at 12 months old and culled after a 7 day chase (n=2). The expression of *Fgf10* persists at in adulthood, in a laminar distribution similar to that observed in early adolescent P27-28(P40) studies, with a majority of Tom$^+$ cells inhabiting the deep cortical layers (A). A majority of Tom+ cells are of a pyramidal morphology (B,C) as well as rare non-pyramidal (B,D). Clusters of Tom$^+$ cells are found within functional regions of the cerebral cortex, such as the visual, somatosensory and auditory regions (E). (C-D Scale 50μm; E scale 100μm). RSGb/c, retrosplenial granular cortex; RSD, retrosplenial dysgranular cortex; V2MM, secondary visual cortex (mediomedial area); V2ML, secondary visual cortex mediolateral area; V1, primary visual cortex; V2L, secondary visual cortex lateral area; S1, Primary somatosensory cortex; AuD, secondary auditory cortex (dorsal); Au1, primary auditory cortex; AuV secondary auditory cortex (ventral).

4.4 Discussion

Based on previous research on the hypothalamus, it was determined that tanycytes derived from the *Fgf10* expressing lineage constitute a population of neural stem/progenitor cells that persist postnatally. The hypothalamus lacks the FGFR1/2-IIIb isoforms that FGF10 normally signals via, and is thus speculated to function via an alternate intracellular mechanism of action within this region (Haan et al., 2013). By utilising the Fgf10^{nLacZ} transgenic mouse line, LacZ$^+$ cells were superficially identified within the postnatal/adult cerebral cortex, as a result it was hypothesised that *Fgf10* expressing cells may constitute a population of cortical neural stem/progenitor cells (Hajihosseini et al., 2008). Due to the neonatal lethality of *Fgf10*$^{-/-}$ mice, little is known in regard to its postnatal function within the CNS (Sekine et al., 1999). Further adding complexity to the research area, it has been shown by previous laboratory members that current commercial anti-FGF10 antibodies are able to recognise other epitopes or fail to detect FGF10. Therefore, lineage tracing models were utilised as a viable alternative to determine the dynamics of postnatal cortical *Fgf10* expression.

4.4.1 Cells derived from the *Fgf10* expressing lineage have neurogenic potential

By use of experimental paradigms determined for hypothalamic studies the *Fgf10*$^{CreERT2/+::Rosa26LoxP\text{-}STOP\text{-}LoxP\text{-}tdTomato}$ mice were pulsed with tamoxifen in early postnatal development (P4-5), and the fate of cells expressing *Fgf10* at this time point traced postnatally. Between P7-P11 the Tom$^+$ lineage significantly amplifies in number within the caudal cerebral cortex to a level retained into P28 and P65, suggesting that if these neurons are indeed new-born they are not transient in existence as has been previously reported in studies on the adult macaque cerebral cortex (Gould et al.,

2001).

At both P7 and P28 variable numbers Tom^+ cells with neuronal morphologies were not $NeuN^+$, indicating that they may be immature neurons, neuroblasts or neural stem/progenitor cells. It should be noted that $NeuN^+$ is not expressed in all neurons within the cerebral cortex, such as Cajal-Retzius cells within layer I. However laminar analysis indicates layer I-II/III account for only a small number of Tom^+ cells at P7 and P28. Between P7 and P28 the ratio shifts, as $Tom^+/NeuN^-$ cells decrease and $Tom^+/NeuN^+$ cells increase, indicating that amplification and maturation of the Tom^+ lineage is neurogenic.

A subset of Tom^+ cells within the cerebral cortex were identified as both glutamatergic neurons ($TBR1^+$) and GABAergic neurons (NPY^+) which in embryonic development are derived from two distinctly separate origins; the embryonic SVZ/VZ and ganglionic eminences respectively. The existence of Tom^+ cell differentiation into both subtypes in the cerebral cortex may indicate there is a common parenchymal progenitor capable of giving rise to both neuronal subtypes postnatally, which may be delineated by *Fgf10* expression.

4.4.2 Proliferation of cortical Tom^+ cells

The 'gold-standard' for analysis of proliferation and neurogenesis is incorporation of BrdU. Pulsing with BrdU at P7 to P11 would have captured any cells actively dividing within these time-points. However, its incompatibility with tdTomato meant other methodology needed to be explored. Previous laboratory members had utilised PCNA as an endogenous proliferative marker, however interrogation of P8 caudal cerebral cortex yielded no Tom^+ and $PCNA^+$ double-labelled cells.

In an attempt to interrogate the potential of Tom^+ neural stem/progenitors the transcription factor Sox2 was utilised as a marker (data not shown), which functions to maintain the characteristics of neural progenitors and thus inhibit differentiation (Graham et al., 2003), however the signal achieved by antibody was unclear and inconsistent with high background. Several additional antibodies were tested including the transcription factor MASH1 (Ascl1) and microtubule associated protein doublecortin (DCX) due to their expression in intermediate progenitors and neuroblasts, in both embryonic and adult neurogenesis (Brown et al., 2003; Kim et al., 2011). However, no clear and conclusive signal was obtained even with analysis of canonical neurogenic regions (data not shown) suggesting non-specificity of antibodies.

Due to the observation of $TBR1^+$ Tom^+ double-labelled cells within the cerebral cortex at P28 it was hypothesised that some of the Tom^+ cells may be proliferative $TBR2^+$ intermediate progenitors. In embryonic cortical glutamatergic neurogenesis $Pax6^+$ radial glia give rise to $TBR2^+$ intermediate progenitors which in turn differentiate into glutamatergic $TBR1^+$ neurons (Englund et al., 2005). In adult hippocampal neurogenesis, TBR2 expression is also highly localised to intermediate progenitors within the dentate gyrus (Hodge et al., 2008). Within the cerebellum $TBR2^+$ cells are found within the internal granule layer specifically within a population of unipolar brush cells and their progenitors (Englund et al., 2006). No $TBR2^+$ expression was observed within the cerebral cortex, with only weak $TBR2^+$ cells within the hippocampus due to high levels of background. Within the cerebellum strong $TBR2^+$ cells were observed indicating the antibody was effective but perhaps not reliable in reporting lower levels of TBR2 protein. Based on the observation of no TBR2 expression within the cerebral cortex, it is unlikely that Tom^+ cells amplify and give rise to the $TBR1^+$ cells observed at P28, and likely give rise to $TBR1^-$ progeny.

4.4.3 FGF10 may be pleiotropic in its function within the cerebral cortex

Several findings indicate that FGF10 may be pleiotropic in its function. The incidence of $NeuN^+$ cells mixed with $NeuN^-$ cells at P7 may be indicative that *Fgf10* is expressed by both new-born neurons and stem/progenitor cells ($NeuN^-$) in a 'salt and pepper' mix within the cerebral cortex. Occasionally glial Tom^+ cells were also observed, often as pairs with blood vessel association, this may indicate the multipotential capacity of cells from the P4-5 *Fgf10* expressing lineage due to existence of Tom^+ neurons and glia. It is tempting to speculate that close association of Tom^+ cells with blood vessels may indicate derivation of this lineage from pericytes, which have been previously identified as capable of differentiating into both neurons and glia *in vitro* (Dore-Duffy et al., 2006). However, given that a majority of Tom^+ cells with blood vessel association are glial in morphology with characteristic end-feet attachments to microvasculature, it is more likely that they are functional astrocytes functioning as part of the blood brain barrier.

In the adolescent and adult mouse brain descendants of *Fgf10* expressing cells present a different laminar distribution to that of the early postnatal mouse, with a vast majority occupying the deep cortical laminae (V and VI). Layer V and VI of the cerebral cortex contain pyramidal neurons that broadly speaking, project both intra-cortically and sub-cortically to regions such as the thalamus, basal ganglia, brain stem and spinal cord. Close relatives of FGF10, FGF7 and FGF22, have been implicated in

inhibitory and excitatory synaptogenesis within the hippocampus respectively. Within the hippocampus it has been reported that FGF22 and FGF7 are secreted from the dendrites of pyramidal neurons to promote differentiation of synapses at presynaptic terminals. Knock-out studies show that *Fgf22*-KO mice have decreased susceptibility to epileptic seizures whereas *Fgf7*-KO have an increased susceptibility (Terauchi et al., 2010). *In vitro* evidence presented within this study implicates *FgfR1-IIIb* and *Fgf2-IIIb* in this process, however previous *in vivo* studies indicate only FgfR1-IIIb is expressed within the hippocampus (Beer et al., 2000; Dabrowski et al., 2015). Initial studies also implicated Fgf10 in synaptogenesis however this was not investigated further (Umemori et al., 2004). Given the prevalence of Tom^+ cells within the deep cortical laminae, projecting outside the cerebral cortex, it is possible that these neurons are in contact with regions such as the thalamus, brain stem and spinal cord where FGFRs are expressed. However, there is no existing literature exploring FGFR1/2-IIIb expression within these regions.

With adult/adolescent tamoxifen pulsing, Tom^+ cells observed were often clustered in deep cortical laminae. Functional clustering, is based on the concept of wiring economy, which states that neurons that are connected should be anatomically close to reduce length of connections (Mitchison, 1992). On a macro-scale it is known that the cerebral cortex is organised into functional regions. However, little is known in terms of the micro-organisation of neurons within them. In the mouse somatosensory cortex, the whisker barrel cortex contains functional units (barrels) of layer IV neurons which correspond to individual whiskers, arranged in an almost identical fashion to the whiskers on the snout (Woolsey and Van Der Loos, 1970). Similarly micro-clustering of neurons has been observed within the motor cortex whereby within clusters, the closer neurons were correlated with each other the shorter the distance between them (Dombeck et al., 2009). It is possible that the observation of tom^+ cells clustered within functional regions of the adolescent/adult cerebral cortex are ‘connected’ in a similar functional manner, based on *Fgf10* expression.

In conclusion, cells derived from an early postnatal (P4-5) *Fgf10*-expressing population amplify postnatally indicating that FGF10 may demarcate a population of neural stem/progenitor/precursor cells within the postnatal rodent cerebral cortex. Preliminary evidence could not document actively dividing cortical Tom^+ cells but does not conclusively rule it out. If Tom^+ cells are neural stem/progenitors they may be slow cycling and largely quiescent in nature, hence PCNA immunolabelling at one time-point, utilising only caudal sections may not be a conclusive indicator of Tom^+ cell proliferation. To detect cell proliferation across a span of time (P7-P11) other thymidine

analogues such 5-Ethynyl-2´-deoxyuridine may have presented a viable alternative to BrdU, as no DNA denaturation step is required for detection (Chehrehasa et al., 2009).

The Tom^+ cells predominantly inhabit the caudal cerebral cortex, and are distributed across all cortical laminae, giving rise to both glutamatergic and GABAergic neurons. In adolescence and adulthood *Fgf10* expression persists but with a varied distribution, leading to the speculation that *Fgf10* expression at different ages may be involved in different processes. However, further detailed investigation of *Fgf10* expression in the adult cerebral cortex was beyond the scope of this project.

5 CONDITIONAL DELETION OF *FGF10* PROPOGATES AMPLIFICATION OF TOM$^+$ CELLS INTO ADOLESCENCE

5.1 Introduction

Upregulation of *Fgf10* has recently been investigated following CNS injury in adult mice (Li et al., 2016). Middle cerebral artery occlusion (MCAO) is a model of ischemic stroke where blood flow supplying the brain is restricted and released to allow reperfusion of brain tissue. By use of FGF10 antibodies it was determined that under normal conditions neurons express FGF10, which can be found localised within nuclei, cytoplasm, cerebrospinal fluid suggesting it is released from neurons into the CNS. Following MCAO the level of *Fgf10* mRNA increased within ischemic areas, and on a protein level within the CSF. Further investigation via injection of FGF10 into the lateral ventricles following MCAO reduced the infarct area and reduced neuronal cell death from apoptosis and neuroinflammation in comparison to MCAO without FGF10 treatment. However, despite the proposed paracrine mode of action the expression of FGF10 specific receptors was not investigated (Li et al., 2016).

Within spinal cord injury similar reductions of inflammation and improved recovery were observed in the presence of FGF10 derived from neurons and microglia/macrophages (Chen et al., 2017). Following spinal cord lesion the level of FGF10, FGF1, FGF2 and FGF7 protein increased as well as the level of FGFR2 (although no distinction was made between IIIb and IIIc splice isoforms). *In vivo* application of FGF10 facilitated an improved functional recovery following spinal cord injury, by significantly decreasing activation and proliferation of macrophages/microglia, and hence release of pro-inflammatory cytokines (Chen et al., 2017). Both the above studies provided insight into the role of FGF10 in pathological contexts, suggesting a function in neuronal survival following injury within the CNS. However little data was collected as to the role of FGF10 in the CNS under physiological conditions.

Postnatally, FGF10 has also been implicated in dendritogenesis of glutamatergic neurons within layer VI of the post-natal mouse whisker barrel cortex (Huang et al., 2017). The whisker barrel cortex is a brain region specifically used to study the remodelling of cortical circuitry. Each individual whisker on rodent snout is represented by a discrete structure in a somatotopic “barrel” map within layer VI of this region (Petersen, 2007; Woolsey and Van Der Loos, 1970) (**Fig. 5-1A**). The architecture of layer VI barrels comprises of “barrel rings” of spiny stellate cells (bSCs) orientated to project their dendrites towards the cell space centre, where clusters of thalamocortical afferent arbours inhabit (**Fig. 5-1B**). Loss of function studies of glutamate receptors, such a NMDARs and mGlut5, show that the organisation of proper dendritic polarity is

dependent on neurotransmission (Espinosa et al., 2009; Huang and Lu, 2018). The disrupted dendritic outgrowth of cortical neurons towards thalamocortical axons following mGluR5 knockout was largely attributed to increasing levels of nerve growth factor (*Ngf*) mRNA, and to a lesser extent *Fgf10* (Huang and Lu, 2018). In postnatal development immediately after birth, thalamocortical afferents reach the layer IV and VI neurons of the somatosensory cortex and by postnatal day 3 begin to segregate into whisker-related clusters, with proper polarity and barrel rings established by P6. After P6, dendrites increase in complexity, elongating and branching in a process that continues throughout the first two postnatal weeks. *In vivo* and *in vitro* analysis demonstrated expression of *Fgf10* and *Fgfr1-IIIb* and *Fgr2-IIIb* within samples of "brain tissue" and cortical neuron primary cell culture, and involvement of *Fgf10* in dendritogenesis in response to glutamatergic transmission *in vitro. In vivo* overexpression of *Fgf10* enhanced dendritogenesis within this. Thus, indicating a potential role for FGF10 in postnatal development of cortical circuitry (Huang et al., 2017).

However, data obtained is inconclusive in determining expression of *Fgfr1-IIIb* and *Fgfr2-IIIb* within the postnatal CNS under physiological conditions, and within the cerebral cortex specifically. Instead, analysis of specific receptor isoform expression is based on RNA from primary cell culture of cortical neurons or brain tissue from unspecified regions (Huang et al., 2017). It is probable that time spent in cell culture conditions and pathological insult will alter the profile of receptor expression within regions under interrogation. Additionally, it is likely that FGF10 functions pleiotropically within the post-natal CNS and cerebral cortex specifically, as many members of the Fgf family do, for example FGF4 has been observed as a mitogenic, angiogenic and survival factor depending on the stage of embryonic development and organ system interrogated (Kosaka et al., 2009).

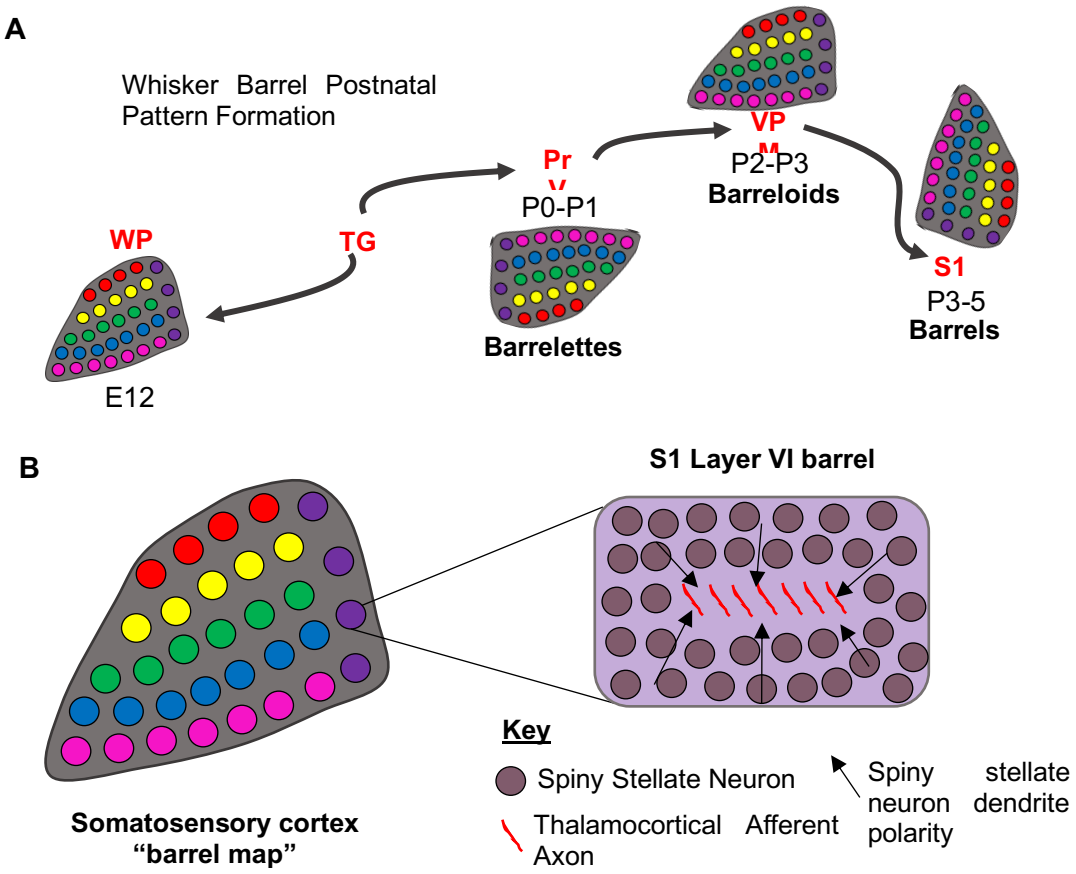


Figure 5-1 The somatotopic organization of the whisker barrels in the rodent somatosensory cortex: Postnatal timeline for emergence of axonal and cellular patterns within the whisker pad, brain stem, thalamus and somatosensory cortex (A). The organization of Spiny stellate neurons and proper polarity in layer VI somatosensory cortex barrels (B). WP, Whisker pad; TG, trigerminal ganglia; PrV, principal trigeminal nucleus of the brainstem; VP, ventroposteromedial nucleus of the thalamus; S1, Primary somatosensory cortex. Adapted from Wu *et al.* 2011, Erzurumulu and Gaster 2012 and Sehara and Kawasaki 2010.

Cells derived from the P4-5 *Fgf10* expressing lineage have been identified within the cerebral cortex and display the potential to amplify in early postnatal life (**Fig.4-4**). In the absence of its receptors (**Fig. 4-1**) the mode through which FGF10 exerts its functions remains largely unexplored. The neonatal lethality of $FGF10^{-/-}$ pups poses an obstacle in determining the role of FGF10 in the postnatal brain (Sekine et al., 1999). Additionally, within the current literature, embryonic analysis of FGF10 knockout mice, focuses on potential paracrine FGF10 signalling via FGFR1/2-IIIb (Ohuchi et al., 2000).

To explore the postnatal role of FGF10 *in vivo,* an inducible Fgf10 knockout mouse was used. The $Fgf10^{CreERT2/Floxed::Rosa26\text{-}tdTomato}$ triple transgenic mouse was developed by addition of an $Fgf10^{floxed}$allele, whereby LoxP sites flank exon 2 of the *Fgf10* locus (Abler et al., 2009). The $Fgf10^{floxed}$ allele is combined with both the $Fgf10^{CreERT2}$ and

Rosa26^{tdTomato} reporter to form the triple transgenic mouse model. The knocked-in CreERT2 construct already renders one copy of *Fgf10* non-functional, as determined by analysis of *Fgf10*^{CreERT2} homozygotes, which die at birth and present with characteristic Fgf10-knockout hallmarks such as absence of limbs (El Agha et al., 2012). Administration of tamoxifen at P4-5 permits the CreERT2 construct produced by Fgf10-expressing cells accesses to the nucleus. As a result, in P4-5 Fgf10^{+} cells the CreERT2 construct activates constitutive tdTomato expression, and excises exon 2 of the single functional *Fgf10* allele, resulting in constitutive Fgf10-knockout. This model results in knockout of *Fgf10* specifically within a population of cells that express Fgf10 at P4 and P5. Subsequent lineage tracing of these cells provides insight into the potential cell autonomous role of FGF10 within the postnatal cerebral cortex (**Fig. 3-1**).

Analysis of the *Fgf10*$^{CreERT2/+}$ mice provided an initial baseline understanding of FGF10^{+} cells in the cerebral cortex, in regard to their distribution, potential to amplify postnatally and cell type expression. This analysis acts as a control, to compare and contrast with the Fgf10 inducible knockout model (Fgf10$^{CreERT2/floxed}$) to gain insight into the role of FGF10 within this population.

5.2 Aims

To further elucidate the role of FGF10 in the postnatal cerebral cortex, by analysis of the lineage derived from *Fgf10* expressing cells following conditional *Fgf10* deletion by use of the *Fgf10*$^{CreERT2/floxed::Rosa26\text{-}tdTomato}$ triple transgenic mouse line. In comparison to *Fgf10*$^{CreERT2/+}$ controls, interrogate any changes in the quantity of tdTomato^{+} cells observed, their distribution within the cerebral cortex, and specific cell types by use of markers such as NeuN and GFAP for neurons and astrocytes respectively. Comparison of inducible knockout data with that obtained from *Fgf10*$^{CreERT2/+::Rosa26\text{-}tdTomato}$ controls will provide insight into the potential role of FGF10 within this population.

5.3 Results

5.3.1 Evidence for deletion of *Fgf10* exon 2

Due to the previously demonstrated unreliability of current canonical anti-FGF10 antibodies in detecting FGF10 specifically it was not possible to assess the impact of tamoxifen induction on levels of FGF10 protein in the *Fgf10*$^{CreERT2/floxed}$ model. In line with ongoing research within the lab on the hypothalamus, Dr Tim Goodman analysed both *Fgf10*$^{CreERT2/+}$ and *Fgf10*$^{CreERT2/floxed}$ genomic DNA in varying conditions with and without tamoxifen pulsing. By use of a panel of primers (**Fig. 5-2**), bands corresponding to *Fgf10* exon 2 deletion were only reliably identified in the RNA samples obtained from Fgf10$^{CreERT2/floxed}$ mice following tamoxifen induction. This indicates that *Fgf10*$^{CreERT2/floxed}$ specifically excises the floxed exon 2, and therefore induces *Fgf10* knockout following tamoxifen treatment (**Fig. 5-2A**).

5.3.2 Lineage tracing of *Fgf10* expressing cells with conditional *Fgf10* deficiency

To recapitulate conditions in Chapter 4 (**Fig. 4-1B**) *Fgf10*$^{CreERT2/floxed}$ pups were pulsed with tamoxifen at P4-5, and brains harvested at P7, P11 and P28. Brains were sectioned and probed with anti-Tomato-dsred, NeuN and GFAP antibodies to lineage trace descendants of *Fgf10* expressing cells with constitutive FGF10 deficiency. For the purposes of quantification all cortical containing sections were analysed, unless stated otherwise.

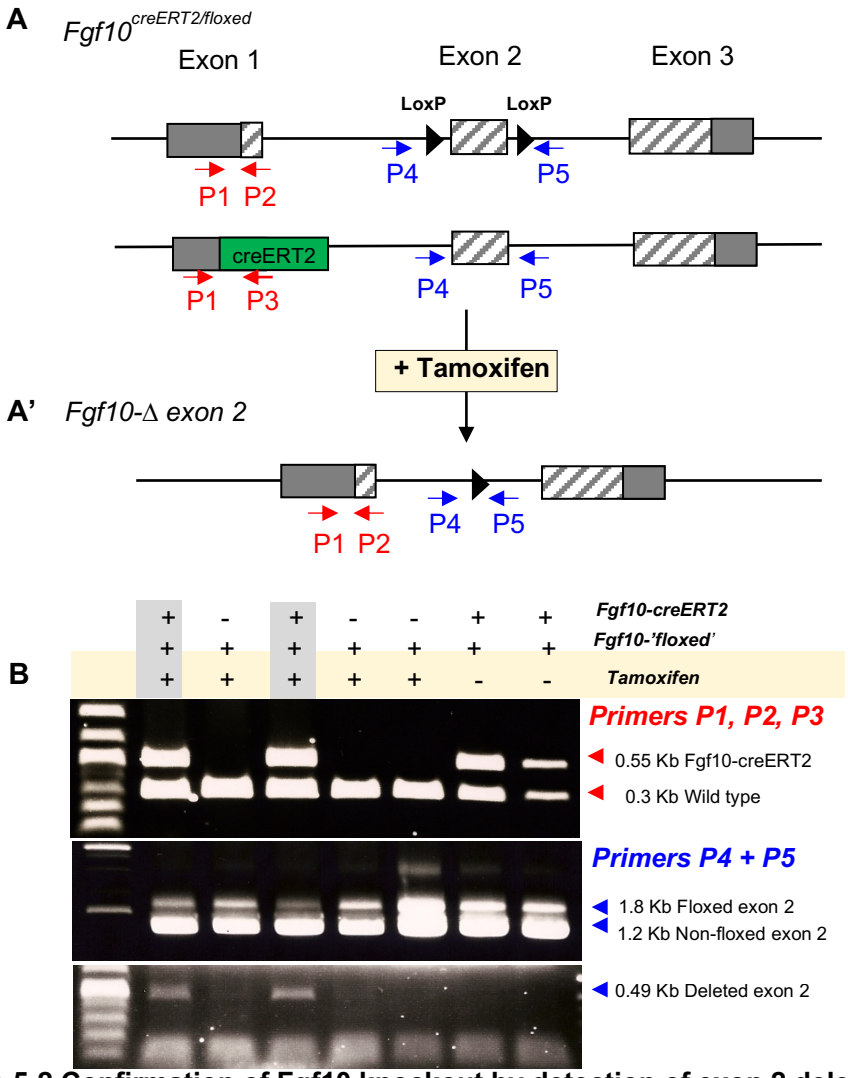


Figure 5-2 Confirmation of Fgf10 knockout by detection of exon 2 deletion band via RT-PCR: A panel of primers recognising regions within exon 1 disrupted by the creERT2 contruct (P1, P2, P3), and exon 2 flanking the LoxP sites (P4-P5) were designed (A). Analysis of DNA extracted from the hypothalamus shows that only in the Fgf10$^{CreERT2/floxed}$ mice with tamoxifen is a fragment corresponding to deleted exon 2 detectable (B). Data collected by Dr. Tim Goodman.

5.3.2.1 Fgf10 deficient Tom$^+$ cells can generate neurons and glial cells postnatally

At P7, P11 and P28 *Fgf10*-deficient Tom$^+$ cells were observed within the cerebral cortex with both neuronal (**Fig. 5-3A,C,E**) and glial (**Fig. 5-3B,D,F**) morphologies, occasionally associated with blood vessels (**Fig. 5-3F**). Often clusters of neurons were observed in columns reminiscent of embryonic radial migration from the VZ and SVZ (**Fig. 5-3A,B**) as well as clusters of neurons within the same cortical laminae and with similar morphologies (**Fig. 5-3C,E**). At P28, Tom$^+$ cells with both interneuron (**Fig. 5-3G**) and pyramidal (**Fig.5-3H**) morphologies were observed, occasionally as isolated cells. This suggests that conditional *Fgf10* knockout does not influence the ability of

lineage traced cells to persist and differentiate into both neurons and glial cells within the postnatal cerebral cortex.

5.3.2.2 Fgf10 deficient Tom+ lineage demonstrate a multipotent potential

As within the *Fgf10*$^{CreERT2/+}$ cerebral cortex, occasionally large mixed clusters of *Fgf10*-deficient Tom^{+} cells were observed in rostral regions of P7 (n=1) and P11 (n=1) brains. Given the scattered distribution of Tom^{+} cells within sections of cerebral cortex such large mixed clusters were striking, consisting of large numbers of Tom^{+} cells, some of which appeared closely associated. The *Fgf10*-deficient Tom^{+} cells within these clusters were often of both neuronal and glial cell morphologies. A small minority of Tom^{+} glial cells expressed GFAP and could hence be classified as astrocytes with clear blood vessel association (**Fig. 5-4**).

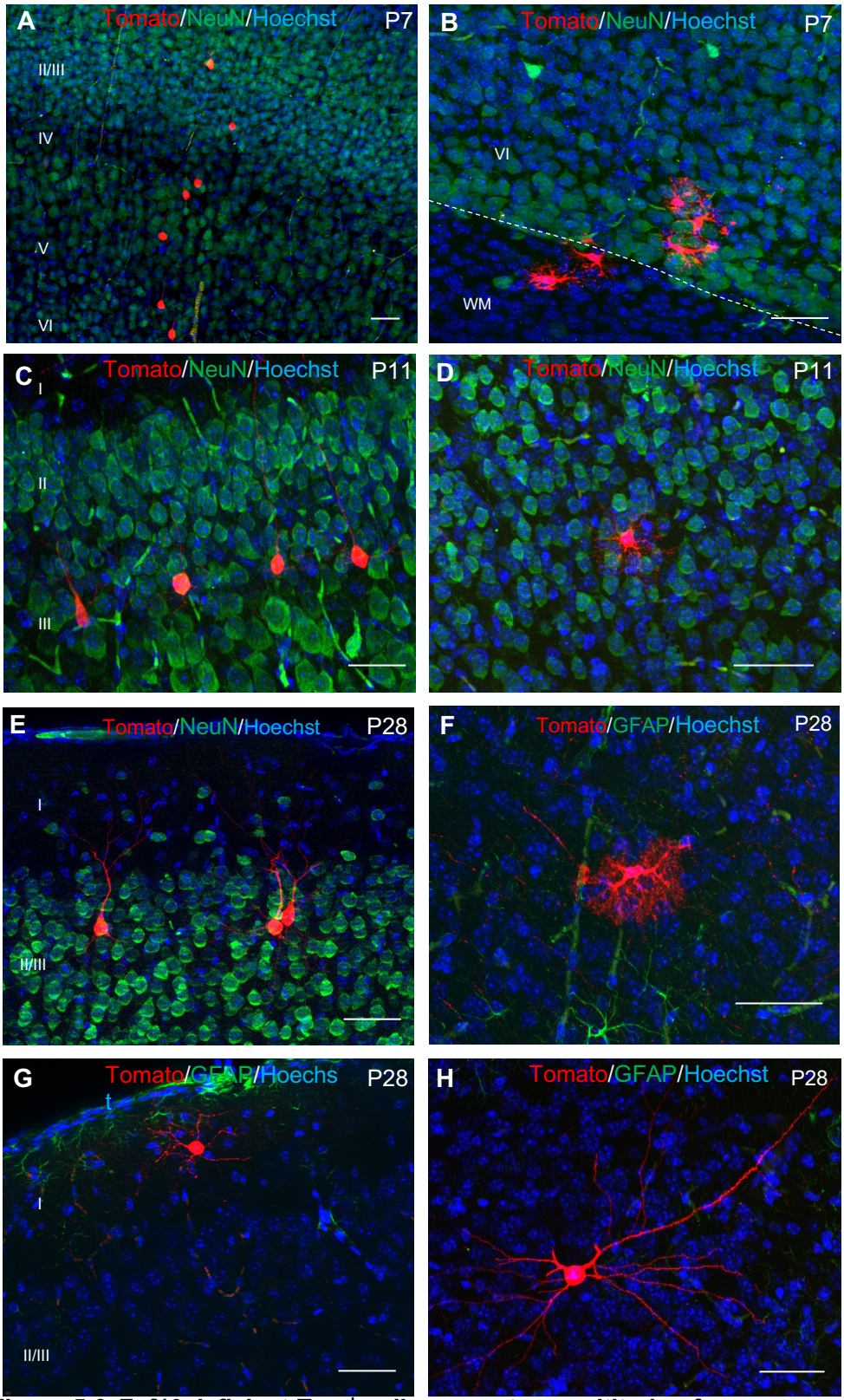


Figure 5-3 *Fgf10*-deficient Tom$^+$ cells generate a multitude of neurons and glia: At P7 Tom$^+$ cells have an immature morphology (A) with some rare glial Tom$^+$ cells (B). At P11 Tom$^+$ cells appear as pairs/clusters with similar morphological characteristics (C). Glial Tom$^+$ cells are also observed (D). At P28 Tom$^+$ cells take on more mature neuronal morphologies (E) rare Tom$^+$ glial cells were also observed with blood vessel association (F). Tom$^+$ neurons of both interneuron (G) and pyramidal neuron (H) morphologies were identified. Scale 50μm.

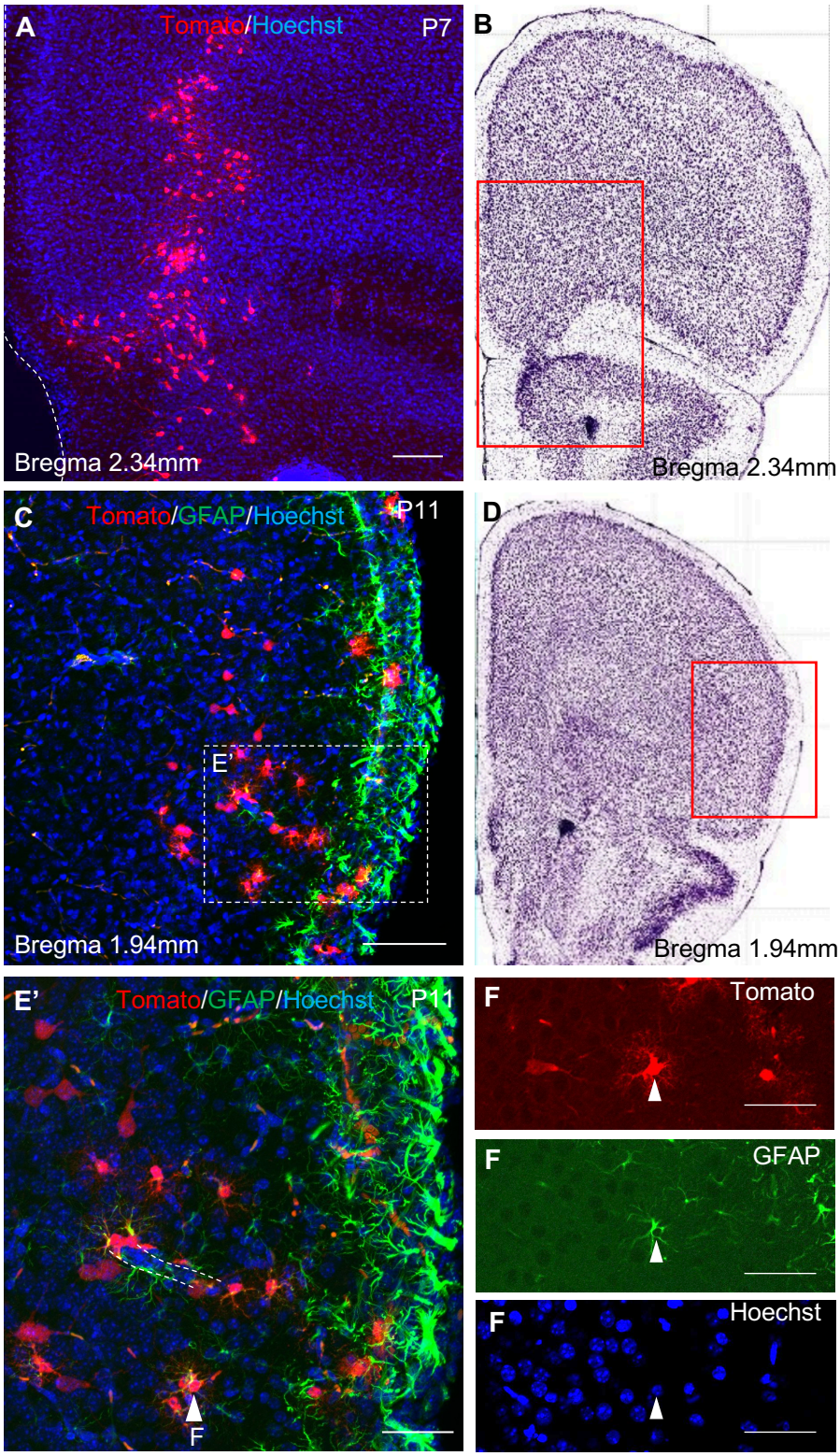


Figure 5-4 *Fgf10*-deficient Tom$^+$ cells present multipotent potential: Rare large mixed clusters of Tom$^+$ neurons, cells with glial morphology are observed in P7 (A-B) and P11 mouse cortices (C-D). Some GFAP$^+$/Tom$^+$ cells are GFAP$^+$ astrocytes with blood vessel association (E). Scale 100μm.

5.3.2.3 Fgf10-deficiency results in two waves of postnatal Tom+ cell amplification

The Tom+ cells with *Fgf10* deficiency significantly increase in number within the cerebral cortex between P7 and P11. A second wave of significant *Fgf10*-deficient Tom+ cell amplification occurred between P11 and P28 (**Fig. 5-5A**) to a level both significantly higher than that observed at P11 and P7. These two waves of Tom+ cell increase is highlighted in images of the retrosplenial cortex (**Fig. 5-5B-D**).

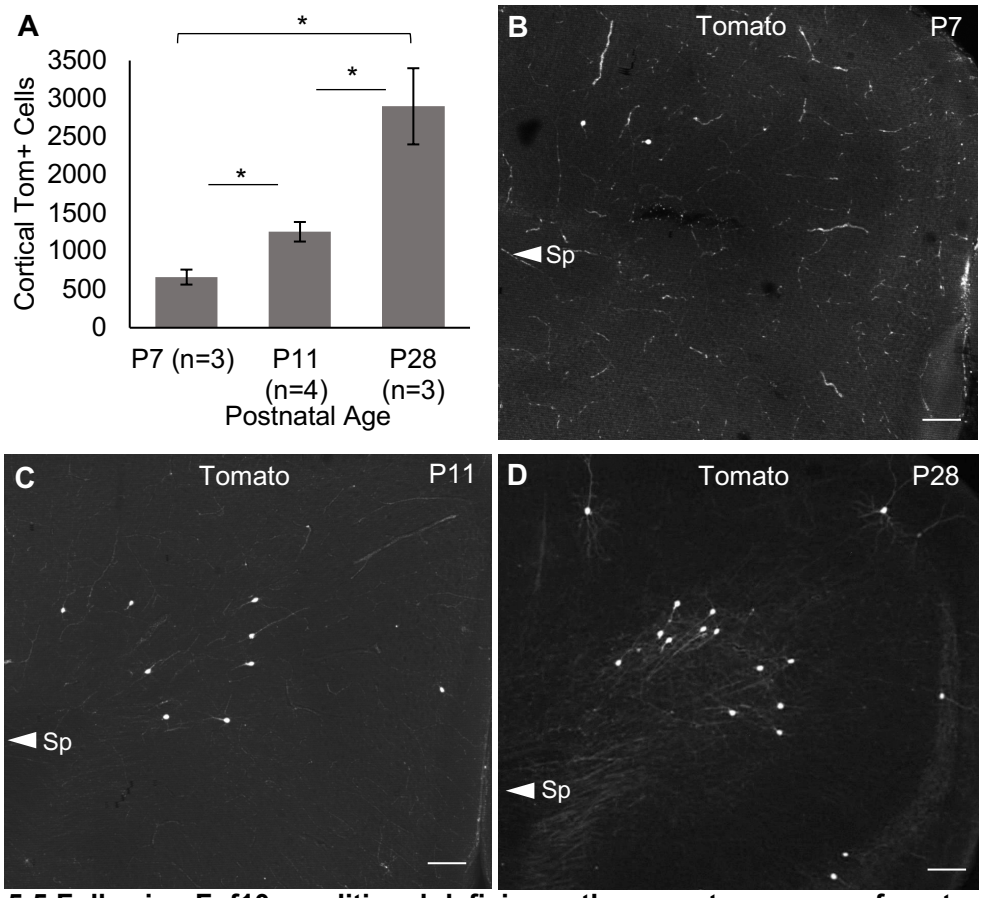


Figure 5-5 Following Fgf10 conditional deficiency there are two waves of postnatal Tom+ cell amplification: Following tamoxifen pulse at P4-5, Tom+ cells significantly increase in number between P7 and P11 (p=0.018), and again between P11 and P28 (p=0.014) to a level at P28 significantly amplified to that at P7 (p=0.012)(A). This Tom+ amplification is clearly visible within the retrosplenial cortex (B-D). Scale 100μm; Sp, Splenium. *= p<0.05.

5.3.2.4 Cortical Tom$^+$ cells amplify along the rostro-caudal axis and within specific cortical laminae beyond P11.

By use of the laminar categories as described in Chapter 4.3.2.2, it was observed that *Fgf10*-deficient Tom$^+$ cells significantly amplify between P7 and P11 within all cortical layers. However, the second wave of amplification between P11 and P28 only reaches significance in the deep cortical layers V-VI. Suggesting that there is either some migration of cortical Tom$^+$ cells into these layers, proliferation of Tom$^+$ cells selectively within deeper cortical laminae, or a reduction of cell death as a result of *Fgf10*-deficiency (**Fig. 5-6A**).

At P7, P11 and P28 *Fgf10*-deficient Tom$^+$ cells were found mostly within caudal cortical regions (Bregma -1.70 to -3.80mm). At variable Bregma regions significantly more Tom$^+$ cells are observed in P11 in comparison to both P7 and P28. However, due to large variation within P28 quantification, significance was not reached within many regions. Based on these trends it appears that amplification of *Fgf10*-deficient Tom$^+$ cells is pronounced between P7-P11 and P11-P28 in the more caudal cortical regions (**Fig. 5-6B**).

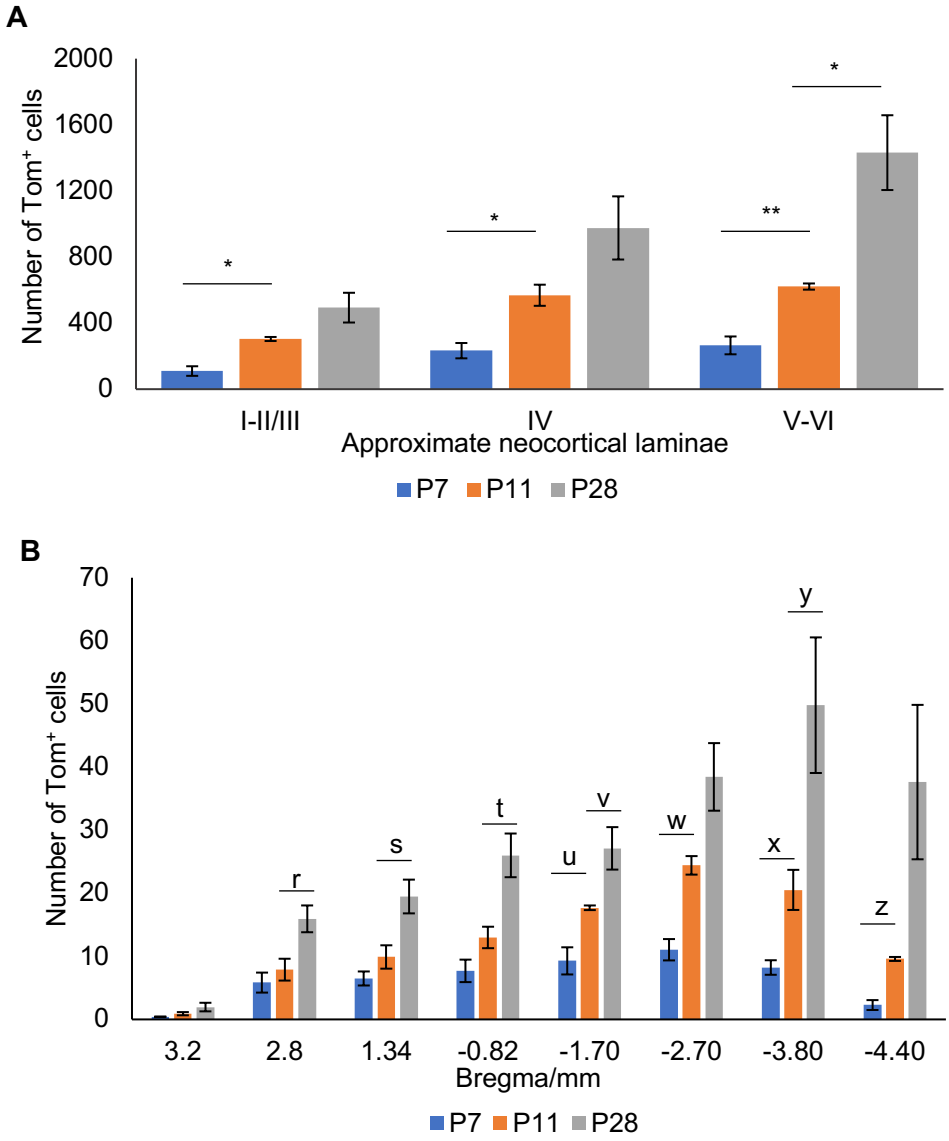


Figure 5-6 Rostro-caudal and laminar distribution of Tom$^+$ cells: *Fgf10*-deficient Tom$^+$ cells are distributed throughout all cortical laminae. Between P7 and P11 they amplify significantly within all laminar categories (I-II/III, p=0.015; IV, p=0.019; V-VI, p=0.0007), however, between P11 and P28 the only significant amplification is within layer V-VI (p=0.023)(A). Cortical Tom$^+$ cells across all postnatal ages analyzed are most concentrated in the caudal cortical regions (B). r, p=0.043; s, p=0.043; t, p=0.028; u, p=0.018; v, p=0.048; w, p=0.04; x, p=0.022; y, p=0.018; z, p=0.045. ** = p<0.001, *= p<0.05.

5.3.2.5 A majority of Fgf10-deficient Tom$^+$ cells differentiate into neurons

To analyse the ability of *Fgf10*-deficient Tom$^+$ cells to differentiate into neurons, NeuN immunolabelling was utilised. As with studies in chapter 3, alternating sections of cerebral cortex were immunolabelled for pan-neuronal marker NeuN. At P7, NeuN$^+$ Fgf10 deficient Tom$^+$ cells account for 70.4% (±2.41%) of the total, compared to 56.88% (±0.41%) in control Fgf10$^{CreERT2/+}$ indicating that at P7 the Tom$^+$/NeuN$^-$ pool at P7 is smaller with Fgf10 deficiency. At P28, 96.65% (±0.23%), of *Fgf10*-deficient Tom$^+$ cells are mature neurons, similar to the proportions observed in *Fgf10*$^{CreERT2/+}$ controls

(96.61% ±0.81%). This indicates that *Fgf10*-deficiency does not influence the ability of Tom^+ cells to differentiate into neurons, however the use of statistical analysis was limited due to insufficient n-numbers (**Fig. 5-7**).

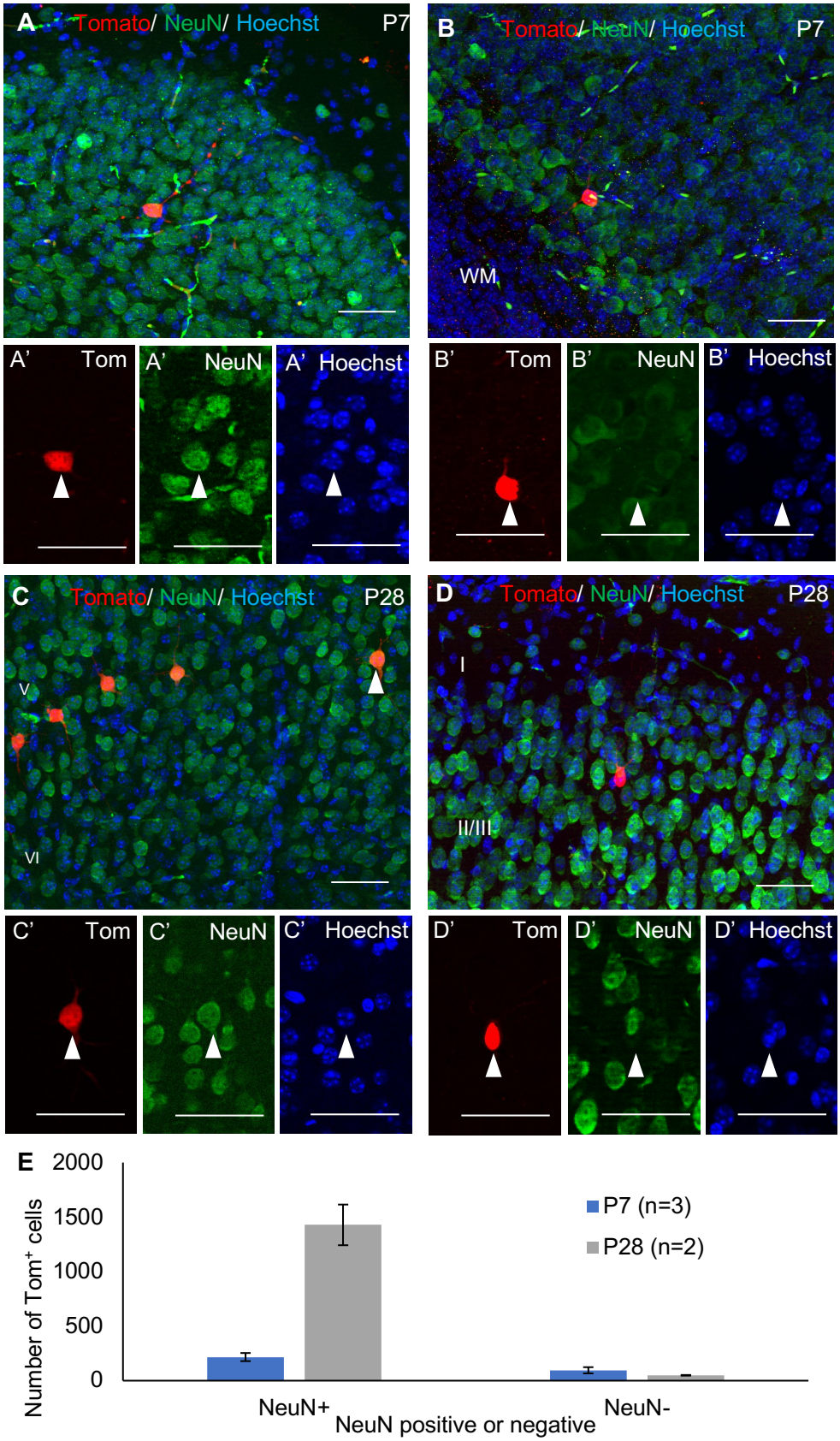


Figure 5-7 A majority of *Fgf10*-deficient Tom$^+$ cells differentiate into neurons: At P7 and P28 both NeuN$^+$ and NeuN$^-$ Tom$^+$ cells with a immature neuronal morphology are observed (A-D). Between P7 and P28 there is a substantial increase in Tom$^+$/NeuN$^+$ cells, and modest decrease in NeuN$^-$ cells, however more experimental repeats are required for statistical analysis (E). Scale 50μm

5.3.2.6 Deficiency of Fgf10 does not influence the distribution of Tom$^+$ cells in the cortex in comparison to Fgf10$^{CreERT2/+}$

Direct comparison of Fgf10$^{CreERT2/+}$ data to Fgf10$^{CreERT2/floxed}$ revealed there is no significant difference between the number of Tom$^+$ cells at P7 and P11 despite *Fgf10*-deficiency. This indicates that both genotypes have the same starting population, and both display an initial wave of Tom$^+$ cell amplification to the same degree. However, at P28 there are significantly more Tom$^+$ cells in the Fgf10$^{CreERT2/floxed}$ cerebral cortex than the Fgf10$^{CreERT2/+,}$ perhaps demonstrating an accumulative threshold effect of Fgf10 deficiency resulting in second distinct wave of Tom$^+$ cells much later in postnatal development (**Fig. 5-8A**). Interestingly the proportion of Tom$^+$ cells in the laminar categories at P28 across both genotypes appears unaffected by *Fgf10* deficiency, indicating that FGF10 is unlikely to have a role in migration of cells from this lineage within the cortical laminae (**Fig. 5-8B**). In terms of distribution of cortical Tom$^+$ cells at P28 throughout the rostro-caudal axis, a majority of Tom$^+$ cells still reside within the caudal cerebral cortex regions in both genotypes (-1.70mm to -3.80mm). Across all Bregma regions, with the exception of the most rostral, there are more *Fgf10*-deficient Tom$^+$ cells than in the control (**Fig.5-8C**). As before, comparison of corresponding Bregma regions containing the retrosplenial cortex regions from both Fgf10$^{CreERT2/+}$ and Fgf10$^{CreERT2/floxed}$ at P28 highlights this increase in Tom$^+$ cell number following conditional *Fgf10*-deficiency (**Fig. 5-9**).

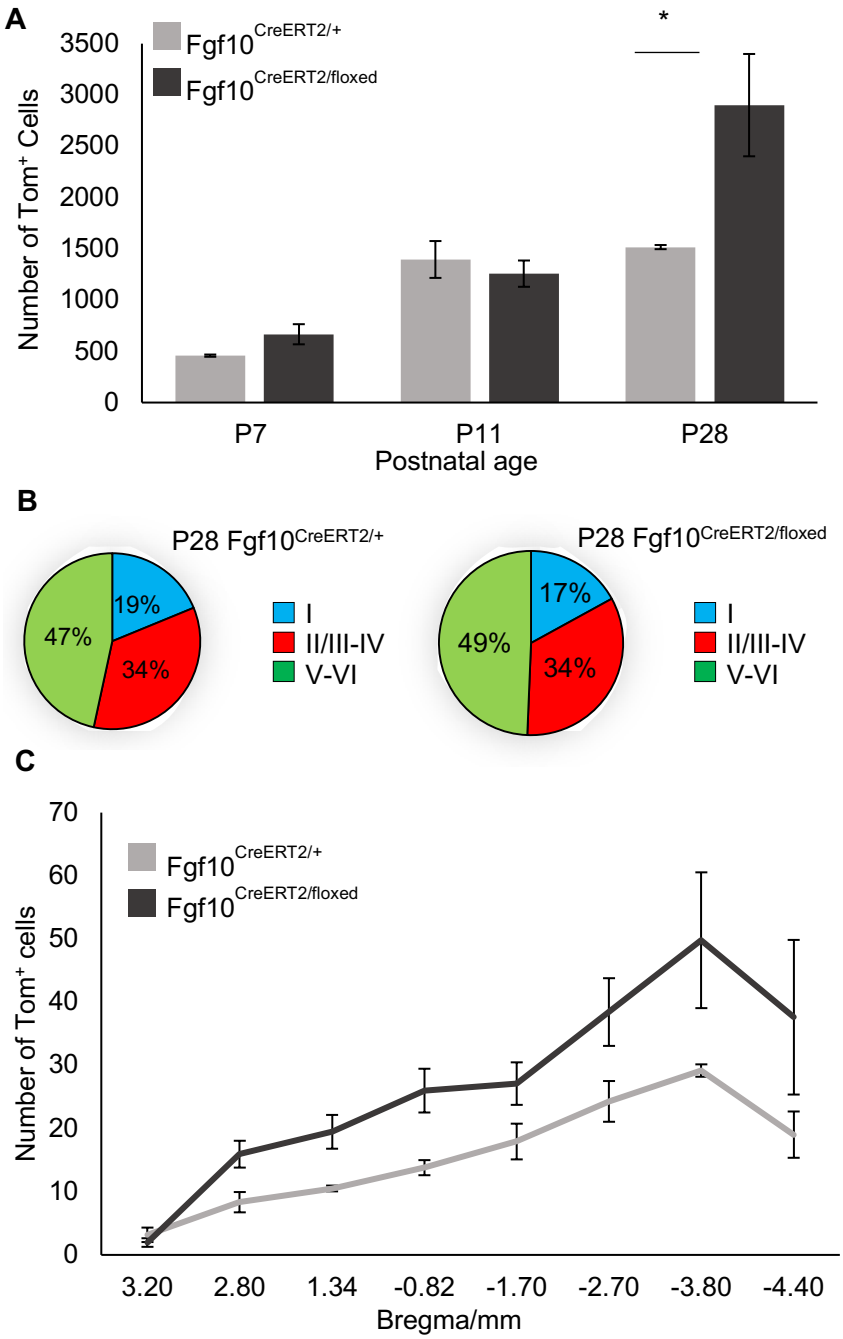


Figure 5-8 Fgf10 deficiency does not influence distribution of Tom$^+$ cells: Between P7 and P11 numbers of Tom$^+$ cells are not significantly different between Fgf10$^{CreERT2/+}$ and Fgf10$^{CreERT2/floxed}$ models (p=0.189 and 0.554 respectively), however at P28 there are significantly more Tom$^+$ cells in the Fgf10$^{CreERT2/floxed}$ cerebral cortex (p=0.012) (A). Laminar distribution at P28 is comparable between genotypes (B) as is their distribution across the rostro-caudal axis (C). *= p<0.05.

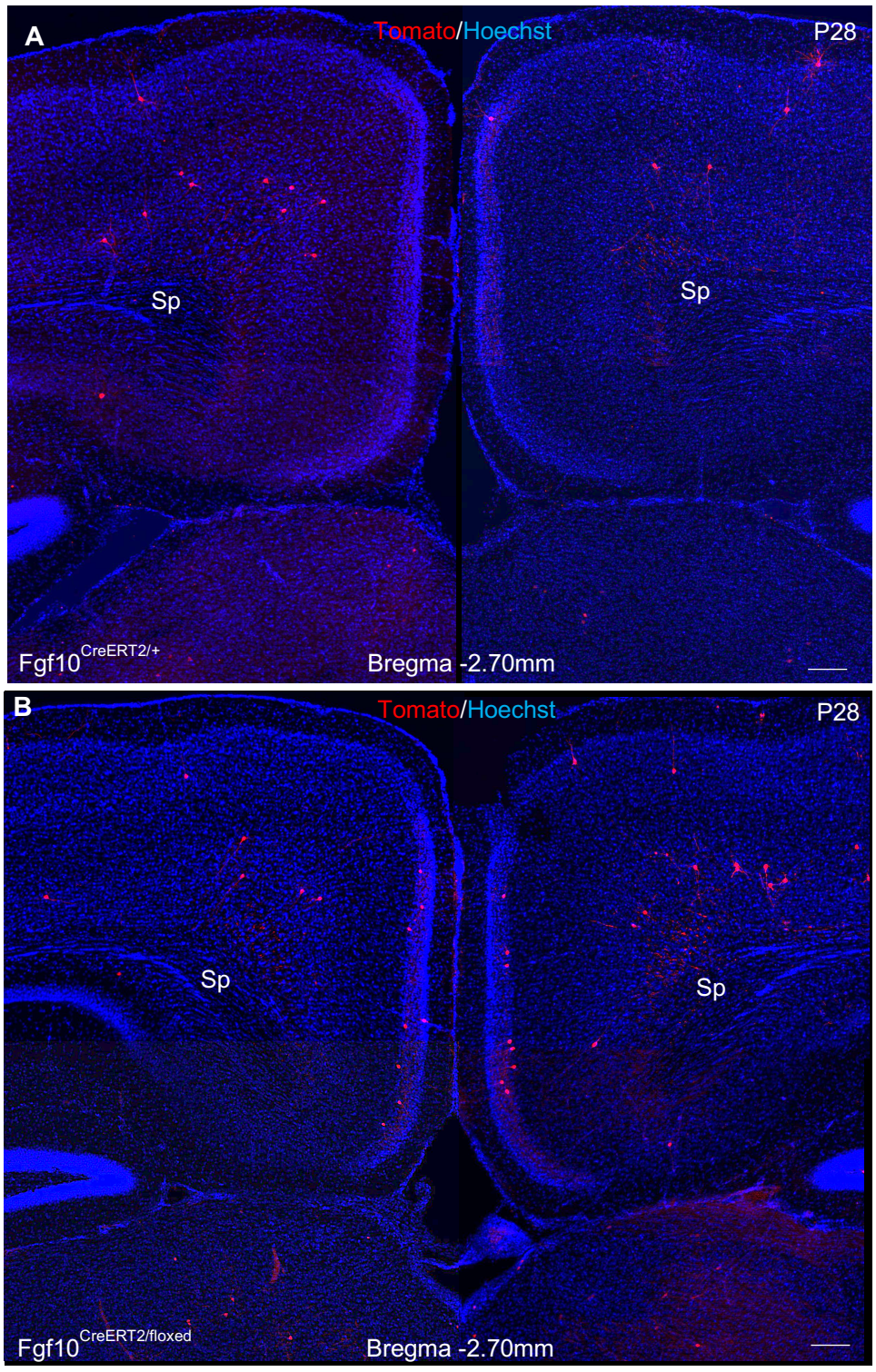


Figure 5-9 Amplification of *Fgf10*-deficient Tom$^+$ cells in the retrosplenial cortex at P28: Comparison of the *Fgf10*$^{CreERT2+}$ (A) and *Fgf10*$^{CreERT2/floxed}$ (B) retrosplenial cortices illustrates the striking amplification of Tom$^+$ cells within this region following conditional Fgf10 deficiency. Sp, splenium. Scale 100μm.

5.3.2.7 Fgf10-deficienct Tom$^+$ cells differentiate into interneurons

Glutamate decarboxylase (Gad67) was utilised as a marker for interneurons within the Fgf10$^{CreERT2/floxed}$ cerebral cortex, due to its role in the synthesis of GABA, it may be used as a pan-interneuronal marker (Pinal and Tobin, 1998; Rudy et al., 2011). Due to the high concentration of *Fgf10*-deficient Tom$^+$ cells within the caudal cerebral cortex, only caudal sections of n=2 P28 Fgf10$^{CreERT2/floxed}$ brains (Bregma -1.70mm to -3.80mm) were studied, as this would capture a majority of Tom$^+$ cells. With *Fgf10*-deficiency, a small minority of cortical Tom$^+$ GABAergic interneurons were identified at P28 (0.47% ± 0.09%) (**Fig. 5-10**). Although these results are not comparable to the *Fgf10*$^{CreERT2/+}$ data using NPY as a marker for a subtype of interneurons (**Fig. 4-7**), it does show that despite *Fgf10*-deficiency Tom$^+$ cells still generate interneurons.

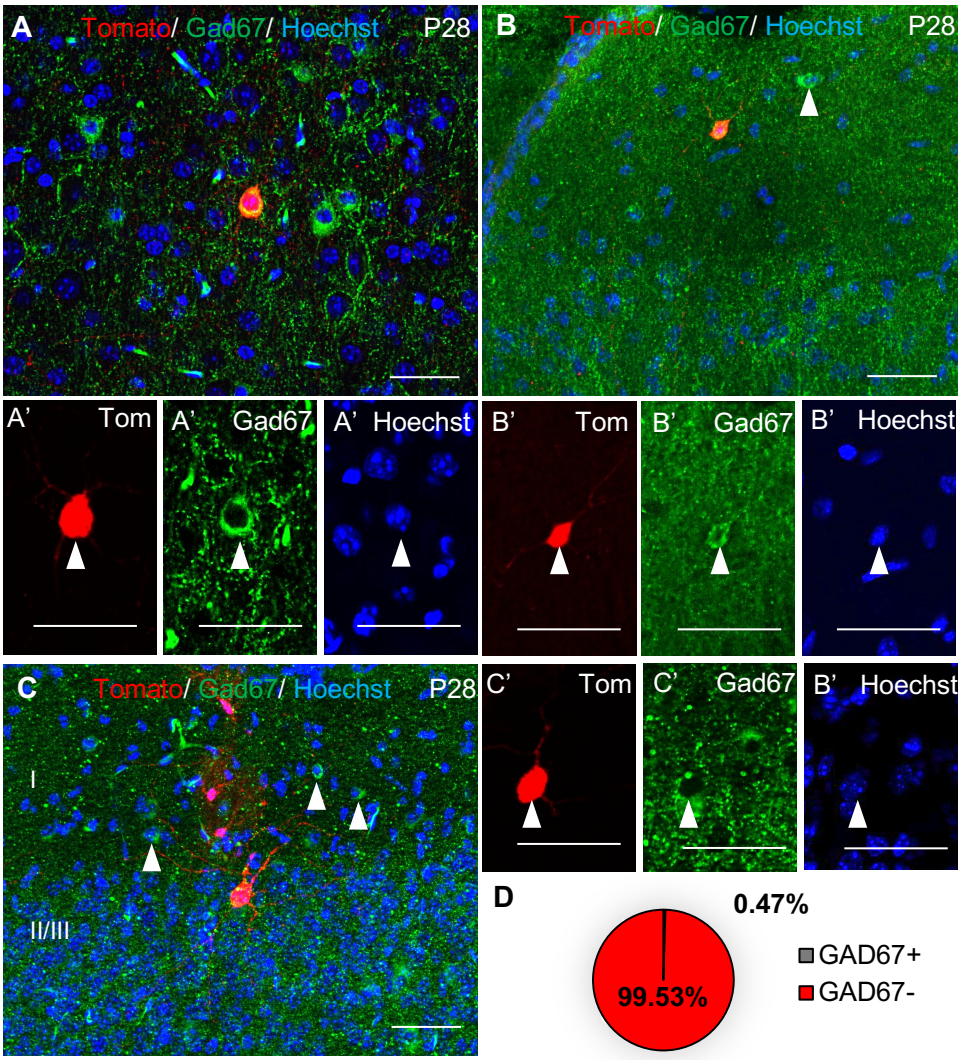


Figure 5-10 A minority of *Fgf10*-deficient Tom$^+$ cells differentiate into Gad67$^+$ interneurons: Tom$^+$ cortical interneurons (Gad67$^+$) are observed with Fgf10 deficiency (A-B), as well as Gad67$^-$ Tom$^+$ neurons (C). The Tom$^+$/Gad67$^+$ interneurons represent a minority of *Fgf10*-deficient Tom$^+$ cells (D). Scale 50μm. A, B and C arrowheads indicate Gad67$^+$ cells that are not Tom$^+$.

5.3.2.8 Fgf10 deficiency results in amplification of a TBR1$^-$ population

To analyse whether caudal *Fgf10*-deficient Tom$^+$ cells were altered in their ability to differentiate into glutamatergic neurons within the cerebral cortex, TBR1 immunolabelling was utilised. As with Gad67 analysis, P28 caudal cortical sections from *Fgf10*$^{CreERT2/floxed}$ brains were analysed (n=3). It was observed that 28.4% (±4.17%) *Fgf10*-deficient Tom$^+$ cells were TBR1$^+$ glutamatergic neurons at P28 (**Fig. 5-11A-C**), however significantly more were TBR1$^-$ (**Fig. 5-11D**). In direct comparison to *Fgf10*$^{CreERT2/+}$ data there was no significant difference between the number TBR1$^+$/Tom$^+$ cells observed in control and *Fgf10*-deficient cortices. Instead it appears that the *Fgf10*-deficient Tom$^+$ cell amplification is restricted to the TBR1$^-$ subtype, of which there are significantly more of in comparison to control at P28 (**Fig. 5-11E**).

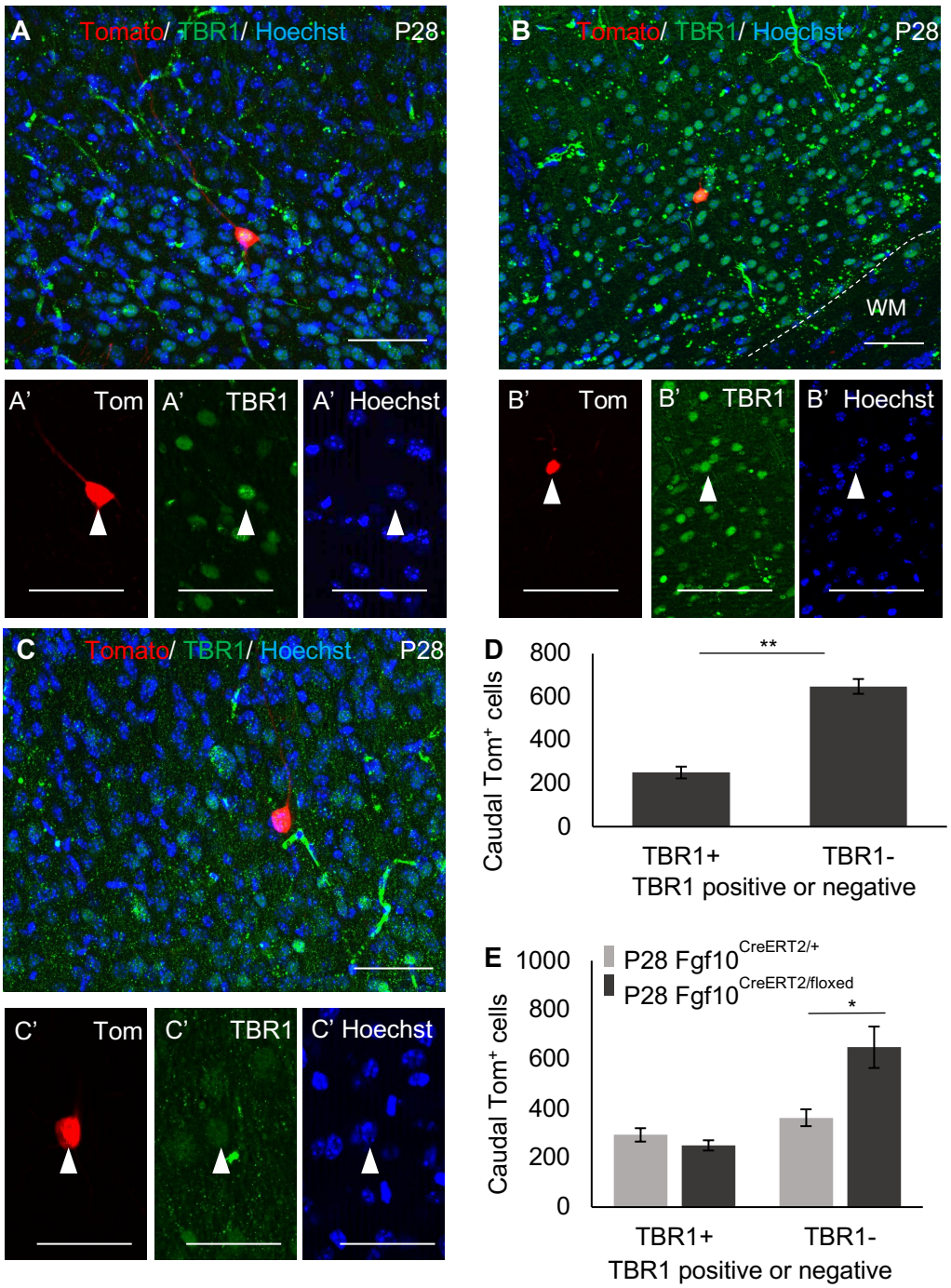


Figure 5-11 Fgf10 deficiency results in an amplification of Tom$^+$ cells determined to be TBR1$^-$: Both TBR1$^+$ and TBR1$^-$ *Fgf10*-deficient Tom$^+$ cells are observed within the cerebral cortex (A-C), with significantly more classified as TBR1$^-$ at P28 (p=0.001) (D). When compared with the *Fgf10*$^{CreERT2/+}$ there is no significant difference between the quantity of Tom$^+$/TBR1$^+$ cells (p=0.281), however the number of TBR1$^-$ cells is significantly higher in *Fgf10* deficiency (p=0.034) (E). Scale 50μm. **= p<0.01; *=p<0.05

5.3.3 Interrogation of Tom$^+$ cell proliferation with *Fgf10*-deficiency

In both *Fgf10*$^{CreERT2/+}$ and *Fgf10*$^{CreERT2/floxed}$ genotypes Tom$^+$ cells were often found in pairs of similar morphology, triplets and clusters greater than four, as well as single cells. By interrogation of Tom$^+$ cell pairs/clusters between P11 and P28 it is possible to gain broad insights in regard to their proliferative capacity and rounds of cell division *Fgf10*-deficient cells may potentially undergo between these timepoints. This was possible due to the observations that Tom$^+$ cells in both genotypes were sparsely distributed, combined with the observation of little to no evidence of Tom$^+$ cell migration between cortical laminae and within the rostro-caudal axis.

In order to analyse cell proliferation of *Fgf10*-deficient Tom$^+$ cells the marker PCNA was used to interrogate the caudal cerebral cortex for evidence of active cell division. Between P11 and P28 there is a significant increase in *Fgf10*-deficient Tom$^+$ cells which is not recapitulated in control *Fgf10*$^{CreERT2/+}$ data. Therefore, this amplification takes place as a result of *Fgf10* deficiency. Timepoints between P11 and P28 (P11, P15, P20 and P28), as well as a timepoint between P7 and P11 (P8) were analysed.

5.3.3.1 Tom$^+$ cells with Fgf10 deficiency likely undergo at least one additional cell division between P11 and P28

Analysis of Tom$^+$ cell clusters (>4), triplets (3), pairs (2) and single cells (1) was carried out for the entire cerebral cortex for entire *Fgf10*$^{CreERT2/+}$ and *Fgf10*$^{CreERT2/floxed}$ brains (n=3). Tomato$^+$ cells within 70µm of each other (based on measurements from the periphery of the somata via ImageJ software) were determined to be part of the same pair/cluster. Distances of more than 70µm was deemed a cut off and indicator for single Tom$^+$ classification (**Fig. 5-12A**).

Between P11 and P28 of the *Fgf10*$^{CreERT2/floxed}$ cerebral cortex there was a significant increase in the proportion of Tom$^+$ cells within pairs (p=0.025), and a trend for an increase in triplets and clusters of four or more that did not reach significance (p=0.64 and p=0.134 respectively). This is coupled with a significant decrease in the proportion of *Fgf10*-deficient Tom$^+$ cells observed as single cell 'clones' (p=0.018). This indicates that *Fgf10*-deficient Tom$^+$ cells are capable of undergoing at least one, cell division between P11 and P28 (**Fig. 5-12B-C**).

Comparably, the control *Fgf10*$^{CreERT2/+}$ brain did not show any significant differences between the proportion of Tom$^+$ cells within pairs, triplets, clusters greater than four and single cell 'clones' (p=0.162, p=0.675, p=0.191 and p=0.145 respectively) (**Fig. 5-12D-E**). This is consistent with the finding that there is no significant increase in Tom$^+$ cell number between P11 and P28 within control tissue (**Fig 4-4**).

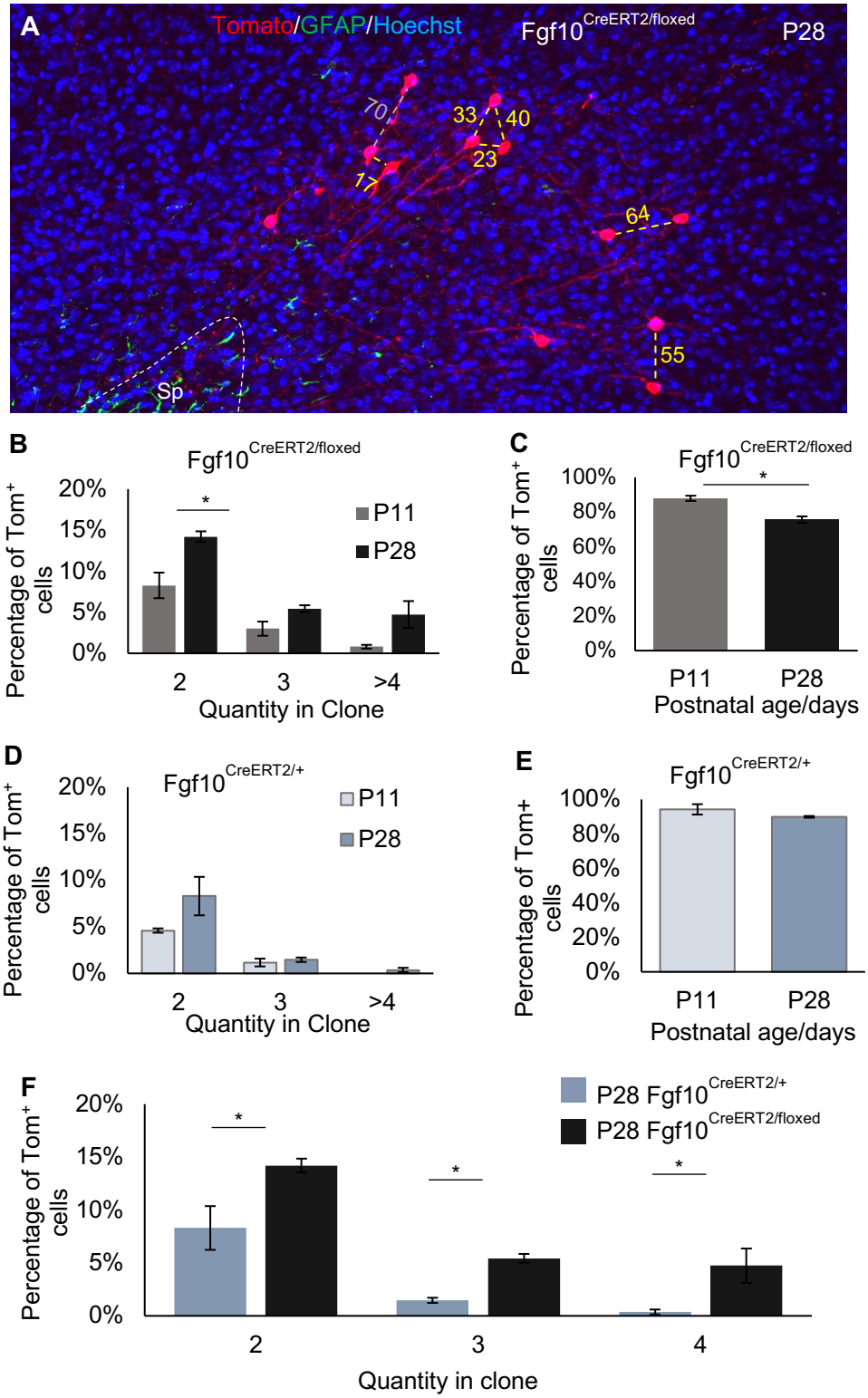


Figure 5-12 *Fgf10*-deficiency increases the proportion of 'clonal' clustered Tom$^+$ cell: The distance between Tom$^+$ cells was determined (A). Between P11 and P28 there is a significant increase in the percentage of *Fgf10*-deficient Tom$^+$ cells in pairs (B), and a significant decrease in proportion of single cell clones ($p<0.05$) (C). In Fgf10$^{CreERT2/+}$ tissue there was no significant differences between Tom$^+$ cells found in pairs or clusters (D) nor between single cell 'clones' (E). Direct comparison of P28 data shows that in the *Fgf10*$^{CreERT2/floxed}$ mice significantly more Tom$^+$ cells are found in pairs and clusters in comparison to *Fgf10*$^{CreERT2/+}$ (F). Measurements in µm. Sp, splenium. Tom$^+$ cells shown as a percentage of total Tom$^+$ cells. *=$p<0.05$.

5.3.3.2 The second wave Fgf10-deficient Tom$^+$ cell amplification occurs in late postnatal development

Analysis of PCNA expression within the cerebral cortex allows for snapshots of cortical proliferation to be captured at the point of culling the animal. Analysis of P8, P11, P15 and P20 *Fgf10*$^{CreERT2/floxed}$ caudal cerebral cortex covers time points where amplification of *Fgf10*-deficient Tom$^+$ cells occurs. In all conditions, the hippocampus was used as a region with high levels of cell proliferation and neurogenesis, and thus PCNA is expressed even into adulthood (Ino and Chiba, 2000; Kumar and Devaraj, 2012). At all ages analysed *Fgf10*-deficient Tom$^+$ cells are found in close association with PCNA$^+$ nuclei which may be reminiscent of a recent cell division (**Fig. 5-13**).

At P11 and P15 *Fgf10*-deficient Tom$^+$/PCNA$^+$ double labelled cells were observed, likely actively proliferating within the cerebral cortex (**Error! Reference source not found.**A-B). These cells were rare, although this study was confined to only caudal cortical regions and therefore may not be entirely representative. Quantification of caudal cerebral cortex Tom$^+$ cells with *Fgf10* deficiency highlights that between P8, P11, P15 and P20 there is no significant difference between Tom$^+$ cell number. However, comparison to P28 shows that even between P20 and P28 there is a significant amplification of *Fgf10*-deficient Tom$^+$ cells ($p<0.001$). This indicates that the second wave of *Fgf10*-deficient Tom$^+$ cell proliferation does not occur until after P20, which was not captured within the parameters of this analysis (**Fig. 5-14**).

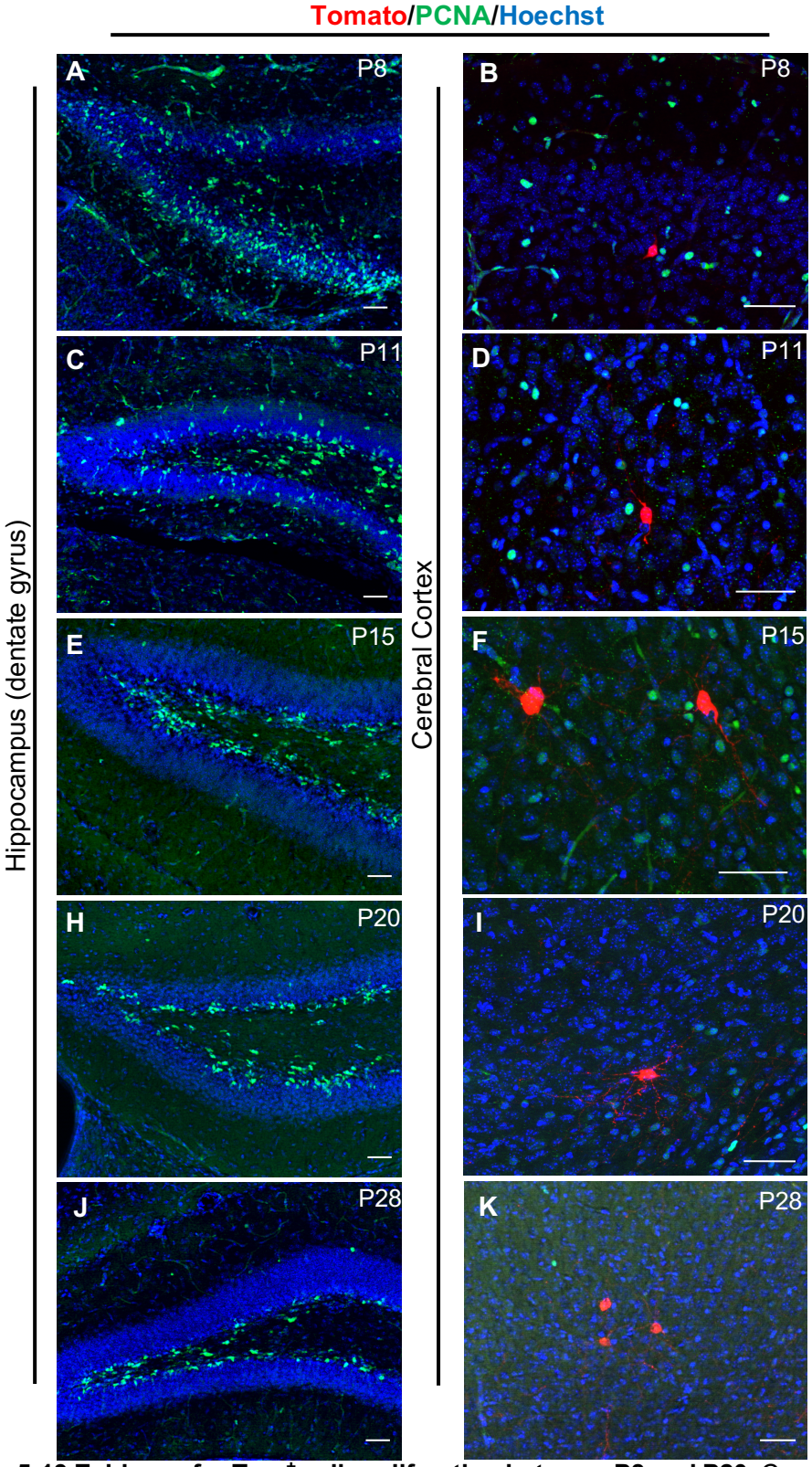
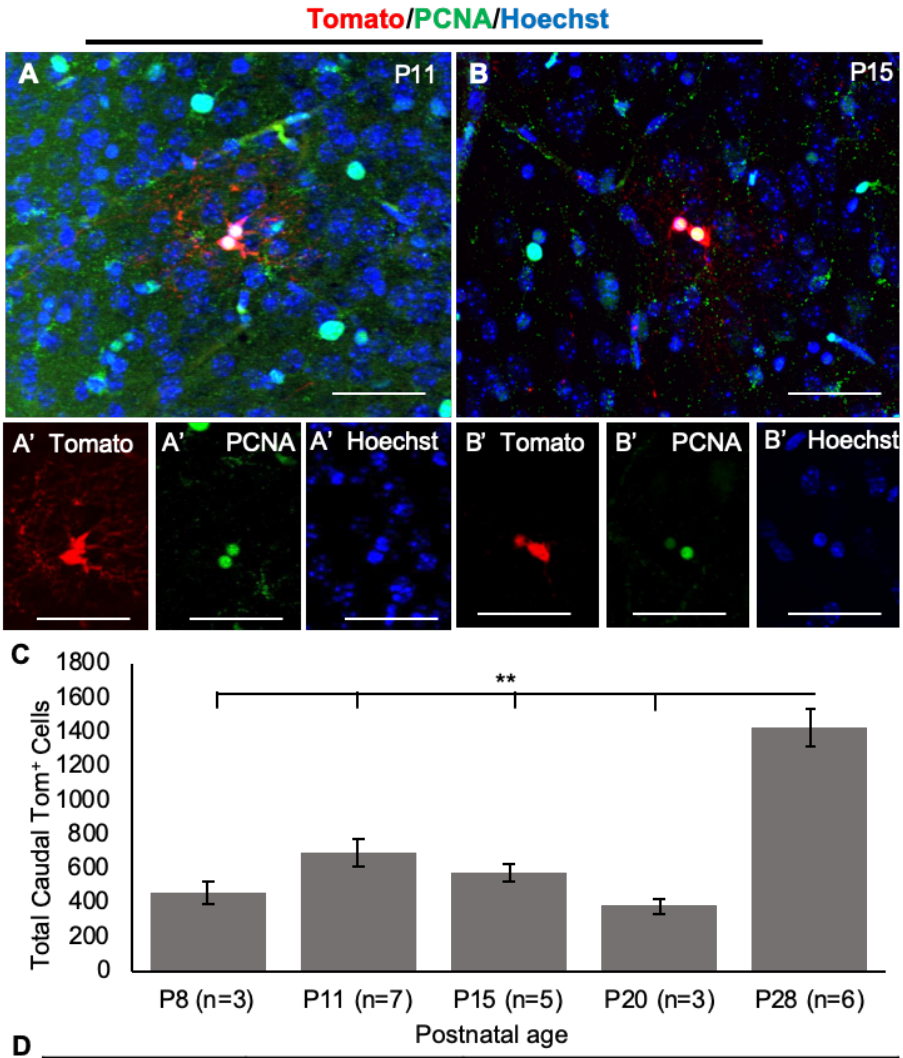


Figure 5-13 Evidence for Tom$^+$ cell proliferation between P8 and P20: Caudal cerebral cortex sections were interrogated for PCNA with the hippocampus as a control. Regularly Tom$^+$ cells were identified in close association with PCNA$^+$ nuclei, reminiscent of recent cell division. Scale 50μm.



D

Comparison	P-value	Significant (S) /Not Significant (NS)
P8-P11	0.313	NS
P8-P15	0.871	NS
P8-P20	0.998	NS
P8-P28	0.0001	S
P11-P15	0.787	NS
P11-P20	0.118	NS
P11-P28	0.001	S
P15-P20	0.572	NS
P15-P28	0.0001	S
P20-P28	0.0001	S

Figure 5-14 Rare actively dividing Tom$^+$ cells are observed at P11 and P15: At P11 and P15 rare Tom$^+$ cells with glial morphology were identified as PCNA$^+$ and thus actively proliferating (A-B). Analysis of caudal Tom$^+$ cell counts show that there is no significant difference between Tom$^+$ cell number between P8-P20, and a statistically significant difference between all postnatal ages and quantity of Tom$^+$ cells at P28, indicating that amplification of *Fgf10*-deficient Tom$^+$ cells occurs beyond P20 (C). The respective p-values from the statistical analysis are shown (D). Scale 50µm. **=p<0.01.

Based on protocol optimised by current laboratory members, a method whereby slices of brain tissue are suspended on inserts in culture was utilised. The fluorescent signal of tdTomato can be observed by live imaging at successive timepoints to trace the fate of *Fgf10*-deficient Tom$^+$ cells *ex vivo* for a short period of time. Based on viability of sections obtained P7 brains were required for set up following P4-5 tamoxifen pulsing. Individual sections were harvested and imaged every three hours with a 12-hour gap overnight due to the need to replace media and ensure tdTomato fluorescence is not quenched (**Fig. 5-15A**).

Preliminary data observed single live division of a *Fgf10*-deficient Tom$^+$ cells within the retrosplenial cortex (**Fig. 5-15B-D**). This suggests that *Fgf10*-deficient Tom$^+$ cells are able to divide, however further analysis centred around this experimental model is needed for definitive conclusions to be drawn.

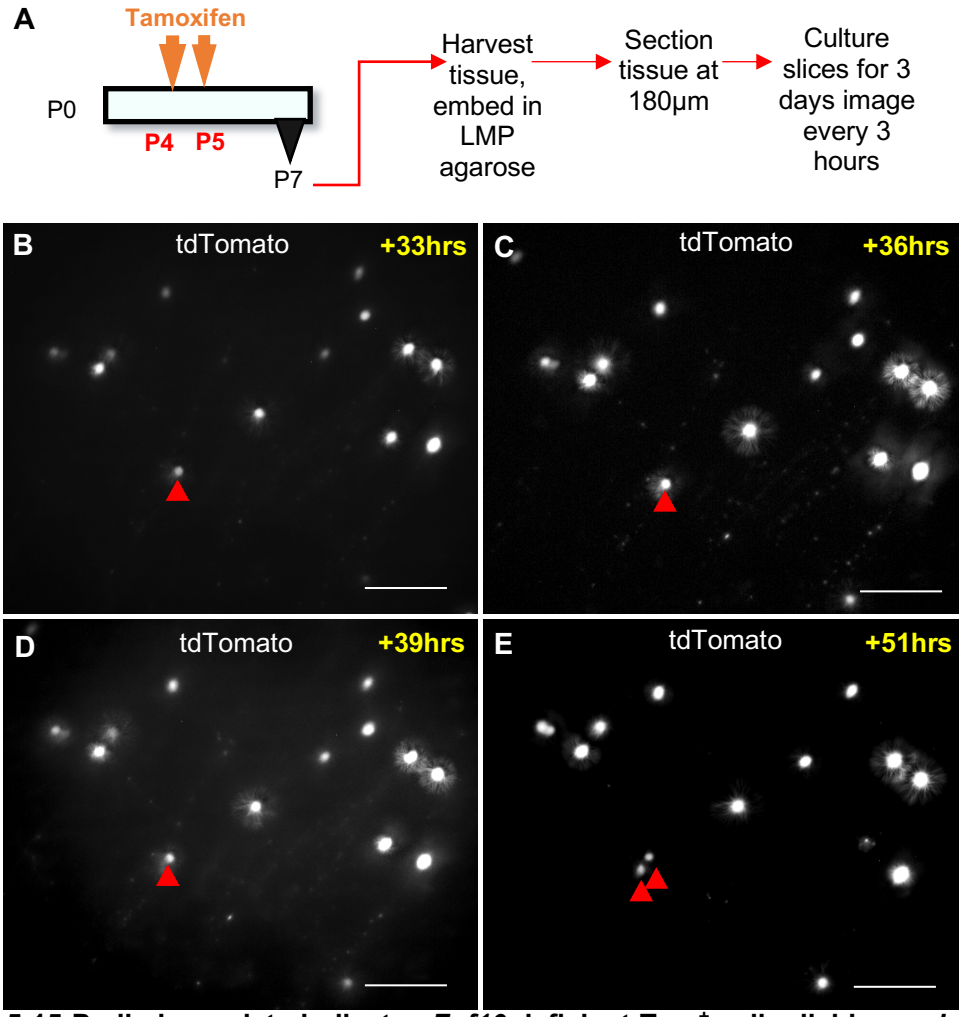


Figure 5-15 Preliminary data indicates *Fgf10*-deficient Tom$^+$ cells divide *ex vivo*: A model whereby slices of brain tissue can be cultured short term was devised by fellow laboratory member Stuart Nayar. Pups were pulsed with tamoxifen P4-5 and sacrificed at P7 for sectioning (A). Preliminary data captures division of *Fgf10*-deficient tdTomato$^+$ cells live (B-E). Scale 100µm.

5.3.4 Preliminary data indicates adolescent conditional *Fgf10* knockout does not impact laminar distribution of Tom$^+$ cells.

As was carried out in Chapter 4.3.4, *Fgf10*$^{CreERT2/floxed}$ mice were pulsed with tamoxifen via intraperitoneal injection in early adolescence (P27-28) and sacrificed at P42. Analysis was carried out to determine the laminar distribution, quantity and morphology of *Fgf10*-deficient Tom$^+$ cells within caudal sections, in comparison to *Fgf10*$^{CreERT2/+}$ controls. In terms of laminar distribution, a majority of Tom$^+$ cells inhabit the deeper neocortical laminar category (**Fig. 5-16A-B**), to a higher degree than that observed in Fgf10$^{CreERT2/+}$. Following conditional *Fgf10*-deficiency in adolescence, Tom$^+$ cells are still observable as interneurons and large pyramidal neurons clustered within deep cortical layers (**Fig. 5-16D-F**). Similarly, to that observed in age matched Fgf10$^{CreERT2/+}$ cortices, no Tom$^+$ glial cells are observed, indicating that in early adolescence absence of *Fgf10* does not influence differentiation into either neuronal or glial lineages. There does not appear to be a difference in total caudal cortical Tom$^+$ cell number between control and *Fgf10*-deficient conditions. However, this is likely to be due to a high degree of variability in *Fgf10*-deficient Tom$^+$ cell counts, which may be a result of inefficient intraperitoneal injection of tamoxifen, and would require repeat experimentation to conclusively determine and run statistical analysis (**Fig. 5-16C**)

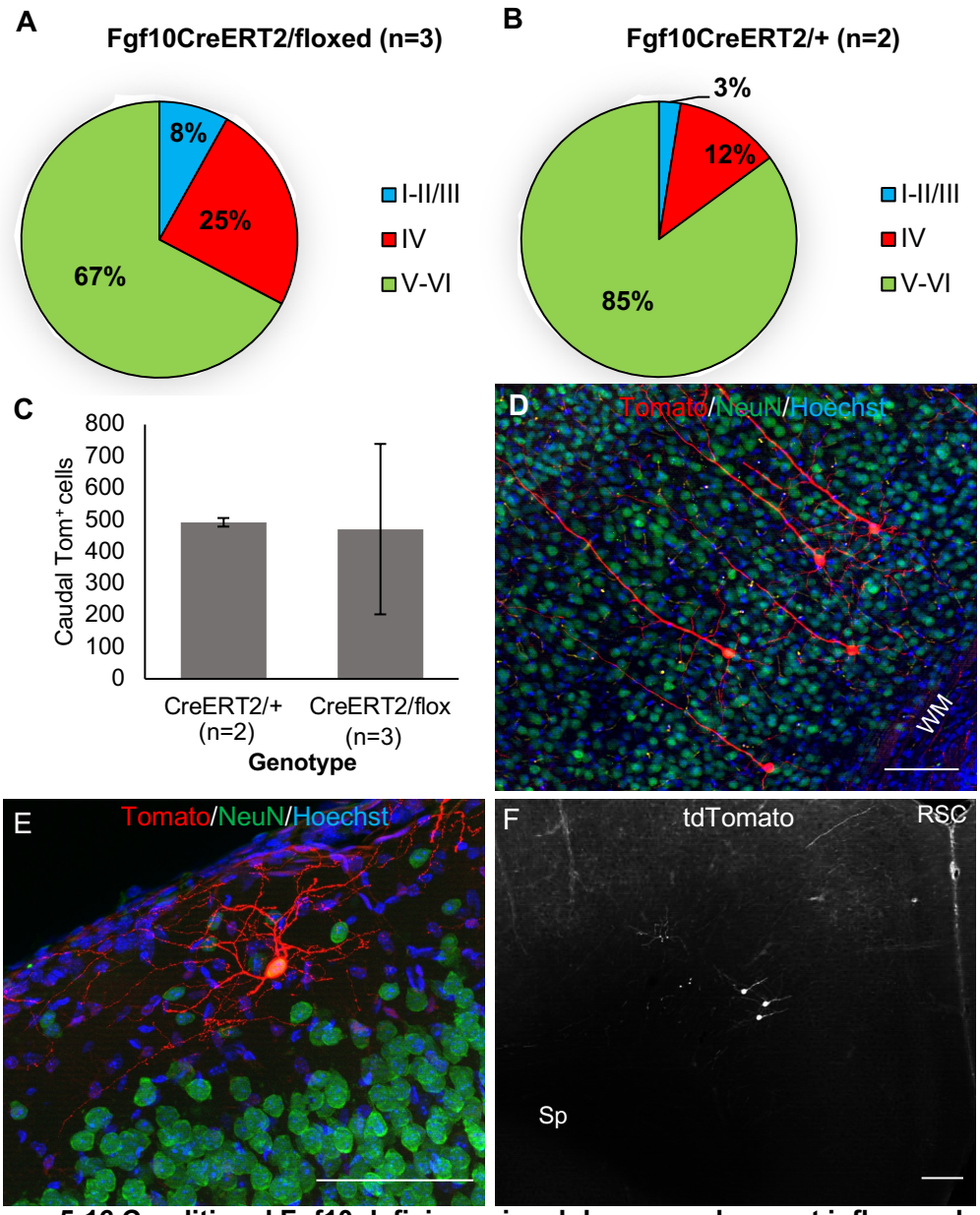


Figure 5-16 Conditional Fgf10 deficiency in adolescence does not influence laminar distribution: Tamoxifen pulsing at P27-P28 renders any FGF10^{+} cell *Fgf10*-deficient and Tom^{+}. This does not influence laminar distribution of Tom^{+} cells in comparison to *Fgf10*$^{CreERT2/+}$ (A-B). Total caudal *Fgf10*-deficient Tom^{+} cells is highly variable, with a high degree of overlap with *Fgf10*$^{CreERT2/+}$(C). Clusters of deep layered Tom^{+} pyramidal neurons (D), Cajal-retzuis interneurons (E) and *Fgf10*-deficient Tom^{+} cells in the retrosplenial cortex are observed (F). Scale 100µm. Sp, Splenium; RSC, retrosplenial cortex.

5.4 Discussion

This study sheds light onto the role of FGF10 within the initially explored P4-5 FGF10^{+} population in Chapter 3. Although varied studies highlight potential roles for FGF10 following pathological insult in the CNS and somatosensory dendritogenesis, its potential role in neurogenesis remains largely unexplored. To interrogate the function of FGF10 in the postnatal cerebral cortex, a triple transgenic model was utilised whereby tamoxifen induced constitutive conditional *Fgf10*-knockout and *tdTomato* expression specifically within the *Fgf10* expressing population at P4 and P5.

At all postnatal ages analysed, cells of neuronal (mature or immature) and glial morphology were observed. A proportion of Tom^{+} cells at P7 are NeuN^{+} and therefore mature neurons. The higher proportion of NeuN^{+} Tom^{+} cells in *Fgf10* deficiency in comparison to the *Fgf10*$^{CreERT2/+}$ control at P28 indicates that the residual NeuN^{-} cells are likely capable of neurogenic proliferation, to increase in quantity by such a large degree by P28. Under physiological conditions the amplification of Tom^{+} cells is largely lineage restricted to cortical neurons (NeuN^{+}). Hence, further analysis should interrogate tdTomato^{+} cells for markers of neuroblasts such as doublecortin rather than radial-glia markers.

Recent research has focused on the markers intrinsic fate restriction of progenitors in embryonic development. For example, broadly speaking the sequential expression of Pax6, TBR2 and TBR1 by RGCs, IPCs and post-mitotic neurons respectively (Englund et al., 2005). In terms of laminar specification, embryonic studies have shown that the zinc-finger transcription factor *Fezf* regulates a decision between subcortical and callosal projection neuron fates within neocortical layer 5 and 6. Callosal neurons connect both hemispheres of the cerebral cortex via the corpus callosum, and require expression DNA binding and chromatin remodelling factor *Satb2* for their specification by repressing the activity of the transcription factor Ctip2 (Alcamo et al., 2008). Subcortical projection neurons on the other hand extend an apical dendrite to layer one, and an axon to regions such as the thalamus, brainstem and spinal cord. The knockout of *Fezf* results in a fate change of subcortical neurons to callosal projection neurons, a process that can be rescued by ectopic expression of downstream transcription factor Ctip2 (Chen et al., 2008). Further analysis into the Tom^{+} cells in regard to their molecular profile should be conducted to analyse if any fate-change is induced following conditional knockout of *Fgf10* in comparison to control.

Between P11 and P28 there is a significant increase in Tom^{+} cells found within deep cortical layers. This could be a result of increased short distance migration of neurons

to deep cortical layers, or that in the absence of FGF10 in deep layer quiescent neuroblasts proliferate, predominantly within layers V and VI. Canonically deep layer neurons are born earliest, as embryonic development progresses progenitors become more restricted to give rise to upper layer neurons (Desai and McConnell, 2000). Studies show that there is a postnatal contribution of neurons specifically into the caudal cerebral cortex by a subset of PDGFRβ expressing cells within the meninges (Bifari et al., 2017). These cells migrate via the ventricular zone firstly into the deep cortical parenchyma and settle between layers II-IV, where it is suggested they remain largely quiescent. A majority of the meningeal derived neurons are $Satb2^{+}$, and are found within the caudal cortical regions, predominantly within the retrosplenial and visual motor cortex, showing a striking similarity to the distribution of Tom^{+} cells where amplification following *Fgf10* deficiency is prominent. At P15 a small fraction of meningeal derived cells within the cortex did not express NeuN, which may indicate they are not yet post-mitotic. It is possible that *Fgf10* has a role in differentiation of this meningeal derived population, which in its absence proliferate either within the meningeal niche, or within the cortical parenchyma itself.

At P7 and P11 the quantity of Tom^{+} cells within both genotypes is not statistically significantly different, indicating that irrespective of *Fgf10*-deficiency, amplification of Tom^{+} cells between P7 and P11 occurs to the same degree. However, between P11 and P28 with *Fgf10*-deficiency there is a second wave of Tom^{+} cell amplification resulting two-fold more *Fgf10*-deficient Tom^{+} cells at P28 . Despite this large increase in Tom^{+} cells with *Fgf10* deficiency, the proportion of which are $NeuN^{+}$ in comparison to $NeuN^{-}$ at P28 is not significantly different to that observed in $Fgf10^{CreERT2/+}$. This further indicates that *Fgf10*-deficiency only influences quantity of lineage-traced cells rather than their differentiation potential.

In 2009 Bandeira et al. noted that there were two waves in rat postnatal development where new neurons were added to the cerebral cortex, between which is a substantial decrease. From P3 to P7 the net number of cortical neurons doubles by neurogenesis, identified by BrdU and NeuN double labelling when pulsed at P4 and culled at P5. In the second postnatal week however the number of cortical neurons decreases (P7 to P15), consistent with known mechanisms of cell death thought to function in regulation of neuronal number and connectivity, in a process known as synaptic pruning (Clarke, 1985). Such regressive events such as programmed cell death, synapse pruning and elimination are required in proper brain development for generation of precise and mature circuitry (Vanderhaeghen and Cheng, 2010). Following this, the neuronal cell number within the cerebral cortex increased again between P15 and adulthood

(Bandeira et al., 2009). In mice, a similar increase in cortical neurons is observed, which continues to P16, following which there their proportion decreases. The study also identifies that at P8 only 65% of cortical neurons expressed NeuN and hence post-mitotic neurons (Lyck et al., 2007). Given that in both cases proliferation preceded neuronal elimination, amplification of Tom^+ cells between P11 and P28 may be a result of in increased survival, or increased proliferation within the cerebral cortex in the absence of FGF10, in line with postnatal cortical expansion.

Analysis of the P28 caudal cerebral cortex showed that a proportion of *Fgf10*-deficient Tom^+ cells are $TBR1^+$ glutamatergic neurons. Direct comparison with control $Fgf10^{CreERT2/+}$ analysis shows that there are significantly more $TBR1^-/Tom^+$ cells in the *Fgf10*-deficient condition. This indicates that amplification of *Fgf10*-deficient Tom^+ cells gives rise to predominately $TBR1^-$ population, whilst the $TBR1^+$ population remains stable and not significantly different from $Fgf10^{CreERT2/+}$. From this data it is not possible to deduce that *Fgf10*-deficient $Tom^+/TBR1^-$ cells are non-neuronal, nor that they are not glutamatergic. However as noted within Chapter 3, this further reinforces the hypothesis that postnatal amplification of the Tom^+ lineage does not follow the canonical embryonic Pax6 – TBR2 – TBR1 progression (Englund et al., 2005). As mentioned previously the new-born $Satb2^+$ cells within the caudal/retrosplenial cortex of either meningeal or VZ origin demonstrate a similar distribution to *Fgf10*-deficient Tom^+ cells. Therefore, *Fgf10*-deficient Tom^+ cells should be probed for expression of Satb2 as a marker of post-mitotic neurons, and progenitor expressed transcription factor Fezf2 in further studies between P11 and P28 (Bifari et al., 2017; Zgraggen et al., 2012).

To interrogate amplification of Tom^+ cells following *Fgf10*-deficiency, as before PCNA was utilised as a proliferative marker, largely due to the incompatibility of BrdU immunolabelling and tdTomato. Often, *Fgf10*-deficient Tom^+ cells were observed in close association with $PCNA^+$ nuclei, which may be suggestive of a recent cell division. At P11 and P15 rare $PCNA^+$ Tom^+ cells were observed following *Fgf10*-deficiency, providing evidence that cells derived from an *Fgf10* expressing lineage are able to proliferate postnatally following conditional *Fgf10*-knockout.

Analysis of cortical PCNA expression following *Fgf10*-deficiency was focused within the caudal cerebral cortex where a majority of Tom^+ cells reside, and between P11 and P20 to capture the second wave of Tom^+ cell amplification. Analysis of caudal cerebral cortex Tom^+ cell number indicated that the amplification between P11 and P28 does not occur until after P20, and therefore will not have been captured by the

parameters of this analysis. Postnatally there is a large contribution of astrocytes and oligodendrocytes to the postnatal cerebral cortex. Specifically $NG2^+$ precursors migrate, proliferate and differentiate into oligodendrocytes throughout postnatal/adult rodent life (Hughes et al., 2013; Kessaris et al., 2006). Interestingly some $NG2^+$ precursors within the cerebral cortex have been shown as capable to differentiate into neurons following injury in a process mediated by Sox2 (Heinrich et al., 2014). It is tempting to speculate that conditional knockout of *Fgf10* in rare Tom^+ cells with glial morphology, which may be $NG2^+$ progenitors result in a fate change of these cells to generate new neurons beyond P20. In order to determine this conclusively, the use of synthetic thymidine analogues such as EdU, which do not require HCl mediated DNA denaturing treatment should be utilised in combination with NG2 specific antibodies. This approach would allow for analysis of proliferation within longer periods of time by daily administration and short chases, rather than 'snap-shots' of proliferation determined by the age of sacrifice.

This study captures the impact of conditional *Fgf10*-knockout specifically within a population of cells which under normal conditions express *Fgf10*. Therefore, the cell autonomous autocrine function of this protein. This is further corroborated by the observation that neither of the FGFRs that FGF10 specifically signals via are expressed within the cerebral cortex at the time of tamoxifen administration (Ch.3 Fig1). However, signalling of *Fgf10* via long-distance sub-cortical connectivity of neurons, and the potential changing landscape of FGFR expression within the postnatal development of the cerebral cortex cannot be ruled out.

In summary, *Fgf10*-deficiency in Tom^+ cells conditional at P4-5 by tamoxifen administration results in two waves of lineage amplification. An initial wave between P7 and P11, in line with that observed in the $Fgf10^{CreERT2/+}$ control, and a second occurring after P20, resulting in significantly more Tom^+ cells when quantified at P28 without an effect on distribution.

6 FIBROBLAST GROWTH FACTOR-10 IS NOT EXPRESSED WITHIN THE EMBRYONIC DORSAL TELENCEPHALON UNTIL LATE DEVELOPMENT

6.1 Introduction

The cerebral cortex develops from the dorsal embryonic telencephalon and is comprised of two broad neuronal subtypes, glutamatergic projection neurons and inhibitory GABAergic interneurons, which are widely believed to be exclusively born embryonically. Projection neurons and interneurons are derived from two distinct origins, the embryonic ventricular and subventricular zones (VZ and SVZ) and ganglionic eminences (GE) respectively. Pyramidal neurons are derived both directly and indirectly (via IPCs) and RGCs, which differentiate from neuroepithelial cells early in embryonic development. These cells have cell bodies which inhabit the ventricular zone and bipolar processes that reach to the ventricular surface and pial surfaces. As well functioning as neural progenitors, RGCs act as a scaffold along which new-born pyramidal neurons may migrate radially along into the cortical plate (Rakic, 2003, 1990, 1974). Interneurons on the other hand are largely derived from a region of ventral telencephalon, the ganglionic eminences (GE), and migrate long distances tangentially into the developing cortical plate. The GE can be subdivided into the medial, lateral, caudal regions, all of which contribute varied heterogenous populations of interneurons to the cerebral cortex, and thus large diversity of cortical interneurons identifiable (Lim et al., 2018; Wonders and Anderson, 2006).

The six layered neocortex of the mouse develops in an inside-out manner, with layer IV containing the oldest neurons, and layer II the newest. Neurogenesis within the mouse cerebral cortex begins at E11.5, after there has been substantial expansion of the progenitor pool, and ceases at E17 (Takahashi et al., 1996). Mouse cortical neurogenesis is followed by generation of astrocytes and oligodendrocytes in a phenomena known as neurogenic-to-gliogenic switch, in which gliogenesis peaks postnatally. (Bayer and Altman, 1991; Qian et al., 2000). Astrocytes are derived from the trans-differentiation of radial glia, at the end of cortical neurogenesis, with a single radial glia being able to give rise to multiple astrocytes within the cerebral cortex (Ge et al., 2012b; Gressens et al., 1992). In the rodent, oligodendrocyte precursors (OLPs) and oligodendrocytes (OLs) enter the cerebral cortex in waves, the first of which is derived from the medial ganglionic eminences, a second from the lateral ganglionic eminences and a third from endogenous precursors within the cortex after birth (Kessaris et al., 2006).

Embryonic cortical development is dependent on a complex symphony of signals. An example of which is the signalling molecule reelin, produced by the Cajal-Retzius cells within layer I/the molecular zone, and GABAergic interneurons spanning layers II-VI.

Reeler mutant mice show an inverted cortical lamination pattern, whereby neurons destined for deeper layers occupy superficial positions, and later born neurons fail to surpass them (Gil-Sanz et al., 2013). The expression of reelin is therefore crucial factor in radial migration. It is known that FGFs have roles in cortical patterning and neurogenesis. For example, FGF8 and FGF17 cooperatively form a gradient of high expression in the anterior cortex and low in the caudal cortex. Reductions in FGF8 and FGF17 activity result in an expansion of caudal cortical tissue at the expense of rostral, hence FGF8 and FGF17 specify positional identity in the developing neocortex (Fukuchi-Shimogori, 2001b; Toyoda et al., 2010).

Transient *Fgf10* expression has been identified within the apical ventricular zone (VZ) between E9.5 and E11.5, a period within which neuroepithelial stem cells transition into radial glia by use of *in-situ* hybridisation (Sahara and O'Leary, 2009). Analysis of *Fgf10*$^{-/-}$ determined that its expression is vital for the proper timing of this transition. In *Fgf10* knockout embryos there is an extended period of symmetric cell divisions of neuroepithelial cells and thus expansion of the progenitor pool, resulting in eventual over-production of neurons specifically in frontal areas. However, detailed analysis of *Fgfr* expression was not determined, although it is known that *Fgfr*s are expressed within the embryonic brain, no clarity was available in regard to the specific splice variants, denoted as IIIb and IIIb isoforms. Furthermore, this conflicts with previous *Fgf10*^{nLacZ} data, which identified no LacZ signal in the dorsal telencephalon at E14.5. Due to the stability of β-galactosidase, it is suitable for transient lineage tracing. Therefore, it is reasonable to surmise that a large population of β-galactosidase^{+} cells would still be detectable at E14, either within RGCs and their descendants at this age if indeed *Fgf10* is transiently expressed by neuroepithelial cells/RGCs (Hajihosseini et al., 2008).

In the hypothalamus, postnatal neural stem cells are demarcated by *Fgf10* expression (Haan et al., 2013), and appear to have an embryonic origin. Expression of *Fgf10* was observed in the embryonic hypothalamus and pituitary using the *Fgf10*^{nLacZ} transgenic mouse strain (Hajihosseini et al., 2008). Recent studies in chick identifies *Fgf10* expressing progenitors in embryonic development which may persist as *Fgf10*$^{+}$ stem/progenitor cells in the postnatal/adult (Fu et al., 2017).

In the adult canonical neurogenic niches, the resident adult neural stem cells are remnants of embryonic neural stem cells. In the subventricular zone of the mouse, adult neural stem cells are generated between embryonic day 13.5 and 15.5, remaining mostly quiescent until they are reactivated in postnatal life (Fuentealba et

al., 2015). Similarly, neural stem cells within the hippocampal dentate gyrus originate from the embryonic ventral hippocampus, relocating into the dorsal hippocampus during late gestation (Li et al., 2013).

In the context of the cerebral cortex, it is important to determine the origin of the postnatal *Fgf10* expressing cells. To obtain more clarity over when and where *Fgf10* expression arises; in the embryonic germinal zone of the dorsal telencephalon, or in basal progenitors that may be retained, scattered throughout the postnatal cortical parenchyma.

6.2 Aims

In order to determine whether, and when *Fgf10* expressing cells first arise within the embryonic dorsal telencephalon, *Fgf10*^{nLacZ} brains were harvested at various embryonic time points. This tissue was sectioned and immunolabelled to detect any β-Galactosidase (β-Gal) expressing cells and hence actively expressing *Fgf10* or derived from an *Fgf10*-expressing lineage. Due to the stability of β-Galactosidase, any β-Gal^{+} cells observed were either actively expressing *Fgf10* at the time of sacrifice or derived from an *Fgf10* expressing lineage.

Further investigation was carried out using the *Fgf10*$^{CreERT2/+:\ Rosa26\text{-}tomato\text{-}dsred}$ double transgenic lineage tracing model. Tamoxifen was administered to the pregnant mother via oral gavage at a range of embryonic time points predicted by plug checking and weight monitoring of females. After allowing 2 days for optimal recombination embryos were harvested and immunolabelled to detect tdTomato (Tom)

In all experiments the hypothalamus was used as a control, due to its known strong embryonic expression of *Fgf10*.

6.3 Results

6.3.1 RT-PCR for the Fgf7 subfamily and Fgf receptors

RNA was isolated from E12 embryo. Briefly, embryos were decapitated, and brains were removed and separated into approximate dorsal and ventral halves with the meninges removed. The RNA obtained was probed with gene specific primers for members of the Fgf7 subfamily (Fgf-3, -7, -10 and -22) and all Fgf receptors, differentiating between IIIb and IIIc splice isoforms of Fgfr1-3. In both dorsal and ventral telencephalon samples, at E12 there was no expression of IIIb isoforms of *Fgfr1* and *Fgfr2*, with only IIIc splice isoforms being expressed. Thus, within the developing brains there is no expression of receptors that FGF10 is able to signal via. Interestingly both dorsal and ventral telencephalon samples showed expression of *Fgf10*. However, it is highly probable that these samples had some degree of contamination, likely from meninges, which are of neural crest/mesoderm origin. It has been shown previously that *Fgf10* is expressed in neural crest derived tissues (Teshima et al., 2016) (**Fig. 6-1**).

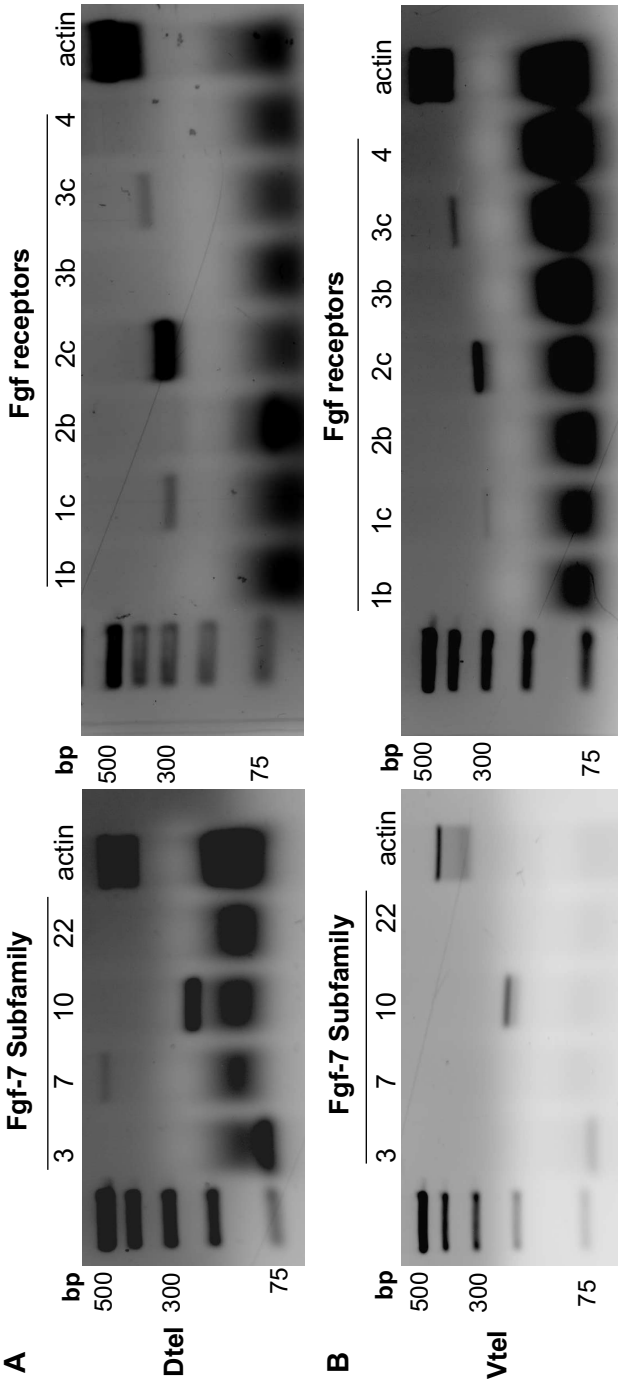


Figure 6-1 Expression of the Fgf7 subfamily and Fgfr's in the embryonic mouse brain: RT-PCR screening of the wild-type E12 dorsal (A) and ventral (B) telencephalon indicating expression of a subset of Fgf7 subfamily members and Fgfrs in early embryonic development.

6.3.2 Fgf10^{nLacZ} lineage tracing

The *Fgf10^{nLacZ}* reporter line was used to identify *Fgf10* expressing cells, and cells derived from an *Fgf10* expressing lineage within the embryonic mouse brain. Previous research indicates that stability of the β-galactosidase protein means this model is capable of transient lineage tracing of *Fgf10* expressing cells.

The β-galactosidase protein may be detected by two methods; Immunolabelling to detect the β-galactosidase enzyme, and by an enzyme-substrate reaction with 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal). Hajihosseini et al had previously identified *Fgf10* expression in the embryonic hypothalamus, but did not comprehensively investigate the developing cerebral cortex (Hajihosseini et al., 2008). In order to improve the temporal clarity of this timeline, a wider range of embryonic *Fgf10^{nLacZ}* tissue was collected for analysis, with a focus on the dorsal telencephalon.

6.3.2.1 Fgf10 is expressed in the embryonic hypothalamus but not the dorsal telencephalon

Robust expression of β-Gal was detected within the developing hypothalamus at E11 (n=2), E13 (n=4), E15 (n=2) and P0.5 (n=3) by immunolabelling. At E11 β-Gal^{+} cells are restricted to the floor of 3rd ventricle lining, a region which will go on to contain β2-tanycytes (**Fig. 6-2E-H**). However, at all embryonic time-points analysed no β-Gal^{+} cells were observed within all aspects of the developing cerebral cortex, including the germinal zones (**Fig. 6-2A-D**). At P0.5 n=1 brain displayed β-galactosidase positive cells within the meninges (**Fig. 6-3**).

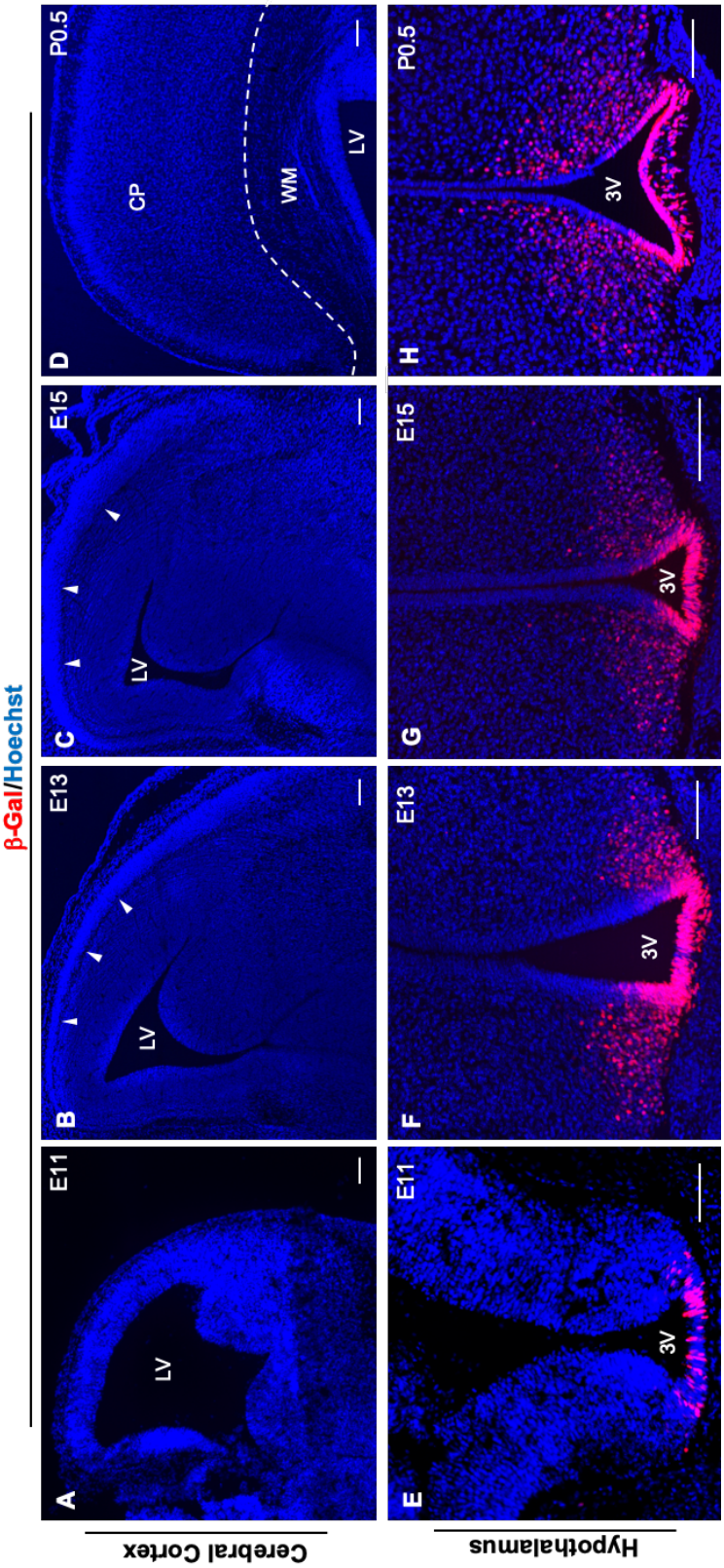


Figure 6-2 Fgf10 expression is not detected in the embryonic cerebral cortex during neurogenesis using the Fgf10^{nLacZ} model: At embryonic ages E11(n=2), E13 (n=4), E15 (n=2) and at birth (P0.5)(n=3), no β-Galactosidase signal is observable within the dorsal telencephalon (germinal zones nor cortical plate) **(A-D)**. Whereas extensive β-Galactosidase signal is detected within the developing hypothalamus **(E-H)**. LV, Lateral ventricle; WM, white matter; 3V, third ventricle; CP, cortical plate. Arrowheads indicate developing cerebral cortex. Scale 100μm.

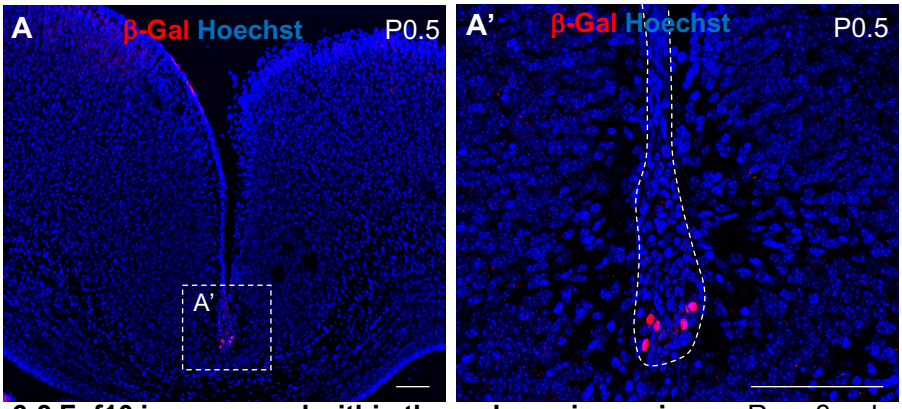


Figure 6-3 Fgf10 is expressed within the embryonic meninges: Rare β-galactosidase^{+} cells are found within the meninges at P0.5. Dashed line showing the pial surface of the cortex Scale 50μm (n=1).

6.3.2.2 Validation of β-galactosidase absence from cerebral cortex

As a secondary method in addition to β-Galactosidase immunolabelling P0.5 (n=2) and P5 (n=2) pups were obtained and stained using X-Gal solution. From previous experimentation with the Fgf10^{CreERT2} lineage tracing mice, it is known that *Fgf10* expressing cells are observable within the cerebral cortex at P4-5. No cortical X-Gal^{+} cells were observed within the cerebral cortex parenchyma and germinal zones at P0.5, confirming observations made by β-Galactosidase immunolabelling. At P5 rare scattered X-Gal^{+} cells were observed throughout the cerebral cortex (**Fig. 6-4**).

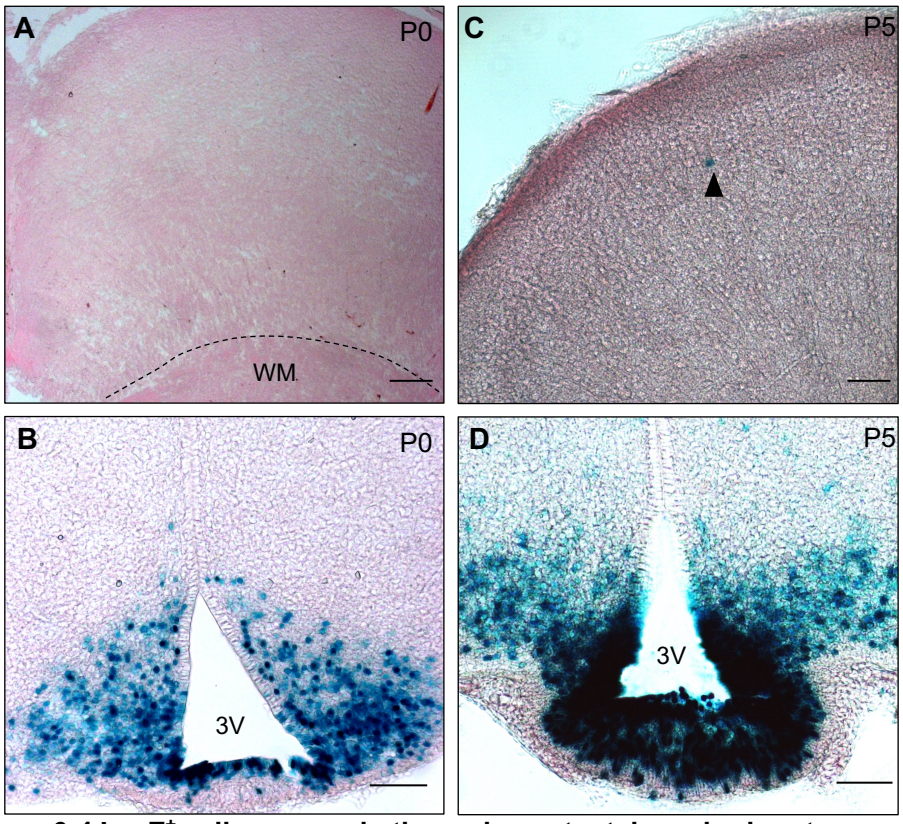


Figure 6-4 LacZ^{+} cells emerge in the early postnatal cerebral cortex parenchyma: X-gal staining of P0 and P5 Fgf10nLacZ tissue. No X-Gal+ cells observed in the P0 cerebral cortex (n=2) (A), and strong X-gal signal in the P0 hypothalamus (B). At P5 scattered single Xgal+ cells were observed in the cortex (n=2) (C), and strong X-Gal staining in the hypothalamus (D). 100μm scale.

6.3.3 *Fgf10$^{CreERT/+::Rosa26\text{-}tdTomato}$* lineage tracing

The *Fgf10^{nLacZ}* model only allows for transient lineage tracing, and it is possible that β-Galactosidase may be diluted out of rapidly proliferating cells that stop expressing *Fgf10*. The *Fgf10$^{CreERT/+::Rosa26\text{-}tdTomato}$* lineage tracing model allows for a more accurate temporal profile of *Fgf10* expression to be elucidated. Administration of tamoxifen results in CreERT2 mediated recombination, and activation of *tdTomato* (Tom) expression in any cell expressing *Fgf10* at the time of pulsing. Oral gavage of pregnant dams with tamoxifen at specific stages of embryonic gestation hence identifies any *Fgf10*$^{+}$ cells at the time of pulse and descendants thereafter. As with the *Fgf10^{nLacZ}* model, the hypothalamus was utilised as a positive control.

6.3.3.1 Short-chase early embryonic development

When embryos were pulsed in early embryonic development, between E8 and E13, and analysed at E15 no Tom^{+} cells were observed within the cortical plate, nor the germinal zones. Within the hypothalamus Tom^{+} cells were observed specifically within the floor of the 3rd ventricle. With later embryonic tamoxifen pulses, Tom^{+} cells remain

restricted to the lining of the 3rd ventricle, suggesting that *Fgf10*-expressing progenitors may be retained within this region (**Fig. 5-5**).

A single E12-E13 tamoxifen pulsed embryo displayed an anomalous phenotype at E15. Exclusively one half of the developing cortical plate showed abundant Tom+ cells, appearing to be migrating from the ventricular zone radially into the cortical plate, and from the ganglionic eminences. The restriction of this phenomena to only one side of the embryo, and the inability to replicate despite several subsequent repeats allows for the conclusion that this pattern of Tom^+ cells within the embryonic cortex is abnormal and anomalous. It's reasonable to speculate that the abhorrent tdTomato expression may be a response to hypoxia experienced by only a half of the embryo (**Fig. 5-6**).

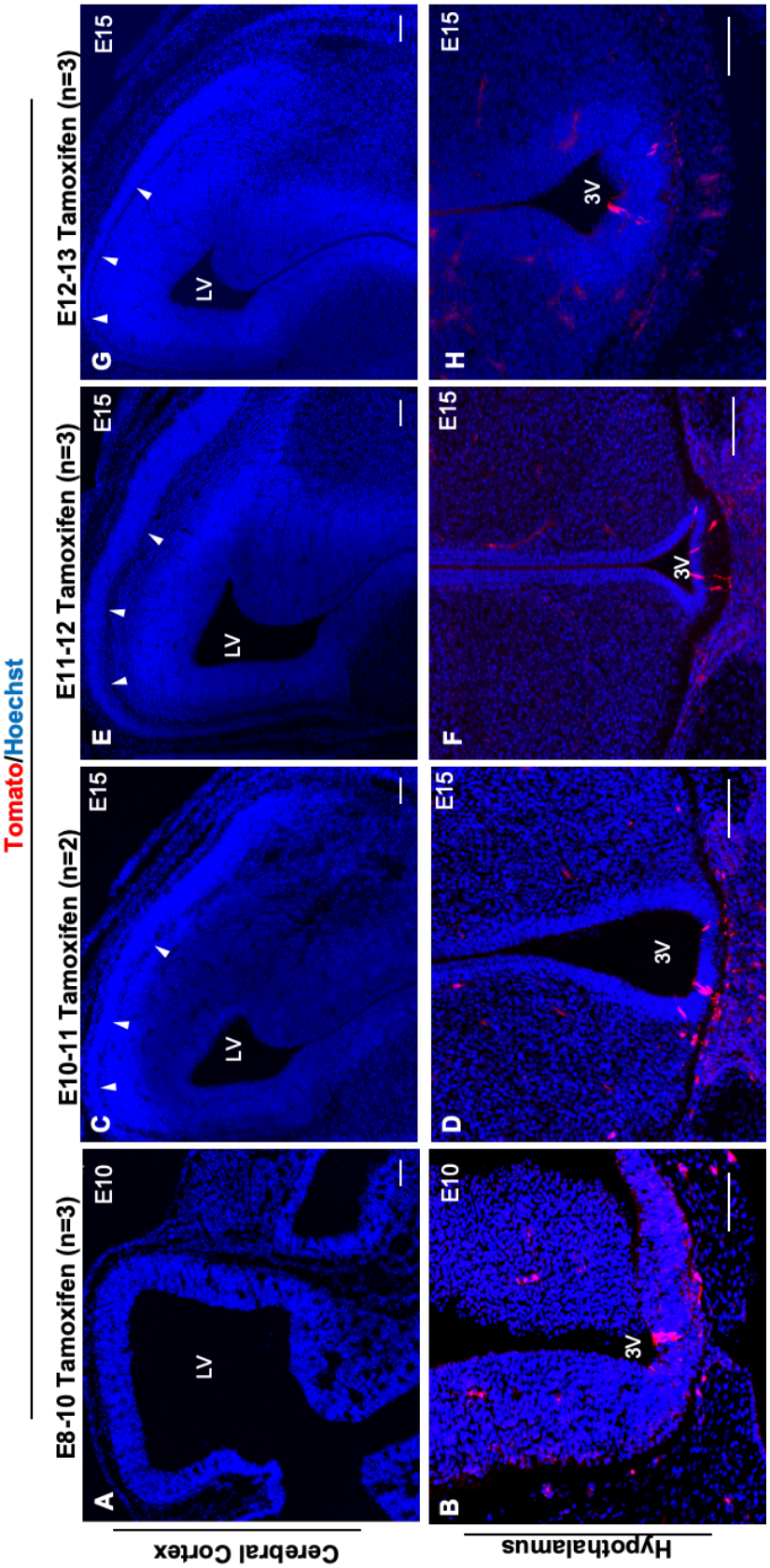


Figure 6-5 Absence of cortical Tom$^+$ cells in as determined by early embryonic tamoxifen induced Fgf10$^{CreERT2/+}$ embryos: No Tom$^+$ cells within the cerebral cortex, but scattered Tom$^+$ cells within the hypothalamic 3V lining when pulsed with tamoxifen at E8-9 (n=3)(A-B), E10-11 (n=2) (C-D), E11-12 (n=3)(E-F), E12-13 (n=3) (G-H). Arrowheads, cortical plate Scale 100μm.

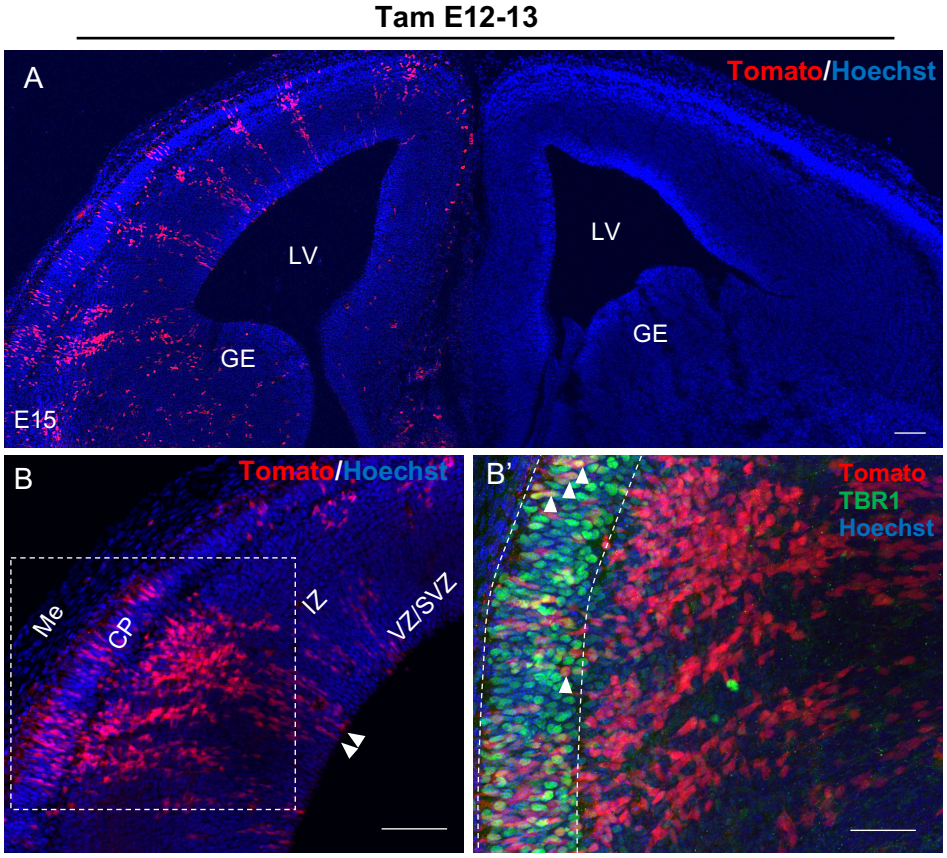


Figure 6-6 Anomalous unilateral expression of tdTomato in the tamoxifen-pulsed *Fgf10*$^{CreERT2/+::Rosa26\text{-}tdTomato}$ brain: In n=1 embryo pulsed at E12-13 and harvested at E15, Tomato+ cells were observed in only half of the embryonic brain (A). The Tom$^+$ cells were detected within the SVZ, VZ, cortical plate and meninges (B). Some Tom$^+$ cells expressed TBR1, a marker of early born cortical neurons (C). SVZ, subventricular zone; VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate; Me, Meninges; LV, lateral ventricle; GE, Ganglionic eminences. Scale 100μm.

6.3.3.2 Long Chase experimentation

A long-chase experiment was performed. Pregnant dams were pulsed with tamoxifen at approximately E9.5 days gestation, and embryos harvested at E18 (n=3). Diffuse Tom$^+$ cells were observed within the hypothalamus, throughout a majority 3rd ventricular lining and the hypothalamic parenchyma, in comparison to the few Tom$^+$ cells observed in the E8-9 pulsed (cull E10.5) embryos within the 3rd ventricle floor. Through analysis of the long and short chase data, it's reasonable to conclude that a small population of *Fgf10* expressing cells are retained within the 3rd ventricular lining, throughout development. These *Fgf10* expressing cells proliferate substantially within embryonic development, giving rise to cells within the 3rd ventricle lining and hypothalamus parenchyma.

Within the cerebral cortex of long-chase embryos no Tom$^+$ cells were observed with the exception of n=1 brain where Tom$^+$ cells were observed within the cerebral cortex

in close association with a blood vessel (**Fig. 5-7**).

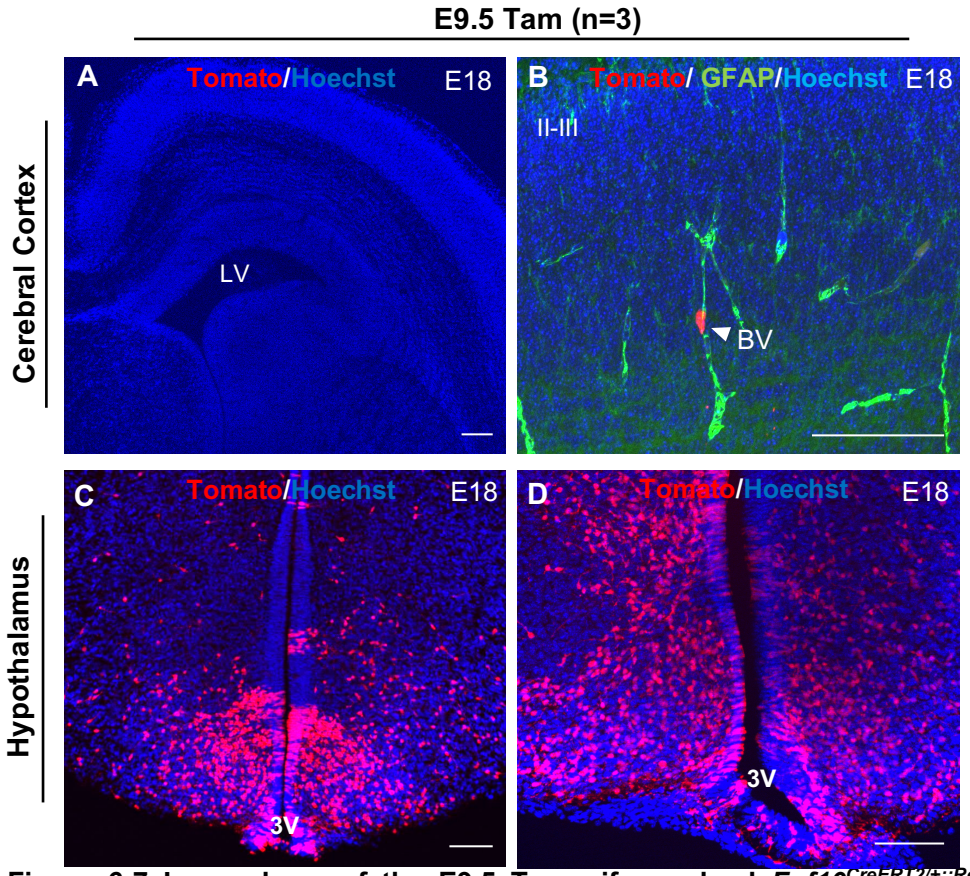


Figure 6-7 Long chase of the E9.5 Tamoxifen pulsed *Fgf10*$^{CreER T2/+}$::*Rosa26-tdTomato* brain: After an early embryonic tamoxifen pulse, at E18 Tom^{+} cells are absent from the cortex (A) with the exception of rare Tom^{+} cells with apparent adhesion to blood vessels (B). In the hypothalamus a minority of Tom^{+} cells at early embryonic stages amplify, resulting in widespread Tom^{+} cells within the hypothalamic parenchyma and 3rd ventricular tanacyte lining (C-D). BV, blood vessel. Scale 100μm (n=3).

6.3.3.3 Short Chase experimentation in late embryonic development

Given the observation of cortical blood vessel associated Tom^{+} cells at E18.5, and β-galactosidase positive meningeal cells at P0.5 it was necessary to cover a later embryonic timepoint. Pregnant dams were pulsed with tamoxifen at approximately E18.5 days gestation and allowed to litter naturally. Pups were culled at P1.5 (n=2). As observed previously, Tom^{+} cells were observed within the hypothalamic 3rd ventricular lining. In contrast to earlier embryonic time-points, Tom^{+} cells were observed within layer II of the cerebral cortex, some of which displayed neuronal morphologies, although with immature processes (**Fig. 5-8**).

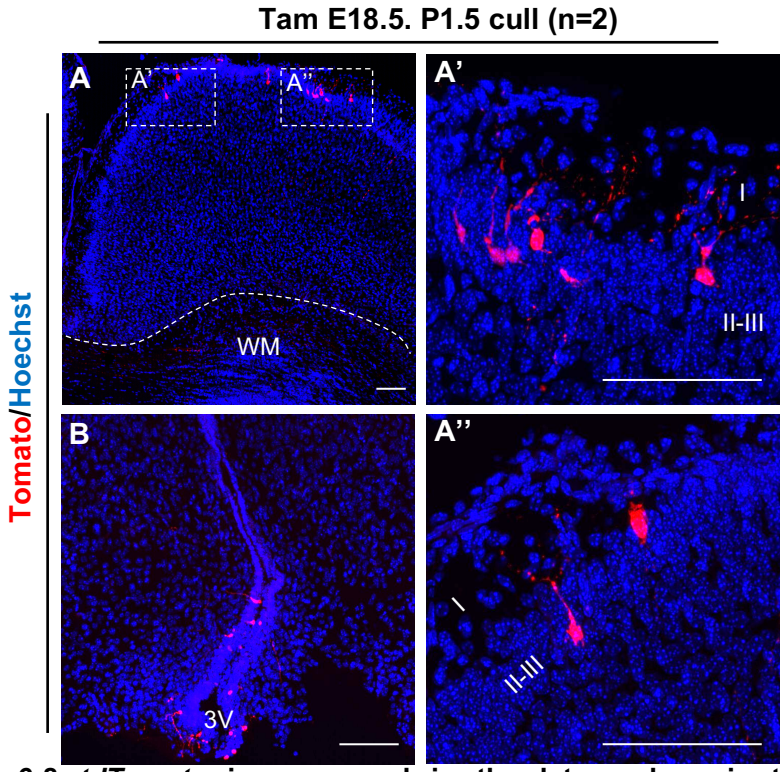


Figure 6-8 *tdTomato* is expressed in the late embryonic tamoxifen pulsed *Fgf10*$^{CreERT2/+::Rosa26\text{-}tdTomato}$ brain: When pulsed at E18.5 and culled at P1.5 Tom^{+} cells are observed within layer 2 of the cerebral cortex (A-A''). Tom^{+} cells are also observed within the 3V lining of the hypothalamus (B). Scale 100μm (n=2).

6.4 Discussion

In order to elucidate the nature of postnatal *Fgf10* expressing cells as potential residual progenitors, it was necessary to interrogate the developing embryo for their origins. The use of transgenic mouse lines to detect *Fgf10* is necessary, given the non-specific nature of FGF10 antibodies commercially available as interrogated in detail by a previous laboratory member.

Due to the ability of the *Fgf10*^{nLacZ} line to accurately report *Fgf10* expression in other tissues such as the lungs, heart and gut (Kelly et al., 2001; Mailleux et al., 2005; Sala et al., 2006). This transient lineage tracing model was selected to investigate whether *Fgf10* expressing cells are present within the dorsal telencephalon at various embryonic timepoints, during early, mid and late neurogenesis. In this model any cells that express the β-galactosidase protein are *Fgf10* expressing or derived from *Fgf10* expressing cells.

Previous research by our laboratory utilised the Fgf10^{nLacZ} model to generate a transient read-out of *Fgf10* expression within the embryonic, postnatal and adult mouse brain, by X-Gal staining. In this model LacZ^{+} cells were only reported as observed in the post-natal cortical parenchyma (Hajihosseini et al., 2008) (**Fig. 2-8**). In order to obtain a clearer picture of *Fgf10* expression in embryonic brain development it was necessary to repeat these studies under heavier scrutiny.

When detailed by immunolabelling, β-Gal^{+} cells were entirely absent from the dorsal telencephalon at all embryonic ages analysed, until P0.5 when there was some evidence of meningeal β-Gal^{+} cells. The meninges are comprised of three distinct layers; the dura mater, arachnoid and pia mater. The meninges envelope and penetrate within the entire mouse brain, with the dura mater being the outermost layer, and pia mater the innermost. In mice the primitive layer of the forebrain meninges is first identifiable between E9 and E10 in mice and derived from neural crest, with the leptomeninges (pia mater and arachnoid) being first identifiable at E13 (Couly and Douarin, 1987). The meninges do not resemble that of an adult until postnatal day 21. (McLone and Bondareff, 1975). In recent years a proportion of cells within the meninges have been identified as stem/progenitor cells, generating new neurons in the perinatal mouse cerebral cortex (Bifari et al., 2017) with the potential to respond to injury (Nakagomi et al., 2012). Studies that knocked out *Fgf10* specifically within the neural crest by use of a *Wnt1Cre* driver mouse line showed phenocopies of many of the *Fgf10*^{null} phenotypes. Although the study only focuses on the development of cranial organs and not all neural crest derived tissues it may be reasonable to

speculate cells derived from an *Fgf10* expressing neural crest lineage may inhabit the meninges.

With the *Fgf10*$^{CreERT2/+::Rosa26\text{-}tdTomato}$ transgenic mouse it was possible to obtain a strict temporal profile of *Fgf10* expression in the embryonic brain by short chases following tamoxifen administration. Through this framework we can conclude that *Fgf10* is not expressed early in embryonic development of the dorsal telencephalon (E8-E13). However, when embryos received tamoxifen late in embryonic development (approximately E18.5) Tom^{+} cells were observed within layer II of the cerebral cortex at P1.5, a region in which later born cortical neurons reside. Given the observation of rare meningeal β-Gal^{+} cells at E18.5 it is possible that Tom^{+} cells with neuronal morphologies are of meningeal origin. In complement to this finding, Bifari et al in identified radial-glia like cells from the PDGFRβ (Platelet Derived Growth Factor Beta) lineage within the meninges, which originate in embryonic development and contribute to caudal layer II and IV neurons without further cell division (Bifari et al., 2017).

In the P1 hypothalamus of the E18.5 tamoxifen pulsed brain, Tom^{+} cells are identified within the 3V lining of the hypothalamus. Thus, *Fgf10* expressing progenitors are retained within the 3V lining throughout embryonic development into early postnatal life.

Analysis of gene expression by RT-PCR, with the ventral and dorsal telencephalon tissue showed expression of *Fgf10* in both instances at E12. However, the high sensitivity of this technique, the difficulty of obtaining a clean and pure sample of cortical tissue, and its conflict with a large body of lineage tracing data allow us to conclude this signal is likely due to contamination. Interestingly, in both dorsal and ventral telencephalon there is no expression of *Fgfr1-IIIb* and *Fgfr2-IIIb*, the receptors through which Fgf10 exclusively binds to in order to exert its paracrine function. Studies have shown in the adult mouse that *Fgfr1-IIIb* is expressed, but regionally restricted to the hippocampus and the cerebellum (Beer et al., 2000) . As referred to previously, Fgf10 has been reported to also operate in an autocrine manner, and possesses two putative nuclear localisation motifs (Mikolajczak et al., 2016), highlighting a potential alternative mechanism of action for FGF10 in absence of its canonical receptors.

In 2009, Sahara *et al* reported *Fgf10* expression within the apical VZ between E9.5 to E13.5 by *in-situ* hybridisation. They hypothesised that FGF10 was crucial for transition of neuroepithelial stem cells into radial glia. Knockout of *Fgf10* led to perturbed accumulation of radial glia by prolonged symmetrical cell division of neuroepithelial

cells, an increase in basal progenitors produced and hence increased neuronal production and a thickening of the rostral cerebral cortex tissue. However, the paper does not identify *Fgfr1* and *Fgfr2* splice isoforms within the embryonic cerebral cortex. The data generated in this study by use of two distinct lineage tracing models conflicts with these findings. If *Fgf10* were expressed in early development by neuroepithelial cells which give rise to RGCs, and hence a majority of cells within the cortical plate, we would have observed widespread β-Gal/X-Gal positive cells throughout the entire cortical plate and ventricular zone as early as E11.5. Furthermore, long-chase lineage tracing of $Fgf10^{CreERT2/+::Rosa26\text{-}tdTomato}$ E9.5 tamoxifen pulsed mice would have yielded a similar wide-spread expression of Tom^{+} cells throughout the cortical plate at E18. The Fgf7 subfamily members *Fgf10* and *Fgf7* are highly sequence homologous, RT-PCR analysis identified *Fgf7* expression within the dorsal telencephalon exclusively. It is tempting to speculate that the *in situ* hybridisation by Sahara *et al.* was erroneously detecting this Fgf7 subfamily member (Sahara and O'Leary, 2009).

In conclusion, *Fgf10* expression within the embryonic brain is restricted to the hypothalamus. In late embryonic development rare *Fgf10* expressing cells are found in the meninges, and layer II of the cerebral cortex at P1.5. Research in 2017 identified radial glia like progenitor cells within the meninges, generated E13.5-E16.5, and migrate into the cortex, differentiating without further proliferation into functionally integrated layer II and IV neurons. Many neurons derived from these meningeal cells are from a PDGFRβ lineage. It is tempting to speculate that *Fgf10* expressing cells within the post-natal cortical parenchyma are derived from embryonic meningeal lineages (Bifari et al., 2017).

7 GENERAL DISCUSSION AND FUTURE DIRECTIONS

Research into the field of adult neurogenesis is dynamic, with increasing evidence outlining the nascent neurogenic potential of regions previously regarded as non-neurogenic in postnatal/adult life. In non-neural organs paracrine FGF10 signalling is implicated in stem cell homeostasis, such as within the lung (El Agha et al., 2014) and in the developing incisor tooth germ (Harada et al., 2002). Within the postnatal/adult central nervous system evidence for *Fgf10* expression was initially collected by use of an *Fgf10*^{nLacZ} lineage tracing mouse. It was observed that regions within which *Fgf10* was expressed overlapped with the speculated non-canonical neurogenic niches, including but not limited to the cerebral cortex and hypothalamus. This led to the hypothesis that *Fgf10* expression demarcates a population of slow dividing or quiescent adult neural stem/progenitor like cells. Further research focused on the hypothalamus showed that cells derived from an *Fgf10* expressing lineage are able to proliferate, adding new neurons to the appetite and energy balance regulating centres of the postnatal/adult mouse brain.

This project sought to investigate whether *Fgf10* expressing cells within the cerebral cortex may also have a postnatal neurogenic potential and gain insight into the role of FGF10 within this region. However, there are difficulties that are encountered in exploring *Fgf10* expression within the cerebral cortex. First, through vigorous protocol testing of several commercially available antibodies against FGF10, it was determined that there was broad non-specific binding across all antibodies tested, postulated to be due to detection of FGF7. As a result, methods such as western-blotting, and those to determine the subcellular localisation of FGF10 were not plausible. Second, the complete knock-out of FGF10 is lethal neonatally, thus rendering it impossible to elucidate its role in the postnatal brain without conditional/inducible approach. Therefore, to analyse *Fgf10* expressing cells within the postnatal cerebral cortex, three lineage tracing models have been utilised; *Fgf10*^{nLacZ}, *Fgf10*$^{CreERT2/+::Rosa26\text{-}tdTomato}$ and *Fgf10*$^{CreERT2/floxed::Rosa26\text{-}tdTomato}$ to investigate the role of this FGF ligand beyond embryonic development.

7.1 Cortical *Fgf10* expressing cells display properties that are consistent with progenitor status

It was observed that P4-5 *Fgf10* expressing cells have a limited potential to amplify in number within the first 11 days after birth, suggestive of postnatal Tom^+ neurogenic proliferation, coinciding with a period of significant cortical volume expansion (Lyck et al., 2007). However, it is not possible to rule-out a contribution of prolonged tamoxifen mediated recombination within these early postnatal ages, which could result in increased numbers of Tom^+ cells not as a result of cell proliferation. There are varied reports documenting differing half-lives of tamoxifen, dependant on the route of administration, dose, length of pulse in days, and tissue analysed. For example, following a substantial oral gavage dose, serum tamoxifen levels reached non-detectable levels within only 4 days (Robinson et al., 1990). A study on two high oral doses of tamoxifen determined that tamoxifen was non-detectable in brain tissue after eight days, a timescale that shortened with smaller doses (Valny et al., 2016). While this data suggests our early postnatal pulse-chase regime is too short, it should be noted that both papers used different mouse strains, different doses and different ages to what were used in this study, therefore may not be comparable.

Postnatally the rodent brain increases in size 6-fold as a result of not only gliogenesis and increased neuronal size, but due to sustained neurogenesis. Within the cerebral cortex of the rat there is a two-fold increase in the number of neurons in the week after birth (Bandeira et al., 2009). Similarly, in the mouse cerebral cortex there is an increase in neuronal number to a magnitude of 80-100% between birth and postnatal day 16 (P16) (Lyck et al., 2007). The timing of the reported postnatal increase in cortical neurons overlaps with the amplification observed of Tom^+ cells in the $Fgf10^{CreERT2/+}$ condition. It is reasonable to speculate that *Fgf10* expressing cells within the cerebral cortex retain neurogenic potential in protracted and limited developmental neurogenesis that continues into postnatal life.

Lyck et al also make the interesting observation that cells in deep cortical layers had delayed acquisition of NeuN antigenicity in postnatal development, and thus could potentially be non-postmitotic, until P16 when they acquired $NeuN^+$ status and hence maturity (Lyck et al., 2007). The identification of $NeuN^-$ Tom^+ cells with an immature neuronal morphology, and a propensity for Tom^+ cells to inhabit the deep cortical layers further corroborates the hypothesis that they represent cells with a neurogenic potential in postnatal cortical expansion.

Analysis of canonical neurogenic niches such as the subventricular zone of the lateral

ventricles and subgranular zone of the dentate gyrus shows that adult NSCs are residual from embryonic development (Li et al., 2013). In the SVZ it was shown that embryonic neural stem cells maintain undifferentiated state and become quiescent until reactivated postnatally (Fuentealba et al., 2015). More recently $FGF10^{+}$ progenitors have been identified within the chick embryonic hypothalamus. It has been speculated that the $FGF10^{+}$ neural progenitor like cells identified within the β-tanycyte lining of the postnatal/adult hypothalamus may be remnants of this embryonic progenitor population (Fu et al., 2017). Surprisingly, *Fgf10* expressing cells were not identified within the embryonic dorsal telencephalon by use of $Fgf10^{nLacZ}$ and $Fgf10^{CreERT2/+}$ models. This may be for one of two reasons, firstly that *Fgf10*-expressing progenitor like cells that exist postnatally are derived from regions outside of the embryonic brain. Secondly, Fgf10 may be a downstream molecule not expressed until birth, as part of a sequential cascade similar to that observed in the progression of radial glia, to IPC to post-mitotic neuron through sequential expression of *Pax6*, *Tbr2* and *Tbr1* respectively.

An embryonic study in 2009 identified a transient *Fgf10* expression in early embryonic development (E9-E13) within the apical ventricular zone, coinciding with the transition of neuroepithelial cells to radial glia. Such regions of Fgf10 expression were not observed with either transgenic mouse models utilised in this study, analysed within the same timepoints. Research conducted by Sahara *et al* (2009) of $Fgf10^{-/-}$ embryos observed an extended period of symmetric cell divisions of progenitors and an increased progenitor number resulting in an eventual over production of neurons within the dorsal telencephalon. Although the study is convincing, our observation that there is no expression of FGF10 in the embryonic dorsal telencephalon is in direct contradiction. If Fgf10 were expressed within early cortical progenitors, widespread constitutive expression of $LacZ^{+}$ or Tom^{+} would be expected throughout the postnatal cerebral cortex. It is still possible that Sahara *et al.* observed the effect and contribution of FGF10 expressing cells derived from outside of the canonical germinal zones, such as those identified within the meninges. High sequence homology between FGF7 and FGF10 may be in part causative of the strong signal observed in embryos by *in-situ* hybridisation techniques (Sahara and O'Leary, 2009).

The meninges have been identified as potential neural stem/progenitor/precursor niche within the postnatal/adult mouse brain. Populations of cells within the meninges express neural stem cell markers such as nestin, NG2, Sox2 as well as neural progenitor markers such as DCX postnatally (Bifari et al., 2015). Subsequent lineage tracing analysis showed that meningeal neural precursors are born embryonically and

remain largely quiescent and migrate into caudal cortical regions in postnatal life, going through a process of differentiation without cell division. Interestingly, meningeal derived neurons specifically contributed to caudal cortical areas such as the retrosplenial cortex and consisted of Satb2^{+} pyramidal neurons (75.8%), and small proportion of Gad67 interneurons (20.5%) at P30. A strikingly similar rostro-caudal distribution as Tom^{+} cells in both control and Fgf10 deficient mouse models (Bifari et al., 2017). Interestingly, evidence of Fgf10 expression as detected in the late embryonic meninges, it is therefore tempting to speculate that *Fgf10* expressing cells may be derived from the meninges embryonically, entering the cerebral cortex after birth where they retain limited proliferative potential within the parenchyma.

Postnatally, Tom^{+} cells were most concentrated within caudal cortical regions at all ages, with large clusters observed within the retrosplenial cortex. The retrosplenial cortex is involved in a range of cognitive functions such as episodic memory and spatial navigation, and is often compromised in disorders which impair memory (Vann et al., 2009). A study showed that within the first postnatal week there is a contribution of new post-mitotic neurons from the SVZ to the retrosplenial cortex, of a subtype that express the transcription factor *Satb2*. In the cerebral cortex, the DNA-binding protein Satb2 is expressed in all post-mitotic neurons that extend axons to the corpus callosum (Alcamo et al., 2008). Given the striking similarities of the new-born neuron morphology in comparison to tdTomato^{+} cells, and the specific high concentration of them in this region, it is reasonable to speculate that cells from the *Fgf10* expressing lineage may have some involvement in the postnatal development of the retrosplenial cortex. The study observed that approximately 15% of the new Satb2^{+} neurons at P15 were born later than E20 based on the BrdU pulsing paradigm used, thus indicating that there is a potential for low level postnatal neurogenesis within this cerebral cortex region (Zgraggen et al., 2012). Given that Bifari et al (2017) determine that meningeal derived neurons enter the cerebral cortex via the SVZ but are not themselves derived from the SVZ implies it may be probable that the Satb2^{+} neurons observed by Zgraggen et al (2012) may in fact be of meningeal origin.

Other studies have noted a modest contribution of new neurons to areas such as the cerebral cortex, amygdala and hypothalamus derived from bone marrow. This study utilised mice that were homozygous for a knockout mutation in the PU.1 gene, which codes for a transcription factor expressed exclusively within the haemopoietic lineage. These mice at birth are deficient in macrophages, neutrophils, mast cells, osteoclasts and B ant T cells, in order to survive require a bone marrow transplant within 48 hours of birth. Despite this in comparison to wild-type PU.1$^{-/-}$ mice display no gross brain

changes. At isolation, donor bone marrow did not express any markers of neuronal or glial lineages including that of $NG2^{+}$ glia. Following bone marrow transplantation at 48-hours post-birth, brains were analysed after 1 month and 2 months. In both time-points donor bone marrow derived neurons were found mostly in the cerebral cortex, hypothalamus and amygdala to name but a few regions. Indicating there is a potential post-natal contribution of new-neurons into the brain from the bone marrow (Mezey et al., 2000).

7.2 Putative Regulators of postnatal cortical neurogenesis – Fgf10 as suppressor.

Analysis of conditional deletion of *Fgf10* within the population of cortical P4-5 FGF10^{+} cells indicates that FGF10 itself may directly supress the duration of neurogenic potential exhibited by this population in a cell autonomous fashion. Given the lack of FGFRs through which FGF10 is capable of signalling via within the cerebral cortex at the time of tamoxifen induction, paracrine FGF10 signalling within the cerebral cortex is improbable. Within the hypothalamus there is no expression of *Fgfr1-IIIb* and *Fgfr2-IIIb* in the regions where FGF10^{+} progenitors inhabit, and a hypothesised cell autonomous role for FGF10 protein. Numerous studies indicate the existence of FGF10 nuclear localisation motifs, and a role for nuclear FGF10 in the pathology of Lacrimo-auriculo-dento-digital (LADD) syndrome (Kosman et al., 2007; Mikolajczak et al., 2016). It is reasonable to speculate that FGF10 may have a role in regulation of gene expression or cell cycle, in a similar mechanism to related Fgf7 subfamily member FGF3, which is postulated to inhibit proliferation by its interaction with NoBP within the nucleus (Reimers et al., 2001).

Following induced conditional knockout of *Fgf10* within the population of cells that express *Fgf10* at P4-5, a second wave of amplification occurs between P11 and P28, despite starting populations of Tom^{+} and *Fgf10*-deficient Tom^{+} cells being comparable and P7 and P11. Literature analysing rodent cortical neuronal cell number from birth to adulthood observed an increase in cortical neurons in the first postnatal week is coupled with a substantial decrease in the second postnatal week. Between P15 and P25 following the period of net neuronal loss, there is a second moderate late addition of neurons to the rat cerebral cortex (Bandeira et al., 2009). Based on this analysis, it is probable that FGF10 has a cell autonomous role in either cell proliferation, survival or differentiation of Tom^{+} cells within the postnatal cerebral cortex.

In the *Fgf10*$^{CreERT2/floxed}$ brains interrogation for evidence of cell proliferation showed only rare Tom^{+} cells with a glial morphology actively proliferating between P11 and P20. The timepoints that Tom^{+}/PCNA^{+} cells were identified coincide with continued postnatal oligodendrogenesis and may be NG2^{+} glia or oligodendrocyte precursor cells (OLPs). Within the cerebral cortex NG2^{+} cells have been shown to express DCX and have a controversial potential to generate new-neurons *in vivo* (Guo et al., 2010; Tamura et al., 2007). It is known that NG2^{+} progenitors are retained within the cerebral cortex into adulthood, contributing oligodendrocytes throughout life. It is plausible that NG2 progenitors following *Fgf10* knockout at P4-5 change fate to produce new-born

neurons during oliogodendrogenesis (Dawson et al., 2003). In regard to cell proliferation following *Fgf10*-knockout, the amplification of Tom^+ cells does not occur until after P20, which was outside the scope of this current experimentation. Although it is possible to speculate the identity of Tom^+ cells which may be proliferating during these timepoints, further experiments would be required to conclusively define this population.

Another possibility is that FGF10 has a role in survival, it is known that there is an over-production of neurons embryonically, which in early postnatal life (<P30) are reduced in number as part of a process known as synaptic pruning, resulting in refinement of cortical circuitry during synaptogenesis. Use of computational modelling suggests that programmed cell death (PCD) reduces the cortical neuron population by 20-30% postnatally (Gohlke et al., 2004). Analysis of mouse postnatal cortical development determined that during the first two postnatal weeks of net-neuronal addition there is a period of minor reduction (Lyck et al., 2007). It is possible that intracellular FGF10 signalling has a role in regulation of cortical neuronal populations, potentially in PCD. It is tempting to speculate that following conditional FGF10 knockout there is increased survival of Tom^+ cells that would otherwise have been lost in this refinement process. Subsequent analysis of cell death by TUNEL would uncover potential clues to this role.

Studies indicate that under normal physiological conditions the cerebral cortex is not extensively neurogenic to the degree of canonical niches such as the SVZ and SGZ. It has been postulated that cues within the local environment of the cerebral cortex negatively regulate neurogenesis. One such family of molecules implicated in this are ephrins, which are secreted by astrocytes in regions outside of the SVZ and SGZ. In mice that were deficient for Ephrin-A2 and -A3 robust increase in numbers of $Ki67^+/BrdU^+/nestin^+$ cells were observed within the cerebral cortex as early as P14, and numerous DCX^+ immature neurons in comparison to wild-type with no differences observed in cell death (TUNEL analysis). Interestingly the expression of ephins within the mouse cerebral cortex is not observed until after P4, a similar time to which *Fgf10* expression is observed in the $Fgf10^{nLacZ}$ mouse (**Fig. 6-4**). It is tempting to speculate that under physiological conditions FGF10 functions intracellularly within a subset of cells within the cerebral cortex to negatively regulate neurogenesis. Given that the $Fgf10^{CreERT2/+}$ mice are hypomorphic, it is possible we are observing dosage related differences in cortical Tom^+ cell number, one with reduced levels of FGF10 activity ($Fgf10^{CreERT2/+}$) and a second with condtional knockout of FGF10 ($Fgf10^{CreERT2/floxed}$).

7.3 The pleiotropic potential of FGF10 in the cerebral cortex

In the literature, FGF10 has been implicated in postnatal dendritogenesis within the somatosensory whisker barrel cortex. However, this study focused on broad knockout of FGFR-1, -2 and -3 which in combination impacted the stability of dendritic patterning, an effect which cannot solely be attributed to FGF10, nor the two specific receptor isoforms FGF10 signals via. Indeed the paper indicates that *Fgr1-IIIb* and *Fgfr2-IIIb* are expressed within the cerebral cortex, however RNA for this analysis was obtained from presumably whole-brain tissue, or primary cortical neuron culture which may have little representation of the receptor expression within the postnatal somatosensory cortex *in vivo* (Huang et al., 2017). *In vitro* FGF10 along with close family members FGF7 and FGF22 have been implicated in synaptogenesis by use of cultures of motor neurons, through being purified from synaptic vesicles (Umemori et al., 2004). Further investigation categorised the action of FGF7 and FGF22 in establishing excitatory and inhibitory synapses respectively within the hippocampus (Dabrowski et al., 2015); a region within which *Fgfr1-IIIb* at least is known to be expressed in adulthood (Beer et al., 2000). Although our study does not detect expression of *Fgfr1-IIIb* and *Fgfr2-IIIb* at the embryonic and postnatal ages analysed, it does not rule out expression of these receptors and hence paracrine signalling of FGF10 in regions outside the cerebral cortex. For example, neurons in the deep cortical layers send corticofugal connections contacting regions such as the thalamus, spinal cord and brain stem, regions where *Fgfr* expression has not been investigated in this study.

In later postnatal timepoints, *Fgf10* expressing cells are still observed within the cerebral cortex, however in a different distribution to that established by early postnatal *Fgf10* expressing cells. A vast majority of Tom^+ cells inhabit the deep cortical layers V and VI in adolescence/adulthood. Within these regions are pyramidal neurons that receive thalamic input and extend processes that reach regions outside the cerebral cortex. This shows that *Fgf10* expression persists within the cerebral cortex through to adolescence, however provides little context to its role within early adulthood. Furthermore, analysis of *Fgfr1-IIIb* and *Fgfr2-IIIb* expression was not investigated beyond P4. Gven the broadly different laminar distribution of Tom^+ cells pulsed in late adolescence in comparison to early postnatal life, it may be possible that FGF10 has a different function in later ages. Additional studies utilising the $Fgf10^{CreERT2/floxed}$ mice to investigate this role were beyond the scope of this project.

7.4 Implications for normal cerebral cortex physiology and repair following injury

In conclusion, the findings of this study provide evidence for the existence of cells with a quiescent neurogenic potential within the postnatal cerebral cortex, demarcated and negatively regulated in terms of their ability amplify by FGF10. However, the mode by which FGF10 conditional knockout results in this amplification is the subject of future studies, whether increased survival, increased cell proliferation or otherwise. The origin of cortical FGF10 expressing cells remains elusive, existing literature on the ability of new-neurons to be born within the cerebral cortex beyond embryonic development indicate the potential of $NG2^+$ glia and meningeal derived neural precursors in this phenomenon (Bifari et al., 2017, 2015). This combined with the observation of rare $\beta\text{-gal}^+$ cells within the embryonic meningeal layer highlights this tissue as the potential origin of cortical *Fgf10* expressing cells.

In terms of normal physiology, the potential of contribution new-born neurons to specific regions of the cerebral cortex postnatally could have an important role in plasticity. As noted in studies of perinatal hypoxia, at early postnatal time-points the cerebral cortex retains some capacity to recover in terms of neuronal number following hypoxic insult (Fagel et al., 2006). In the hippocampus integration and generation of new-born neurons is not only ongoing as part of neuronal turnover, but is also key to plasticity within the hippocampus throughout life in response to experience and external influences (Ge et al., 2008). During postnatal development milestones such as the opening of the eyes contribute to experience dependant-maturation of cortical circuitry, a phenomenon that new-born cortical neurons may contribute to.

In rare brains of both $Fgf10^{CreERT2/+}$ and $Fgf10^{CreERT2/floxed}$ large mixed clusters of Tom^+ neurons and $GFAP^+$ glia are observed, which may be indicative of multipotent potential of this lineage postnatally. The rare occurrence of large clusters, restricted to a single section or cortical region, leads to the assumption that it is in response to a localised event such as mechanical or ischemic injury. This research may uncover a potential pathway to focus on in improving the ability of the cerebral cortex to incorporate viable new-born neurons, or a method through which to amplify proliferation of endogenous cells with neurogenic potential by conditional FGF10 knockout. In the literature, FGF10 has been implicated as having a role in survival following cortical ischemia in a paracrine manner within the adult mouse brain. Such analysis utilised anti-FGF10 antibodies and reported localisation of FGF10 within the cytoplasm and the nuclei of neurons, and also within the cerebrospinal fluid. Levels of FGF10 have been shown

to be upregulated following middle cerebral artery occlusion (MCAO) ischemic injury. Additionally, injection of FGF10 protein directly into the lateral ventricles reduces subsequent apoptosis, attributed to activation of the PI3K/Akt pathway (Li et al., 2016). It should be noted that this study utilised adult mice, for which the dynamics of FGF10 signalling was not extensively investigated within this study, nor the FGFR1-IIIb/FGFR2-IIIb profile. Assuming the validity of FGF10 immunolabelling, this study collects further evidence for a pleiotropic role for FGF10 within the cerebral cortex, due to the observation of both nuclear, cytoplasmic and secreted FGF10 proteins.

7.5 Future Studies

Initial future studies should first and foremost focus on obtaining a valid and specific anti-FGF10 antibody. As a result, it will be possible to observe the subcellular localisation of FGF10 and evaluate further the hypothesised nuclear role of FGF10 in the postnatal cerebral cortex. Use of an anti-FGF10 antibody would also allow for identification of cortical FGF10^{+} cells at P4-5, and classification by immunolabelling for NG2 and PDGFRβ progenitors of potential meningeal origin. Assays such as co-immunoprecipitation will enable the determination of protein-protein interactions of FGF10 within the cerebral cortex, thus providing further clues as to the dynamics of FGF10 in this brain region.

The potential cell proliferation of *Fgf10*-deficient Tom^{+} cells should be further interrogated between P20 and P28, by the use of EdU, for which detection utilises a Click-Chemistry reaction. This does not require denaturing treatment wiht HCl and should therefore be compatible with anti-Tom immunolabelling. Likewise, to evaluate the potential role of FGF10 knockout in neuronal survival, TUNEL analysis should be conducted to interrogate any differences in PCD of Tom^{+} cells following conditional *Fgf10*-knockout.

Several papers observe a postnatal contribution of Satb2^{+} neurons to caudal cortical regions (Bifari et al., 2017; Zgraggen et al., 2012). Caudal Tom^{+} cells should be interrogated for expression of the transcription factor Satb2 to further elucidate the cortical neuronal subtypes and gain clues as to whether they could be a similar population derived postnatally. Further analysis should be conducted into the meninges and bone marrow to determine whether Tom^{+} cells can be detected within these tissues, and if they are capable of differentiating into neurons by culturing *in vitro,* to determine the origin of *Fgf10* expressing cells.

Finally, the potential of Tom^{+} cells in both *Fgf10*$^{CreERT2/+}$ and *Fgf10*$^{CreERT2/floxed}$ cerebral cortex to respond to either ischemic injury by middle cerebral artery occlusion or stereotaxic ‘stab’ mechanical injury should be investigated. Given the occurrence of large mixed clusters of Tom^{+} neurons and GFAP^{+} astrocytes, speculated as a result of injury, the ability of Tom^{+} cells to proliferate and differentiate in response to injury should be analysed and compared between both genotypes.

8 ABBREVIATIONS

5HT3aR	Ionotropic Serotonin Receptor 5HT3aR
3V	Third Ventricle
AB	Acid Box
AKT	Protein Kinase B
Arc	Arcuate Nucleus
AMCA	Aminomethylcoumarin
ANR	Anterior Neural Ridge
ASCL1	Achaete-scute family bHLH transcription factor
Au1	Primary Auditory Cortex
AuD	Secondary Auditory Cortex (Dorsal)
AuV	Secondary Auditory Cortex (Ventral)
B-Gal	Beta-Galactosidase
bHLH	Basic Helix-Loop-Helix Transcription Factor
BLBP	Brain Lipid Binding Protein
BMP	Bone Morphogenetic Protein
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
BV	Blood Vessel
CA	Cornu Ammonis
CD133	Prominin-1
CGE	Caudal ganglionic eminences
cKO	Conditional knockout

CR	Calretinin
CrERT2	Tamoxifen dependant Cre-recombinase
CNS	Central Nervous System
CP	Cortical plate
CRKL	CRK-like protein
CSF	Cerebrospinal Fluid
CSFR1	Colony Stimulating Factor 1 Receptor
CTIP2	COUP-TF-interacting protein 2
CV	Cardiovascular
Cxcl12	C-X-C motif chemokine 12
DCX	Doublecortin
DG	Dentate Gyrus
DLX	Distal-less
DMN	Dorsomedial Nucleus
DA	Dopamine/Dopaminergic
EDTA	Ethylenediaminetetraacetic acid
EdU	5-Ethynyl-2´-deoxyuridine
EGL	External Granule Layer
Emx2	Empty Spiracles Homeobox 2
ERK	Extracellular signal–regulated kinase
eSVZ	Embryonic Subventricular Zone
EtOH	Ethanol
Ets	E26 transformation-specific transcription factor

FACS	Fluorescence Activated Cell Sorting
Fezf	Fez family zinc finger protein
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FRS2	Fibroblast growth factor receptor substrate 2
FLRT3	Fibronectin leucine rich transmembrane protein 3
GABA	Gamma-Aminobutyric acid
Gad67	Glutamate Decarboxylase-67
GE	Ganglionic Eminences
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GLAST	Glutamate Aspartate Transporter 1
Grb2	Growth factor receptor-bound protein-2
GSx2	Genetic-Screened homeobox 2
Gt	Goat
HBSS	Hanks Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HFD	High Fat Diet
HSPG	Heparan Sulphate Glycosaminoglycans
iFGF	Intracellular Fibroblast Growth Factor
IGL	Internal Granule Layer
IKKβ	Inhibitor of nuclear factor kappa-B kinase

	subunit beta
IP	Intraperitoneal
IPC	Intermediate Progenitor Cell
IZ	Intermediate Zone
KLPH	Klotho/lactase-phlorizin hydrolase-related protein
LADD	Lacrimo-auriculo-dento-digital (syndrome)
LGE	Lateral Ganglionic Eminences
LMP	Low Melting Point
MAP-Kinase	Mitogen-Activated Protein Kinase
Me	Meninges
ME	Median Eminence
MCAO	Middle Cerebral Artery Occlusion
MGE	Medial Ganglionic Eminences
MHB	Mid-hindbrain boundary
Ms	Mouse
MZ	Marginal Zone
NCAM	Neural Cell Adhesion Molecule
NE	Neuroepithelial cell
NeuroD	Neuronal Differentiation bHLH transcription factor
NeuN	Neuronal Nuclear Antigen
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells

NG2	Neuron-glial antigen 2
Ngf	Neural Growth Factor
Ngn	Neurogenin
NGS	Normal Goat Serum
NMDARs	N-methyl-D-aspartate receptor is a glutamate receptor
NoBP	Nucleolar FGF3 binding protein
NOS	Nitric oxide synthase
NPC	Neural Progenitor Cell
NPY	Neuropeptide Y
NSC	Neural Stem Cell
OB	Olfactory Bulbs
OCT	Optimal Cutting Temperature Compound
OLP	Oligodendrocyte Progenitor
OL	Oligodendrocyte
oIP	Outer Intermediate Progenitor
Olig2	Oligodendrocyte Transcription Factor 2
OTX1	Orthodenticle Homeobox 1
PAX6	Paired Box 6
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction

PDGFR	Platelet Derived Growth Factor Receptor
Pea3	Polyoma Enhancer Activator 3
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PiR	Piriform Cortex
PLC-gamma	Phospholipase C-gamma
PoA	Preoptic Area
PP	Preplate
PPEPs	PLP-promoter-expressing NG2 progenitors
Prox1	Prospero Homeobox 1
PrV	Principal trigermianl nucleus of the brainstem
PSA-NCAM	Polysialylated-neural cell adhesion molecule
PV	Parvalbumin
RA	Radial Astrocyte
RAS	Rat sarcoma GTPase
Rb	Rabbit
RGC	Radial Glia Cell
RMS	Rostral migratory stream
Rmx2	
RSC	Retrosplenial Cortex
Rt	Rat
S1	Primary Somatosensory Cortex
SCA67	Spinocerebellar ataxia type 27

SDS	Sodium Dodecyl Sulphate
SEF	Similar Expression to FGF
SGZ	Subgranular Zone
Shh	Sonic Hedgheog
Sox	Sex determining region Y-box
Sp	Splenium
SP	Subplate
SPRY	Sprouty
SST	Somatostatin
STAT	Signal Transducer and Activator of Transcription
sVEGFR	Secreted Vascular Endothelial Growth Factor Receptor
SVET1	Subventricular Expressed Transcript 1
SVZ	Subventricular Zone
TAG-1	Transient axonal glycoprotein 1
TBI	Traumatic Brain Injury
TBR1	T-box Brain 1
TBR2	T-box Brain 2
TE buffer	Tris-EDTA buffer
TG	Trigerminal Ganglia
TM	Transmembrane Domain
Tom	tdTomato
TUJ1	Neuron-specific Class III β-tubulin

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
V1	Primary Visual Cortex
V2L	Visual Cortex Lateral Area
V2MM	Secondary Visual Cortex
V2ML	Secondary Visual Cortex Mediolateral Area
VEGF	Vascular Endothelial Growth Factor
vIP	Ventricular Intermediate Progenitor
VIP	Vasoactive Intestinal Polypeptide
VMN	Ventromedial Nucleus
VZ	Ventricular Zone
WM	White Matter
WP	Whisker Pad
X-Gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside

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