



**INVESTIGATIONS INTO THE REGULATION OF
SUBVENTRICULAR ZONE NEUROBLAST MIGRATION
BY PROTEIN KINASES.**

A thesis submitted to the Department of Physiology,
Anatomy and Genetics, in partial fulfilment of the
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By

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Abstract

The subventricular zone (SVZ) of the mammalian brain serves as one of only two sources of adult born neurons. Adult born neural progenitors – known as neuroblasts – acquire the ability to migrate and travel large distances to their destination in the olfactory bulb. Disruption of neuroblast migration is associated with learning and memory deficiencies and, following injury, neuroblasts are re-routed to promote neurodegeneration. While a lot of research has attended to augmenting the production and survival of neuroblasts, the body of evidence for pharmacological targets or compounds that promote migration is comparatively sparse.

This thesis set out to identify novel strategies to modulate neuroblast migration for brain repair by studying proteins known to modulate migration and identifying new ones through compound screening.

Firstly, an explant migration assay from the mouse SVZ was used to investigate the potential to use growth factors to stimulate neuroblast migration. This confirmed that insulin-like growth factor 1 (IGF1) and IGF2 regulate neuroblast migration, as previously reported by other research groups. The role of IGF2 is investigated further using a mouse model in which the binding of IGF2 to IGF2R is disrupted, resulting in increased proliferation in the embryonic cortical SVZ, brain overgrowth and perinatal lethality.

In the second half of this thesis I try to tackle one of the major bottlenecks limiting the search in for pharmaceutical interventions targeting neuroblast recruitment: the lack of high-fidelity in vitro migration assays. Drawing concepts from existing in vitro migration assays and cerebral organoid models, I developed a novel neuroblast spheroid migration assays that permits the investigation of large numbers of interventions, concurrently, in 3 dimensions.

Using the spheroid assay I successfully screened 1012 small molecule kinase inhibitors for their effects on neuroblast migration. Several compounds were identified that significantly increased or decreased neuroblast migration. Two genes: MUSK and PIK3CB were selected from the screen as putative biological targets and genetic knockdown of these genes validated that interruption of their activity increased neuroblast migration. In the future these compounds could be studied further to explore their potential for augmenting the recruitments of new neurons to sites of injury so support neuroregeneration, or for decreasing invasion of brain malignancies.

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Authorship Statement

I primarily designed the project in this thesis under the supervision of Prof. Francis Szele. Dr Bass Hassan consulted on the experiments for chapter 3 and Dr Daniel Ebner consulted on chapters 4 and 5.

In Chapter 2, Dr Nick Davies helped to complete the screen of growth factors on the migration of neuroblasts from SVZ explants and the qPCR analysis of IGF family genes expression.

In Chapter 3, Dr Marvat Surakhy generated the Igf2rl1565A mouse model and completed all the necessary husbandry to provide the embryos required for this chapter. Dr Nick Davies and Dr Fernando Garcia-Moreno assisted with the staining and quantification of the embryo brain sections

In Chapter 4, Dr Val Millar assisted in optimising the RMS spheroid assays for use on a high content imaging system. She also developed all of algorithms for automatically analysing the images generated.

In Chapter 5, Dr Daniel Ebner and Dr Val Millar helped in designing the kinase inhibitor screen. Mr Ben Cheung assisted with some of the analysis of the screen data and genetic validation of the biological targets.

All other experimental procedures, quantifications and analyses were done by myself with generous guidance from members of the Szele Lab and the greater scientific community.

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Abbreviations

¹⁴ C	carbon ¹⁴
APP	Amyloid precursor protein
ATP	Adenosine Triphosphate
AURKC	Aurora C kinase
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
BP	Binding protein
CK	Casein Kinase
CDK	Cyclin-Dependent Kinase
CGE	Caudal Ganglionic Eminence
CMS	Congenital myasthenic syndromes
CNS	Central nervous system
CRISPR	Clustered regularly interspaced single polynucleotide repeats
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
DCX	Doublecortin
DDR1	Discoid domain receptor 1
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DYRK	Dual specificity Yak 1-related kinase
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
EPO	Erythropoietin
FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GBM	Glioblastoma Multiforme
GCK	Germinal centre kinase
GDNF	Glial cell-line derived neurotrophic factor

GFAP	Glial-fibrillary acidic protein
GFP	Green fluorescent protein
GRK	G protein-coupled receptor kinase
GSK3	Glycogen synthase kinase 3
HBSS	Hanks Balanced Salt Solution
HCS	High content screening
HTS	High throughput screening
HUNK	Hormonally up-regulated Neu-associated kinase
HVc	hyperstriatum ventrale, pars caudalis
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IN	Intranasal
INSR	Insulin receptor
IR	Insulin receptor
IRS	Insulin receptor substrates
Isx	Isoxazole stem cell modulator of small molecules
LGE	Lateral Ganglionic Eminence
LIF	Leukemia inhibitory factor
MAP	Mitogen-activated protein
MAPK/ERK	Mitogen-activated protein kinase/extracellular signal-related kinase
MASH1	Distal-less homeobox (Dlx)-2 and Achaete-scute homolog 1
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MGE	Medial Ganglionic Eminence
MINK	Misshapen-like kinase
MMP-9	Matrix metalloproteinase-9
MMS	Medial migratory stream
MUSK	Muscle specific kinase
NAD	Nicotinamide adenine dinucleotide
NGF	Nerve growth factor
NPCs	Neural progenitor cells
NSCs	Neural stem cells
OB	Olfactory bulb
PB	Phosphate buffer

PBS	Phosphate buffered saline
PCP	Planar cell polarity
PDGF	Platelet derived neurotrophic factor
PFA	Paraformaldehyde
PI	Propidium iodide
PI3K/Akt	Phosphoinositide 3-kinase/Protein Kinase B
PK	Protein kinase
PKIS	Published Kinase Inhibitor Set
PLK	Polo-Like Kinase
PML	Promyelocytic leukemia
PPP	Picropodophyllin
PSA-NCAM	Polysialylated form of neural cell adhesion molecule
PTEN	Phosphatase and tensin homolog
RGCs	Radial glial cells
RNA	Ribonucleic acid
RTKs	Receptor tyrosine kinases
RMS	Rostral migratory stream
SGFs	Smart Growth Factors™
shRNA	Short hairpin RNA
SDF1	Stromal-derived factor 1
SVZ	Subventricular zone
SGZ	Subgranular zone
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
Tuj1	Neuron-specific Class III β -tubulin
VEGF	Vascular endothelial growth factor
VZ	Ventricular zone
Wnt	Wingless-type MMTV integration site family

Chapter 1 Introduction

1.1 Adult Neurogenesis

In his book *Degeneration and Regeneration of the Nervous System*, Ramón y Cajal wrote that: “In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated” (Ramon y Cajal, 1928). It became deeply established in the neurosciences, as well as by the general public, that no new neurons are added to the brain once development is complete. Any early reports of neurogenesis in the adult brain were dismissed as other mitotic cell types from unknown sources (Levi, 1898; Allen, 1912). It was not until the 1960s that advances in technology brought with them a change in the established dogma. (3H)-thymidine pulses and autoradiographs were first used by Altman (1962), revealing for the first time regions of proliferating neurons in postnatal rat brains. Goldman and Nottebohm (1983) built upon this work, first detailing the development of new neuronal niches in the hyperstriatum ventrale, pars caudalis (HVC), of the ventricular zone in healthy adult canaries. This led the way for numerous research groups to demonstrate that neurogenesis occurs continuously throughout life in specific regions of the adult mammalian brain (Ming and Song, 2011). Adult neurogenesis has since been localised to two regions of the adult brain: the subventricular zone (SVZ) of the forebrain lateral ventricles (Altman & Das, 1966; Altman, 1962; Lois & Alvarez-Buylla, 1993), and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965; Kaplan and Bell, 1984) and potentially several other putative niches (Lin & Lacovitti, 2015).

1.2 Architecture of the rodent subventricular zone

Neurogenesis is not a single event but a complex multistage process encompassing the proliferation of neural stem cells, their survival, migration, differentiation, and functional integration into the developed neuronal circuitry (Ming and Song, 2011). The rodent SVZ lies adjacent to ependymal cells lining the wall of the lateral ventricles. Next to the ependymal cells are slow-cycling stem cells with astroglial properties, called type B1 cells. These cells derive from embryonic radial glia and are typically distinguished by the expression of glial-fibrillary acidic protein (GFAP) and the filament protein Nestin. B1 cells divide symmetrically to produce two transit amplifying cells, or type C cells, which decrease production of GFAP and begin expressing

the distal-less homeobox (Dlx)-2 and Achaete-scute homolog 1 (MASH1) (Obernier *et al.*, 2018). Type C cells divide up to four more times before giving rise to the highly motile neuroblasts, or type A cells. Neuroblasts characteristically express the polysialylated form of neural cell adhesion molecule (PSA-NCAM) and the early neuronal marker doublecortin (DCX) (Alvarez-Buylla and García-Verdugo, 2002). Neuroblasts, B1 and C cells are separated from the rest of the striatum by a second set of GFAP+ cells, B2 cells. Neuroblasts in the rodent brain initiate the migratory route by establishing chains of migration along the rostral migratory stream (RMS), the route to the olfactory bulb (OB) (Altman, 1969). Here the cells detach from the RMS, migrate radially to the outer layers, and terminally differentiate into different subtypes of OB interneurons, with six independent cell types characterised, dependent on their origin along the axes of the SVZ (Merkle *et al.*, 2007).

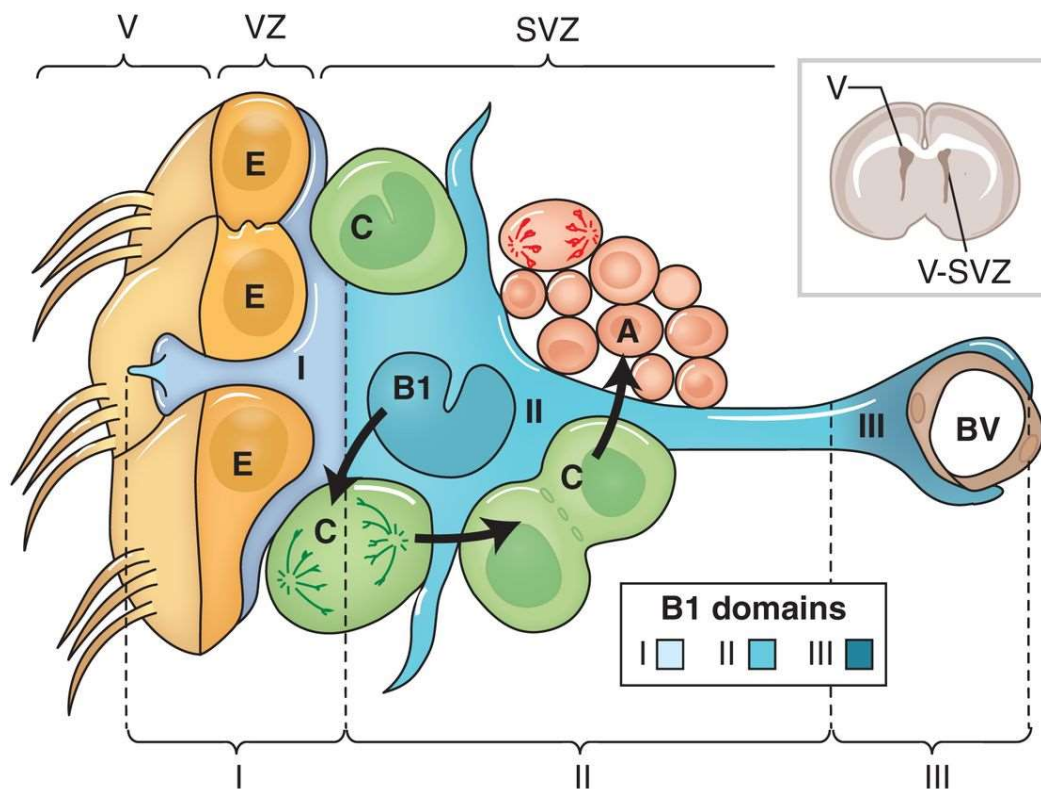


Figure 1 Cytoarchitecture of the rodent subventricular zone (Image taken Lim and Alvarez-buylla, 2016) Type B1 cells (blue) are the astrocytes that serve as the SVZ stem cell. These can divide and produce type C cells (green), which are rapidly dividing, transit amplifying cells. Type C cells give rise to type A cells (red), the migratory neuroblasts.

1.3 The subventricular zone in the human brain

Several studies have conclusively proven the existence of neurogenesis in the human brain (see Bergmann *et al.*, 2015 for review). Nevertheless, neurogenesis in the human SVZ remains a hotly debated topic. The cytoarchitecture of the adult human SVZ is significantly different to the one observed in rodents. The ventricular wall of the human lateral ventricle is lined by a single row of ciliated ependymal cells, but unlike rodents, this borders a hypocellular gap layer containing an extensive network of astrocyte processes. Adjacent is a dense “ribbon” of GFAP expressing astrocytes. Intraoperative samples from the astrocyte ribbon have shown these cells can proliferate *in vitro* and function as multi-lineage neural progenitors (Sanai *et al.*, 2004). This study also found rare PSA-NCAM+ cells with a migratory morphology in the SVZ. However, it has been argued that despite retaining the potential to make new neurons, the primary function of the SVZ ribbon astrocytes is to serve as a source for new glial cells (Menn *et al.*, 2006).

The presence of a migratory path comparable to the rodent RMS is still controversial in adult humans. Migrating cells have been described along the astrocytic ribbon (Quiñones-Hinojosa *et al.*, 2006; Curtis *et al.*, 2007), however these cells are usually found as individuals or pairs and evidence of a physical structure connecting SVZ to the olfactory bulb is still lacking (Paredes *et al.*, 2017). Recently, a much talked about histological analysis of the adult human hippocampus failed to identify any evidence of adult neurogenesis. Interestingly, however, they used the SVZ as a positive control and did identify several DCX+ migrating neuroblasts in the young adults (ages 20-30) examined (Sorrells *et al.*, 2018). In the human infant, extensive migration of neuroblasts has been described and captured in an elegant series of time lapse imaging in post-mortem brain slices (Paredes *et al.*, 2017). In addition to migrating along an RMS akin to the rodent brain to the olfactory bulb, two other collections of neuroblasts were described that are not found in the rodent brain, or in other primates. A sub-population migrate along the medial migratory stream (MMS) destined for the ventral medial prefrontal cortex, an area important for social decision-making and risk processing (Sanai *et al.*, 2011). There is also an ‘arc’ of neuroblasts around the anterior pole of the lateral ventricle that migrate radially into the cerebral cortex (Paredes *et al.*, 2017). Another study of the postnatal development of the human SVZ revealed many young neuroblasts immediately adjacent to the ependyma in infants. There are also several EGFR expressing cells in the infant SVZ alleged to be neural stem cells; although these cells were rarely found after 2 years old (Sanai *et al.*, 2011). DCX+ neuroblasts in the adult

human SVZ are rare and only a small portion express proliferative markers (C. Wang *et al.*, 2011). Postmortem studies of the post-stroke human brain show increased putative neuroblasts around the infarct area in the striatum (Jin *et al.*, 2006; Macas *et al.*, 2006). Although the source of these neuroblasts has not been precisely determined, they accumulate in regions close to blood vessels, suggesting a possible role of vasculature in the successful neuroblast recruitment (Jin *et al.*, 2006).

The inability to consistently birthdate cells in human specimens has stimulated the development of new strategies, such as the use of carbon ¹⁴ (¹⁴C) (Spalding *et al.*, 2015). This approach takes advantage of the increased atmospheric levels of ¹⁴C during the height of atomic bomb testing between the 1940s and 1960s. Cells that were dividing during these years would be expected to have elevated levels of ¹⁴C in their DNA. Work using ¹⁴C birthdating concluded that there is little to no postnatal neurogenesis in the adult human OB (Bergmann *et al.*, 2012). Interestingly new neurons were identified in the human striatum, a brain region adjacent to the SVZ and involved in movement and cognition (Ernst *et al.*, 2014). Remarkably, this study also demonstrated that postnatally generated striatal neurons are preferentially depleted in patients with Huntington's disease, thus linking impairment of adult neurogenesis with neurodegenerative diseases. Striatal interneurons have also been shown to be exclusively generated during development so striatal neurogenesis remains contentious (Wang *et al.*, 2014).

1.4 Embryonic neuronal migration.

During embryonic development, the predominant neurogenesis is that of cortical projection neurons arising from the VZ and SVZ of the dorsal telencephalon. At the onset of neurogenesis, around embryonic day (E) 10.5 in the mouse, neuroepithelial cells in the VZ stop dividing symmetrically and become PAX6+ neural progenitor cells known as radial glial cells (RGCs). RGCs can give rise to projection neurons both directly and via Eomesodermin expressing (TBR2+) intermediate progenitor cells. The first cohort of neurons migrate out of the VZ towards the cortical plate around E11 in mice, using the radial glia filaments acting as a scaffold (Nadarajah and Parnavelas, 2002). Radial glia cells extend from the lateral ventricles, through the VZ and out into the cortex, directing and supporting the migration of these young neurons (Greig *et al.*, 2013). The development of the cerebral cortex progresses with successive waves of

migration that position neurons within the different layers in the cortical plate (Hatten, 1999). Once in their final position they differentiate and integrate into glutamatergic neuronal networks; each expressing layer specific markers (Figure 2A).

There also exists a smaller subpopulation of inhibitory GABAergic interneurons, which must migrate long distances to reach the cortex using a distinct mode of migration called tangential migration (Figure 2B). Interneurons are a highly heterogeneous and diverse neuronal population that arise from progenitor pools within ganglionic eminences of the developing telencephalon. The Medial Ganglionic Eminence (MGE) is the predominant source of cortical and striatal interneurons; while the Caudal Ganglionic Eminence (CGE) gives rise to mainly hippocampal interneurons. The adult SVZ derives from the Lateral Ganglionic Eminence (LGE) that gives rise to most, if not all, interneurons that migrate rostrally and populate both the glomerular and the granule cell layers of the OB (Ayala *et al.*, 2007).

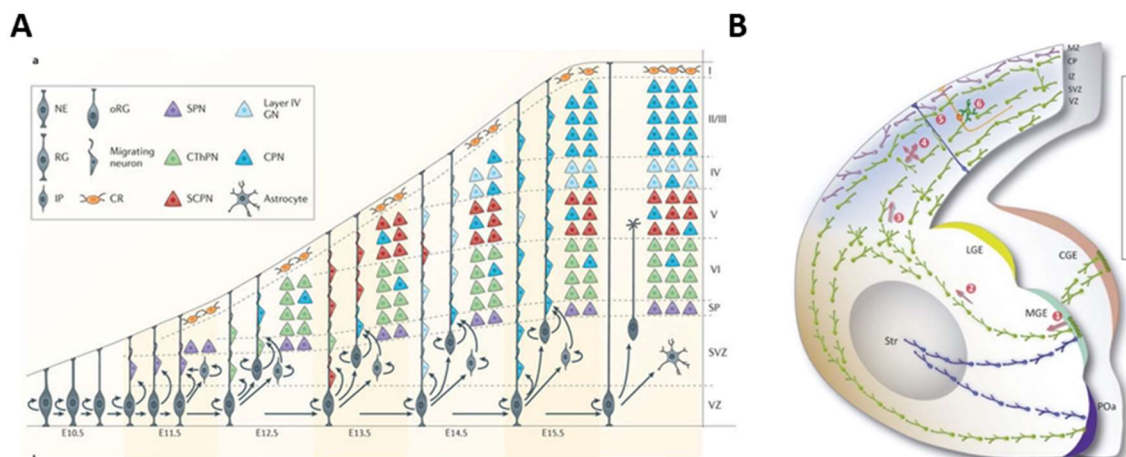


Figure 2 Image of embryonic brain development (Images taken from Greig *et al.*, 2013)

(A) Schematic of neurogenesis in the mouse neocortex during embryo development. Neurogenesis begins around E10, reaching a peak around E15. Radial glia lining the ventricular zone divide to become intermediate progenitors, which give rise to non-mitotic neural precursors. Using the radial glia as scaffolds the neural precursors migrate radially to become cortical projection neurons. The majority of neurogenesis has completed around E17, at which point radial glia release their apical attachment to the ventricle and begin to give rise to astrocytes. **(B)** Schematic representation of the migratory paths of interneurons in the developing mouse brain. Interneurons arise from the ganglionic eminences. Cells in the medial ganglionic eminence (MGE) migrate tangentially to neocortex, while cells originating from the lateral ganglionic eminence (LGE) migrate rostrally to the olfactory bulb. The tangentially migrating interneurons travel perpendicular to the radial glial cells.

1.5 Neuroblast migration in the adult brain

In the postnatal rodent brain, the environment and mode of migration of neuroblasts are distinct from those in the embryonic brain. Neuroblasts generated in the adult mouse SVZ migrate over a long distance (about 8 mm) at a high speed (100 $\mu\text{m}/\text{h}$), toward the OB (Lois and Alvarez-Buylla, 1994; Alvarez-Buylla and García-Verdugo, 2002; Nam *et al.*, 2007). Over 30,000 neuroblasts are estimated to be produced each day in rodents (Alvarez-Buylla and García-Verdugo, 2002). Unlike neuronal migration during development adult neuroblasts migrate without guidance of glial or axonal processes (Doetsch *et al.*, 1997). Instead they form elongated cell aggregates called “chains,” in which each cell migrates along the others in the chain (Lois *et al.*, 1996). As seen in Figure 3 below, the chains of neuroblasts aggregate to form the RMS as they migrate towards their destination in the OB (Doetsch and Alvarez-Buylla, 1996).

At the second postnatal week, the type B2 astrocyte processes progressively encase and separate the forming neuroblast chains into compartments, giving rise to the hallmark glial tunnel of the adult RMS by about week four postnatally (Alves *et al.*, 2002; Peretto *et al.*, 2005). The role of the ensheathing glial tunnel remains to be elucidated, although guidance for neuroblast migration and assisting chain formation have been suggested. *In vitro*, time-lapse microscopy revealed that neuroblasts self-organise into chains and moved rapidly (122 $\mu\text{m}/\text{h}$) in the absence of the encasing astrocytes (Wichterle *et al.*, 1997), suggesting that glial processes are not required for cell migration. In addition, chain formation *in vivo* takes place before the establishment of glial tunnels (Law *et al.*, 1999), indicating that astrocytes are not necessary for chain formation. Neuroblasts migrate along the RMS in a saltatory manner with rapid changes in morphology, speed and direction; complete reversals of direction have also been described (Nam *et al.*, 2007). Migration speed is therefore inherently variable but estimates from two-photon microscopy of slice cultures estimate speeds between 50-105 $\mu\text{m}/\text{h}$ (Nam *et al.*, 2007; James *et al.*, 2011; Young *et al.*, 2012).

Once neuroblasts reach the olfactory bulb, they switch to migrate radially as individual cells then integrate as GABAergic neurons in the olfactory granule cell layer, or dopaminergic neurons in the periglomeruli (Lois and Alvarez-Buylla, 1994). The cellular motions remain saltatory but speeds have been shown to reduce to between 5-65 $\mu\text{m}/\text{h}$ (De Marchis *et al.*, 2001; Bovetti *et al.*, 2007). Functional GABAergic input to newly migrated neurons is first seen as early as 3 days after arrival in the olfactory bulb (Panzanelli *et al.*, 2009). However, as much as 70% do not survive the period

of synaptogenesis and undergo programmed cell death (Petreanu and Alvarez-Buylla, 2002). Neurons that do integrate in rodent brains have been found to survive for up to 19 months.

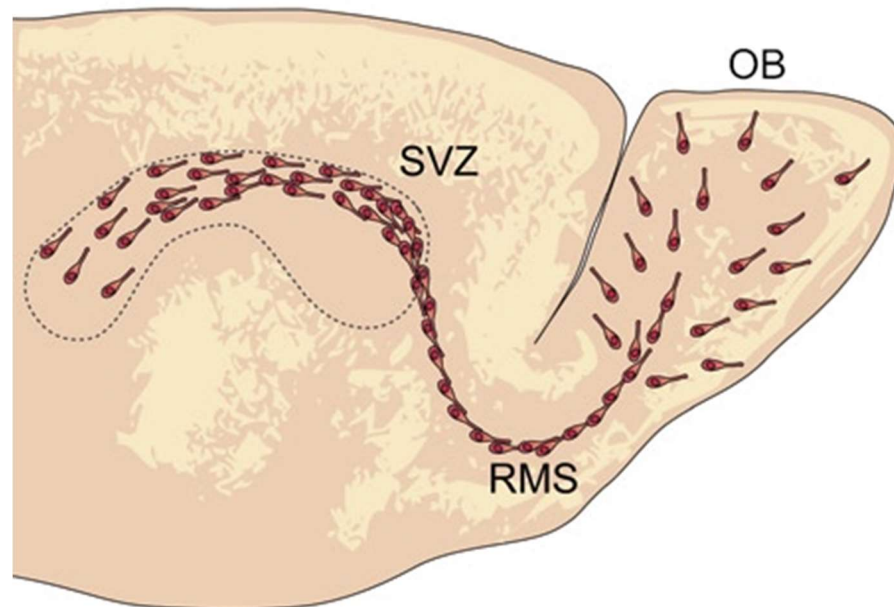


Figure 3 Illustration of neuroblasts migrating along the rostral migratory stream (Image taken from Sawada *et al.*, 2011).

Neuroblasts are generated in the adult SVZ from non-motile C cells. The neuroblasts self-aggregate to form elongated chains that migrate tangentially through the rostral migratory stream (RMS) toward the olfactory bulb. After reaching the olfactory bulb neuroblasts detach from the chain and migrate radially as individual cells into the outer layer where they differentiate into interneurons.

1.6 Mechanisms of neuroblast migration

The mechanisms and dynamics of neuroblast migration from the SVZ into the site of integration in the OB have been extensively studied but are still not fully understood. Elucidating the mechanism that drives this migration is difficult due to the long distance of this migration and the complex route through established brain tissue.

Migrating neuroblasts have a bipolar shape with a relatively long leading process and shorter trailing process. Migrating neuroblasts move in a saltatory manner by repeating three steps: (1) extension of the leading process, (2) movement of the centrosome, and (3) nuclear movement and retraction of the rear of the cell (Kaneko *et al.*, 2017). In the first step, the neuroblast initiates movement by extending its leading process, which points to its destination along the neighbouring cells. They search for guidance signals by vigorously and continuously extending multiple, diverging branches from the leading process to better sense and align with the source of the

orienting gradients (Ming & Song, 2011). The branch that best aligns with the net gradient of the guidance cues is stabilized, while other branches retract, and the nucleus moves in the direction of the stabilized leading process. Neuroblasts can also alter migratory direction by reversing their polarity; that is, by converting the trailing process directly into a leading process while the previous leading process retracts like a trailing process (Martinez-Molina *et al.*, 2011). Once the migratory direction is decided, interneurons advance forward by performing a repeated cycle of two-phase nucleokinesis (Yanagida *et al.*, 2012). First, as the leading process is stabilized, organelles including the centrioles and Golgi apparatus within the perinuclear cytoplasm form a presomal swelling and extend into the leading process. In the second phase, the nucleus translocates toward the presomal swelling as the trailing process retracts toward the new position of the cell soma. This two-phase nucleokinesis results in the characteristic saltatory mode of neuroblast migration, alternating between a resting phase, when the leading process is actively extending and exploring, and a moving phase, when the cell soma translocates in a new direction (Lois *et al.*, 1996).

The unique regulatory mechanisms that orchestrate chain migration have started to be disentangled. The directed migration of neuroblasts is dependent on the concerted action of multiple extracellular factors, cell adhesion/extracellular matrix and axon guidance molecules, growth factors, and neurotransmitters (Lalli, 2014).

Active cytoskeletal modification is required for neuroblast migration in both the adult and embryonic brain. Cyclin-dependent kinase 5 (CDK5) is necessary to establish chain formation of new neurons in the RMS, and also regulates the speed and direction of migratory action (Hirota *et al.*, 2007). The phosphorylation of drebrin on S142 by CDK5 maintains leading process stability for polarized migration along the RMS (Sonogo *et al.*, 2015). Protein kinase C (PKC) dependent phosphorylation of fascin on Ser39 regulates actin bundling and the polarized morphology of migratory neuroblasts (M Sonogo *et al.*, 2013). The expression of PSA-NCAM and β 1-integrin on the membrane surface, matrix metalloproteases, and other matrix molecules such as laminins are also integral to the migration process (Murasu and Horwitz, 2002; Bovetti *et al.*, 2007). These matrix proteins are important in regulating the adhesion of neurons in forming the chain, allowing cells to move past one another whilst staying within the RMS. The vasculature has also been shown to direct the migratory route of new neurons, with chains forming close associations with blood vessels (Whitman *et al.*, 2009).

Other important signals in directing migration are controlled by the directional flow of cerebrospinal fluid (CSF) in the lateral ventricle, which establishes a concentration gradient of diffusible proteins (Sawamoto *et al.*, 2006). Chemorepellents secreted by the choroid plexus guide neurons against the concentration gradient, whereas secreted factors such as netrin1 (Murasue and Horwitz, 2002), glial cell-line derived neurotrophic factor (GDNF) (Paratcha *et al.*, 2006), and brain-derived neurotrophic factor (BDNF) (Chiaramello *et al.*, 2006) direct migrating neuroblasts towards the olfactory bulb. Astrocytes, which form the glial tubes, for neurons to migrate through, are also important in regulating the migratory process through both the uptake of the neurotransmitter GABA (Bolteus and Bordey, 2004) and the endothelial cell-derived BDNF (Chiaramello *et al.*, 2006). Maintenance of this glial tube is also dependent upon neuron-derived soluble protein Slit1, which induces expression of RMS astrocyte receptor Robo and influences the distribution and morphology of these cells (Kaneko *et al.*, 2013). Migration of neurons in the adult brain may be more difficult due to the decreasing numbers of neural stem cells with increasing age. Since neural stem cells (NSCs) secrete Slit1, essential for glial tube maintenance, it is possible that there is not enough Slit1 present in the adult RMS to maintain glial tubes and chain structures to allow efficient migration to the site of integration (Nguyen-Ba-Charvet *et al.*, 2004; Kaneko *et al.*, 2013).

1.7 Diseases implicated in neuroblast migration.

1.7.1 Neurodevelopmental Disorders

Neurological syndromes caused by disruption of neural migration are amongst the most devastating human disorders. Failure of neuronal migration causes severe developmental abnormalities in the layering of the cerebral cortex and results in the human disorder lissencephaly, which means “smooth brain.” Moreover there are several genes that are required for neuronal migration and known to be important in certain neurological disorders, but are largely ignored in studies of the disease process, such as NRG1 (Flames *et al.*, 2004), DISC1 (Kamiya *et al.*, 2005), or the β -amyloid precursor protein APP (Young-Pearse *et al.*, 2007). Table 1 summarises genes involved in neuronal migration that are associated with a syndrome (Evsyukova *et al.*, 2013).

Table 1. Neuronal disorders linked to mutations in genes that disrupt normal neuronal migration. Table adapted from Evsyukova *et al.*, 2013.

Syndrome	Gene	Defect
Periventricular heterotopia	<i>FLNA</i>	Decreased Filamin A affinity for actin cytoskeleton leading to cell-adhesion defect, disrupted onset of neuronal migration (Sheen <i>et al.</i> , 2004)
	<i>ARFGEF2</i>	Impaired vesicular trafficking of adhesion molecules and promotion of Filamin A phosphorylation (leading to a decreased affinity for actin), disrupted neuronal migration (J. Zhang <i>et al.</i> , 2013).
Subcortical band heterotopia	<i>DCX</i>	Disrupted microtubule bundling. Defect in nucleokinesis, disrupted maintenance of neuronal migration (Kappeler <i>et al.</i> , 2006).
	<i>RhoA</i>	Destabilization of actin in radial glial cells and disrupted radial glia scaffold, leading to radial migration defect. Disruption of multipolar-to-bipolar transition, neurite extension, and locomotion of radially migrating neurons (Cappello <i>et al.</i> , 2012).
Type I lissencephaly	<i>TUBA1A</i>	Defects in the tubulin folding and heterodimer assembly pathway. Disrupted radial migration, abnormal lamination of hippocampus and cortex (Keays <i>et al.</i> , 2007).
	<i>PAFAH1B1</i>	Destabilization of microtubules and defective centrosome functions. Defect in nucleokinesis and disrupted maintenance of radial and tangential migrations of neurons (Pilz <i>et al.</i> , 1999).
	<i>ARX</i>	Direct target of Dlx2 transcription factor. Disrupted tangential migration of cortical interneurons (Marcorelles <i>et al.</i> , 2010).
	<i>RELN</i>	Disruption of multipolar neuron polarization, terminal phase of neuronal migration, and inside-out layer formation (Jossin and Cooper, 2011).
Type II lissencephaly	<i>Pomt1</i>	O-Mannosyltransferase mediating biosynthesis of O-mannosyl glycans. Mutation leads to hypoglycosylation of α -dystroglycan, defective basal lamina and glial endfeet-pial membrane interactions, overmigration of cortical neurons (Hu <i>et al.</i> , 2013).
	<i>Dmd</i>	Member of the dystrophin-glycoprotein complex (DGC), linking DGC to actin. Disruption of radial glia scaffold and basal lamina leading to overmigration of cortical neurons (Pawlisz and Feng, 2011).
Polymicrogyria	<i>TUBB2B</i>	Tubulin heterodimer assembly defect. Disrupted radial migration: accumulation of late-born neurons in SVZ (Jaglin <i>et al.</i> , 2009)
	<i>TUBA8</i>	Variant of α -tubulin with unknown function. Mutated in the autosomal recessive syndrome characterized by generalized polymicrogyria and associated optic nerve hypoplasia (Abdollahi <i>et al.</i> , 2009)
	<i>GPR56</i>	Defective basal lamina and glial endfeet-pial membrane interactions, malpositioning of neurons in the developing cortical plate (Li <i>et al.</i> , 2008)
Schizophrenia	<i>NRG1/ERBB4</i>	Decreased PI3K recruitment and activation. Disrupted tangential migration of cortical interneurons (H. Li <i>et al.</i> , 2012).
	<i>DISC1</i>	Abnormal dynein function. Disrupted Disc1–NDEL1, Disc1-ErbB interactions. Accumulation of late-born neurons in the SVZ. Neuronal migration defects in the hippocampus (Seshadri <i>et al.</i> , 2010).
	<i>POU3F2</i>	Regulation of the expression of the p35 and p39 regulatory subunits of Cdk5 in migrating cortical neurons. Neurons are unable to express Dab1, defective migration of cortical neurons (Potkin <i>et al.</i> , 2009).
	22q1.2 deletion	1.5- to 3-megabase hemizygous deletion on human chromosome 22. Leads to multiple CNS defects, including interneuron migration deficits (Meechan <i>et al.</i> , 2012).
Autism	<i>CXCR4</i>	Altered SVZ interneuron migratory stream. More interneurons in the VZ, with frequent radially oriented leading processes (Zhang <i>et al.</i> , 2013).
	<i>CNTNAP2</i>	Neuronal transmembrane protein member (neurexin superfamily) involved in neuron-glia interactions. Disrupted CNTNAP2 activity leads to ectopic neurons in corpus callosum, lamination defects, and decreased number of interneurons (Peñagarikano <i>et al.</i> , 2011).
	<i>CACNA1C and CACNB2</i>	L-type Calcium channel subunits. Potential changes in terminal phase of interneuron migration (Smoller <i>et al.</i> , 2013)
	<i>SOX5</i>	Transcription factor. Inactivation leads to disrupted preplate partition and laminar positioning defects of subplate and deep-layer neurons (Rosenfeld <i>et al.</i> , 2010)
Juvenile myoclonic epilepsy	<i>EFHC1</i>	Microtubule-associated protein. JME mutations disrupt the morphology of radial and tangential migrating neurons and disrupt their migration (accumulation of radial migrating neurons in the SVZ, reduced number of interneurons reaching the cortex) (De nijs <i>et al.</i> , 2012).
Depression	<i>Slc6a4</i>	Serotonin transporter. Disruption of 5-HT6 leads to aberrant interneuron migration and placement in the cerebral wall (Riccio <i>et al.</i> , 2009).
Mental retardation	<i>PAK3</i>	Rho GTPase effector, expression decreased in migrating interneurons, increased when they reach the cortex. Deletion disrupts tangential migration of cortical interneurons. Overexpression of PAK3 leads to arrest of interneuron migration (Peippo <i>et al.</i> , 2007).
Alzheimer's Disease	<i>APP</i>	Accumulation of late-born neurons in deep cortical layers (SVZ/IZ) caused by abnormal Dab1 activation (Young-Pearce <i>et al.</i> , 2007)
Joubert syndrome	<i>Arl13b</i>	Disrupted cilia formation and ciliary localization of signalling receptors. Aberrant patterns of tangential migration of interneurons (Higginbotham <i>et al.</i> , 2012).
Dyslexia	<i>DYX1C1</i>	Disruption of cilia morphology and signalling, defect in radial migration (Hoh <i>et al.</i> , 2012).

1.7.2 Adult Brain Injury

A remarkable feature of adult neurogenesis is the migration of neuroblasts to sites of injury. It has been observed that following a brain injury neuroblasts will migrate and are able to differentiate into functional neurons where the damage occurred (Alvarez-Buylla and García-Verdugo, 2002; Arvidsson *et al.*, 2003; Ramaswamy *et al.*, 2005; Thored *et al.*, 2006; Yamashita *et al.*, 2006; Leker *et al.*, 2007; Lindvall and Kokaia, 2015). The numbers of these new neurons that successfully integrate is small, and typically not sufficient to restore full function so augmenting the migration and survival of neuroblasts has attracted considerable interest in recent history (Lindvall and Kokaia, 2015).

Adult brain injury, such as stroke and trauma, have been well studied in rodents, such as by middle cerebral artery occlusion (MCAO) commonly used to model focal brain ischemia. Increased proliferation of NSCs has been observed in the SVZ between 7 and 14 days after MCAO in rodents, with these cells found to migrate directly to the striatum following the MCAO in rats (Jin *et al.*, 2003; Kreuzberg *et al.*, 2010). Unlike in normal adult neurogenesis, following ischemia neuroblasts are reported to migrate directly to sites of injury from the SVZ, rather than through the RMS into the olfactory bulb. This process was observed from 1 week through to 16 weeks after injury (Thored *et al.*, 2006). Moreover, the neuroblasts from the SVZ can migrate to the cortex as well as the striatum, and differentiate into mature neurons (Kreuzberg *et al.*, 2010).

For functional restoration of the brain to occur after stroke it is imperative that neurons can reach the site of damage. There is some evidence that radial glial cells are located in both the SVZ and the ischemic striatum after stroke, where they can direct migrating neuroblasts (Zhang *et al.*, 2007). Migration post-injury is also aided by the close association between neuroblasts and the vasculature on their migratory journey. Chains of neuroblasts have been shown to interact with endothelial cells resulting in the chains organising in a mediolateral direction so that neuroblasts move parallel to blood vessels (Yamashita *et al.*, 2006). Following insult, increases in blood vessel density and endothelial cell proliferation are exhibited for the first 2 weeks, and new neuroblasts remain close to blood vessels for up to 16 weeks as they continue the neurogenesis process towards the ischemic damage (Thored *et al.*, 2007).

Multiple treatment approaches for stroke associated with improved functional recovery are linked to improved neurogenesis. Erythropoietin (EPO), a successful therapy in animal models but

showing mixed benefits in clinical trial (Souvenir *et al.*, 2016), is a neuroprotective agent administered to the stroke-damaged brain, which has also been shown to promote neurogenesis. Moreover, endogenous EPO has also been shown to be involved in mediating neurogenesis after stroke. Mouse models with conditional knockdown of the EPO receptor subjected to cortical stroke reduced NSC proliferation in the SVZ, decreased levels of migration of neuroblasts to the site of injury and impaired functional recovery (Yip *et al.*, 2011).

Especially interesting from a therapeutic perspective are extracellular signalling proteins that could be useful in a clinical setting. Intrastratial administration of GDNF has been reported to increase cell proliferation in ipsilateral SVZ, to recruit neuroblasts into the striatum after MCAO, and to promote survival of neurons once they have differentiated (Kobayashi *et al.*, 2006). GDNF also showed positive effects in a patient with Parkinson's disease where it induced sprouting of dopaminergic neurons following intrastratial infusion, suggesting this could be a treatment approach to be utilised in multiple neurodegenerative disorders (Douglas-Escobar *et al.*, 2012).

Causal links between neurogenesis and the vasculature have been identified. Angiogenesis related proteins, such as stromal-derived factor 1 (SDF1) and angiopoietin 1. SDF1 acts as a tropic signal for neuroblasts to direct their migration to the ischemic area, and SDF1 has been shown in rodent models to improve post-stroke neuroblast migration and recovery in behavioural tasks (Ohab *et al.*, 2006). In the region of the stroke SDF1 levels are increased via production by reactive astrocytes and activated microglia (Robin *et al.*, 2006). The SDF1 receptor, CXCR4, is expressed on neural progenitors, and initiates the migration of neuroblasts (Robin *et al.*, 2006). This mechanism demonstrates how ischemic damage and inflammation can regulate migration of neuroblasts.

Monocyte chemoattractant protein-1 (MCP-1) and osteopontin are also inflammation-induced proteins that can trigger neuroblast migration through a similar mechanism to SDF1. The expression of the MCP-1 gene is also upregulated in reactive astrocytes and activated microglia, and MCP-1 binds to its receptor CCR2, which is found on new neuroblasts after stroke. Mice lacking MCP-1 or CCR2 showed decreased neuroblast migration into the striatum after stroke than in the wildtype animals (Yan *et al.*, 2007). Osteopontin is upregulated in microglia in the brain following stroke and stimulates the integrin β 1 receptor on neuronal progenitor cells, promoting migration in the striatum. Again, reducing osteopontin in the brain by introduction of a neutralising

antibody against the protein significantly reduced neuroblast recruitment to the striatum (Yan *et al.*, 2009b). In contrast to the neuroblasts in the RMS, which is a permissive environment for migration in the adult brain, those in the damaged striatum need to move through developed neuronal circuitry to reach the damaged area. Lee *et al.* (2006) found that levels of the extracellular protease matrix metalloproteinase-9 (MMP-9) increases after stroke and colocalises to DCX, a neuroblast marker, indicating remodelling of the ECM facilitates migration towards the lesion. An inhibitor for MMP reduced neuroblast migration in the mouse striatum, suggesting a role for extracellular proteolysis, the main function of MMP, in the regulation of neuroblast migration following ischemic damage.

1.7.3 Brain Tumours

Neuronal migration is not necessarily regenerative and there are mounting efforts to identify strategies to block migration from neural malignancies. Glioblastoma Multiforme (GBM) is both the most common and most deadly form of brain cancer. Despite extensive research, outcomes for GBM have been largely unchanged since the introduction of temozolomide in 2005. Although still debated, increasing evidence is pointing to a role of the SVZ in GBM development. Between 50-60% of GBM tumours contact the SVZ and this is correlated with unique recurrence and migration patterns (Jafri *et al.*, 2013). Tumours contain a subpopulation of cancer stem cells that display similar characteristics and markers to SVZ stem cells, such as nestin and CD133 (Lim *et al.*, 2007). In animal models, GBM can be induced in the SVZ with the genetic manipulation of single oncogenic genes: IDH1, Nf1, Trp53, and Pten (Jacques *et al.*, 2010; Alcantara Llaguno *et al.*, 2015; Bardella *et al.*, 2016).

GBM tumours are highly invasive and the process of GBM invasion is similar to neuroblast migration, with GBM cells using the same migratory routes as neuroblasts (Arvidsson *et al.*, 2003), and likely sharing many similar mechanisms. GBM tumours associated with the SVZ demonstrate a tangential migration pattern that differs from tumour growth observed away from the SVZ (Amodeo *et al.*, 2017). Genetic profiling of invasive GBM tumour cells found several genes associated with SVZ neuroblast migration, including cMyc and AnnexinA2 (Haskins *et al.*, 2013). Recently, Amodeo *et al.* (2017) identified a common pathway to both processes in the CNS, through the regulator of forebrain development, the nuclear scaffold protein promyelocytic

leukaemia (PML). As well as increasing neuroblast migration in the adult rodent brain, PML is also activated in human primary GBM cells. The study found the pro-migratory function to be independent of its effect on cell proliferation and found it to act through Polycomb repressive complex 2 (PRC2)-mediated repression of Slits, genes known to regulate axon guidance. This study demonstrated a role for PML and Slit genes in both normal NPC and GBM cell migration in humans and rodents (Amodeo *et al.*, 2017). Slits are known to guide axon projections and neuroblast migration during development but Slit genes have now been found to attract vascular endothelial cells and promote tumour-induced angiogenesis (Wang *et al.*, 2003). Prior to this study neuronal guidance cues had been shown to also regulate the guidance of leukocytes during their migration, and furthermore leukocyte factors had been found to affect neurons (Wu *et al.*, 2001; Rao *et al.*, 2002). These studies together indicate the conserved role of Slits in cell migration across multiple cell types, from neuroblasts to vascular endothelial cells, in both healthy development and pathological processes (Wang *et al.*, 2003).

DCX is another protein that is well characterised in neurogenesis but is also thought to play a role in migration mechanisms of cancer cells. DCX in the CNS is important in stabilising microtubules to support migration, and it has been suggested that unstable interactions between DCX and microtubules, causing disrupted movement of cells, may initiate the unregulated migration of cancer cells (Ayanlaja *et al.*, 2017). DCX is expressed in human GBM (Daou *et al.*, 2005), gangliogliomas (Becker *et al.*, 2002), and cortical tubers (Lee *et al.*, 2003), and the expression of DCX in brain tumours is concentrated at the invasive front, supporting the role of this protein in migration (Ayanlaja *et al.*, 2017). These studies have common findings in demonstrating genes that affect migration of neuroblasts in the brain can also have roles in cell migration in pathological processes in the CNS and other tissues.

1.8 Pharmacological manipulation of neuroblast migration

With mechanisms underlying neuroblast migration beginning to be elucidated, pharmacological compounds to enhance or block these processes can be investigated. Since some of the genes regulating migration in pathological processes are known (as described in Table 1; Evsyukova *et al.*, 2013), therapeutic strategies to return these to physiologically normal levels can be identified.

However, to augment regeneration following injury, novel mechanisms of action need to be identified.

The neurogenic response depends on the careful orchestration of several cellular processes. First there must be activation and expansion of neural progenitors, secondly these cells must survive in the current differentiation state, the cells must migrate to the site of injury, differentiate into the necessary neuronal subtype, and finally integrate into the existing neuronal networks. Without each of these steps working precisely the new cells cannot replace the lost cells in the neuronal architecture and will not recover the damage at the site of injury. Many therapeutic strategies are focused on stimulating the birth of new cells. However, most studies in rodent models have not determined the specific stage in the neurogenesis process that is being targeted by the therapy of interest. Indeed, recent findings of neurogenesis following stroke have shown that production of neuroblasts is likely not the bottleneck in this process, and that focus should be turned instead to the survival of neuroblasts, the directed migration, and the differentiation into functional neurons (Lindvall and Kokaia, 2011). As many as 50% of neuroblasts and newborn neurons die through apoptosis triggered by the cells to eliminate redundant or incorrectly connected cells, greatly reducing the numbers that eventually integrate into the brain networks (Lu *et al.*, 2017).

Few studies have focused on the promotion of migrating neuroblasts as a treatment angle (Cui *et al.*, 2009; Zhang *et al.*, 2010). The distances that separate sites of origin from ischemic lesions in the adult brain result in migratory routes that are orders of magnitude longer than in the embryo and contain more complexities, such as extensive vascularization and dense regions of white matter, mature dendrites, axons, and synapses. This impediment, together with the low rate of survival and differentiation of neuroblasts, result in approximately only 0.2% of neurons damaged in a rodent stroke model getting effectively replaced (Arvidsson *et al.*, 2003). Therefore, increasing the number of cells reaching the site of injury is essential to augment functional recovery.

1.8.1 Growth Factors & Neurotrophic Factors

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors have been shown to function as key regulators of important cellular processes, including proliferation, differentiation, survival and cell migration. Many growth factors and their corresponding RTKs have been identified as potential modulators of SVZ neurogenesis (see Table 2). In addition myriad studies have shown that administration of growth and neurotrophic factors can augment the numbers of SVZ-derived neuroblasts and neurons at sites of injuries in animal models (Lindvall and Kokaia, 2011). The blood–brain barrier (BBB) protects the brain by allowing the entrance of only specific molecules and proteins, but at the same time, it presents an important obstacle for the delivery of medications to the CNS. As a result, intranasal (IN) delivery has attracted the attention of researchers and clinicians as an alternative, non-invasive, and effective route for protein therapeutic delivery. Endocytosis by nerve processes in the nasal epithelium is thought to account for the majority of drug absorption (Lioutas *et al.*, 2015).

While promising, growth factor therapy is still in its infancy. Unfavourable pharmacokinetics, poor blood brain penetration and adverse effects associated with systemic administration will need to be overcome. Many of these growth factors have common intracellular pathway targets, which can result in unexpected combinatorial responses and altered expression of endogenously produced factors. Further research is, therefore, needed to determine and optimise the ideal growth factor based therapy to promote the progenitor migration for brain regeneration.

Table 2. Growth factors and their receptor tyrosine kinases associated with SVZ neurogenesis

Receptors	Physiological function in SVZ	Therapeutic potential in injury
Insulin-like Growth Factor-I (IGF)		
InsR, IGF1R	IGFR expressed in olfactory bulb and RMS. IGF1R deletion disrupts neuroblasts exit from SVZ (Hurtado-Chong <i>et al.</i> , 2009). Ectopic expression of IGF <i>in vivo</i> causes chemoattraction of neuroblasts to source (Maucksch <i>et al.</i> , 2013).	IN delivery increases proliferation of NG2+ neural precursors, attenuated brain damage and improved some behavioural deficits in neonatal brain injury model (Cai <i>et al.</i> , 2011; Lin, Rhodes and Cai, 2011)
Epidermal Growth Factor (EGF)		
EGFR, ErbB4	EGFR is expressed on B cells and C Cells and regulates proliferation in the SVZ (Doetsch <i>et al.</i> 1997). EGFR has also been found to be expressed at low levels on neuroblasts. Exposing brain slices to TGF α , an agonist of EGFR, decreased the percentage of migrating cells in the RMS by approximately 40% (Kim <i>et al.</i> , 2009).	IN delivery of HB-EGF in perinatal injury model enhances generation of oligodendrocytes, diminishes structural abnormalities and alleviates behavioural deficits (Scafidi <i>et al.</i> , 2014; Cantarella <i>et al.</i> , 2007)
Fibroblast Growth Factor (FGF)		
FGFR1, FGFR2	FGF2 is found in high concentration in B cells but not C cells. It is essential for stem cell proliferation and maintenance and is predicted to mediate neuroblast migration via an interaction with anosmin (García-González <i>et al.</i> , 2010).	IN delivery of FGF increased proliferation in SVZ, recruitment of DCX+ progenitors and improved neurological functional recovery in rat MCAO model of stroke (Cheng <i>et al.</i> , 2011)
Vascular Endothelial Growth Factor (VEGF)		
FLT1, VEGFR2	SVZ progenitors express FLT and FLT null mice have increased proliferation but fewer neuroblasts in the RMS. VEGF expressed by SVZ astrocytes and stimulates neuroblast migration from SVZ explants via VEGFR2 (Zhang <i>et al.</i> , 2003). However, VEGF may modulate neuroblast migration indirectly by modulating vasculature (Bozoyan <i>et al.</i> , 2012).	IN delivery reduces infarct area and increases microvessels in rat MCAO stroke model (Yang <i>et al.</i> , 2010).
Brain Derived Neurotrophic Factor (BDNF)		
TrkB, p75NTR	BDNF expressed by SVZ vasculature, TrkB found on RMS astrocytes, while neuroblasts express p75NTR (Snappyan <i>et al.</i> , 2009). BDNF overexpression increases neuroblast numbers in RMS (Grade <i>et al.</i> , 2013). BDNF stimulates neuroblasts motility <i>in vitro</i> but not migration distance (Chiaramello <i>et al.</i> , 2007)	IN delivery alleviates injury in rate MCAO stroke model (Jiang <i>et al.</i> , 2011) and increases migration of progenitors from SVZ (Alcala-Barraza <i>et al.</i> , 2013)
Glial Derived Neurotrophic Factor (GDNF)		
GFRα1, RET	GDNF, NCAM and GFR α 1 expressed along RMS and olfactory bulb. GDNF has chemoattractant role on neuroblasts, potentially via activating Cdk5 (Paratcha <i>et al.</i> , 2006)	IN delivery protects against neural apoptosis in rat model of Parkinson's Disease (Yue <i>et al.</i> , 2017)
Nerve Growth Factor (NGF)		
TrkA	NGF and TrkA expressed in SVZ/RMS. Found to modulate migration and differentiation of neuroblasts (Shamloo <i>et al.</i> , 2015) and oligodendrocyte precursor cells (Triaca <i>et al.</i> , 2004)	IN delivery improves outcome in MCAO model of stroke in rat (Cheng <i>et al.</i> , 2009; Zhu <i>et al.</i> , 2011) and traumatic brain injury in rat (Tian <i>et al.</i> , 2012) and in human infant (Chiaretti <i>et al.</i> , 2017).
Hepatocyte Growth Factor (HGF)		
cMet	High levels of cMET expression in neuroblasts but not astrocytes (Garzotto <i>et al.</i> , 2008). <i>In vitro</i> HGF has been shown to increase proliferation, decrease differentiation and be a chemoattractant. Inhibition <i>in vitro</i> increases neuroblast production (T. W. Wang <i>et al.</i> , 2011).	Unknown
Neuregulins (NRG1-NRG3)		
ErbB4	ErbB4 highly expressed on C cells and neuroblasts (Anton <i>et al.</i> , 2004). ErbB4 deletion results in RMS disruption. NRG signalling proposed to influence switch from tangential to radial migration in olfactory bulb.	Unknown

1.8.2 Small Molecules

An exciting development in the goal to enhance endogenous neurogenesis is the discovery that many existing drugs have been shown to increase neurogenesis both in animal models and humans. For example, the antidepressants Sertraline and Fluoxetine, the antiepileptic drug Ethosuximide, the antipsychotic Haloperidol, and the Alzheimer's Disease drug Memantine have all been shown to increase adult neurogenesis (see Davies *et al.* (2015) for review). This leads to the intriguing possibility that some of their biological effects may arise from the production of new neurons. There is now considerable interest in discovering novel compounds that promote the proliferation, survival, differentiation, maturation and migration of neural stem cells.

An *in vivo* screen of hippocampal neurogenesis identified P7C3 and its derivative P7C3A20 (Pieper *et al.*, 2010). The authors went on to demonstrate that these molecules work through inhibiting apoptosis of new born neurons, an effect mediated through the NAD salvage pathway. In 2010 Wurdak *et al.* published a phenotypic screen for compounds promoting neural differentiation of mouse neural precursor cells using the expression of markers for stem cells (Nestin), neurons (Tuj1) and astrocytes (GFAP). They identified the molecule KHS101, which promotes neurogenesis *in vivo* at the expense of proliferation. Starting from the unexpected result that compounds screened for cardiac regeneration induced a neuronal phenotype, Schneider *et al.* (2008) identified a series of 5 isoxazoles, they named Isx (isoxazole stem cell modulator of small molecules). Additional experiments selected Isx9, a potent stimulator of cell proliferation and neuroblast differentiation without depleting the neural stem cell pool that improves spatial memory in adult mice (Petrik *et al.*, 2012). Kim *et al.* (2012) identified several GSK3 β inhibitors as potent inducers of neuronal differentiation, which are believed to work via that activation of the Wnt/ β -catenin pathways. One of these compounds, SB216763, has subsequently been shown to increase neurogenesis and decrease ischemic damage in an animal model of stroke (Piccin, Tufford and Morshead, 2014). A similar screen seeking to discover compounds that promote dopaminergic differentiation also concluded that the neurogenic compounds identified consisted of three main classes: statins, TGF- β inhibitors and GSK3 inhibitors (Rhim *et al.*, 2015). A research team at GSK subsequently adapted the Kim *et al.* (2012) protocol to use human neural progenitor cells and identified a potent promoter of neural differentiation, with a yet unannotated biological target (Lin *et al.*, 2015). Edaravone, a free radical scavenger widely used in Japan for acute ischemic strokes, has also been shown to modulate both hippocampal and SVZ

neurogenesis via upregulation of BDNF production (Okuyama *et al.*, 2015). A subsequent study also identified edavarone, along with 5-methyl-7-methoxyisoflavone and lovastatin as candidate compounds to stimulate proliferation and maturation of oligodendrocyte precursor cells (Eleuteri *et al.*, 2017).

Most of the reported promotion of neurogenesis is dependent on increasing the levels of survival or differentiation of cells. In contrast, very little progress has been made in identifying small molecules that modulate progenitor migration. As will be discussed in detail in Chapter 4, this is due in a large part to the lack of suitable *in vitro* assays limiting research to single genetic interventions. For example, a promising recent study utilised a potent inhibitor of the Down's Syndrome related dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) to rescue aberrant radial migration and dendritic spine development in a mouse model of the disease (Nakano-Kobayashi *et al.*, 2017). Two separate studies employed the same embryonic organotypic brain slice culture, which identified the Src family kinase inhibitor PP2, the PKC inhibitors BIM1 and Ro318220, and 11 novel nonannotated compounds as disruptors of cortical radial migration (Zhou *et al.*, 2007; Jossin *et al.*, 2003). However, to my knowledge, the search for small molecules compounds that stimulate neural progenitor migration is still an open challenge.

1.9 Aims

This thesis set out to identify novel strategies to modulate neuroblast migration for brain repair. The aim is to add to the knowledge described in the Introduction by studying proteins known to modulate migration, and identifying new ones through compound screening, to confirm proteins that increase neuroblast migration from the SVZ that could be therapeutic targets for recovery after stroke or brain injury.

My studentship is partially supported by Silver Creek Pharmaceuticals who are developing a new class of protein therapeutics engineered to overcome the short half-lives and off-target effects of naive growth factors. Known as Smart Growth Factors these are fusion proteins consisting of a targeting arm to concentrate the drug in the tissue of interest, a half-life modulator to prolong exposure, and a modified growth factor signalling arm tuned to elicit the desired signalling predicted by computational modelling. In chapter 2 of this thesis I set out to establish a discovery platform to instruct future development of Smart Growth Factors for the stimulation of endogenous

neuroblast migration. Through this I determined that, of the growth factors previously reported to modify neuroblast migration, only IGF1 and IGF2 significantly stimulate migration when used in isolation. Due to conflicts in commercial interests and personnel changes the co-development with Silver Creek ceased at this point.

In chapter 3 I investigated the role of the IGF2R receptor in the developing brain using a novel mouse model in which the binding of IGF2 to IGF2R is eliminated through a single point mutation. We show that this mutation results in perinatal lethality, increased brain and body weight and an increase in proliferation in the VZ and SVZ of the neocortex.

In chapter 4 I aim to develop a medium to high throughput platform for studying compounds or genes that modulate neuroblast migration. The phenotypic screening assay developed is based on 3D spheroids and enables the rapid and robust testing of both molecular and genetic manipulations on neuroblast migration.

In chapter 5 I complete a screen of ~900 small molecule kinase inhibitors using the new phenotypic migration assay. Several compounds were identified that significantly increase and decrease migration and the knock down of two genes: MUSK and PIK3CB were validated to increase the migration of SVZ neuroblasts.

Chapter 2 Insulin-Like Growth Factor 1 increases migration of neuroblasts *in vitro*

2.1 Introduction

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors that have been shown to function as key regulators of important cellular processes, including proliferation, differentiation, survival and cell migration. Each RTK binds to one or more soluble or membrane-bound ligands, such as growth factors, that promote the formation of multi-component signalling complexes and the activation of 'immediate-early' signalling.

Many growth factors and their corresponding RTKs have been identified as potential modulators of subventricular zone neurogenesis. These factors include basic fibroblast growth factor (FGF2), epidermal growth factor (EGF), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and platelet derived neurotrophic factor (PDGF) (see Table 3). There has been increasing interest in the potential to use growth factors as biotherapeutics for neuroregeneration after both adult and neonatal brain injury. IN delivery of IGF1, EGF, NGF, BDNF, FGF1 and VEGF have all resulted in a positive therapeutic outcome in mouse models, which has often been cited as at least partially down to increased recruitment of SVZ neuroblasts (Schäbitz *et al.*, 2007; Cheng *et al.*, 2009, 2011; Alcala-Barraza *et al.*, 2013; Ju *et al.*, 2014; Scafidi *et al.*, 2014). However, relatively little systematic data is available on the diversity of signalling responses mediated by RTKs signalling in the different cell types in the SVZ.

Table 3. The effects of growth factors on neurogenesis

Cytokine	Effects On Neurogenesis				References
	Proliferation	Migration	Differentiation	Survival	
Basic fibroblast growth factor (FGF2)	↑		↓	↑	(Frinchi <i>et al.</i> , 2008; Woodbury and Ikezu, 2014)
Epidermal Growth Factor (EGF)	↑	↓			(Kim <i>et al.</i> , 2009; Wang <i>et al.</i> , 2013)
Glial Derived Neurotrophic Factor (GDNF)		↑		↑	(Kobayashi <i>et al.</i> , 2006; Paratcha, Ibáñez and Ledda, 2006)
Insulin Like Growth Factor 1 (IGF1)	↑	↑	↑	↑	Ziegler <i>et al.</i> , (2015); Hurtado-Chong <i>et al.</i> , 2009
Vascular Endothelial Growth Factor (VEGF)	↑	↑			(Ma <i>et al.</i> , 2012; Greenberg and Jin, 2013)
Nerve Growth Factor (NGF)		↑	↑	↑	(Saito, 2004; Bath and Lee, 2010)
Heregulin/neuregulin	↑	↑			(Ghashghaei <i>et al.</i> , 2006)
Platelet Derived Neurotrophic Factor (PDGF)	↑	↓	↑		Jackson <i>et al.</i> , 2006; Sato <i>et al.</i> , 2016
Bone Morphogenetic Protein 4 (BMP4)		↓	↓		(Cole <i>et al.</i> , 2016)

There are two major signalling pathways triggered by RTKs. In the MAPK/ERK pathway, the activation of the receptor activates the small GTPase Ras which recruits Raf to the membrane where it is activated. Raf subsequently activates MEK and MEK activates ERK. The second major RTK mediated pathway is the PI3K/Akt. Growth factors activate the lipid kinase PI3K through either direct PI3K recruitment to the active RTK or indirect recruitment involving adaptor proteins. PI3K generates phosphatidyl inositol 3,4,5 tri-phosphate (PIP3), which recruits the protein kinase AKT to the plasma membrane where it is activated by 3-phosphoinositide-dependent kinase 1 (PDK1). Notably, several downstream proteins, such as the Glycogen synthase kinase 3 (GSK-3) and Cyclin dependent kinases (CDKs), are often positioned as converging nodes of the MAPK/ERK and PI3K/Akt pathways, such that cross talk between the two pathways coordinates the balance between self-renewal and differentiation (Rhim *et al.*, 2016).

2.1.1 Insulin-like growth factor signalling family.

The IGF family includes ligands, receptors, IGF binding protein (IGFBPs) and an ever-increasing list of signalling modulators. Classically, there are three ligands: Insulin, IGF1 and IGF2, three cell surface receptors: insulin receptor (IR), IGF1R and IGF2R and at least seven IGFBPs (Figure 4). The IGF1R binds IGF1 with high affinity and both IGF2 and insulin with lower affinities (approximately 10-fold and 100-fold, respectively). Binding of IGFs to the IGF1R induces a conformational change in the receptor that results in autophosphorylation and the activation of the insulin receptor substrates (IRS-1 through -4) and Src-homology collagen protein (Shc) (LeRoith *et al.*, 1995). These in turn activate the intracellular signalling pathways including PI3K/Akt and MAPK/ERK. IR exists in two isoforms: IR-B and IR-A, both of which can form IR/IGF1R hybrids that preferentially signal with IGF1 and IGF2. Only IGF2 binds to the IGF2R and while there are some reports that the IGF2R may signal via the MAPK/ERK pathway, it is largely agreed that its primary function with respect to IGF signalling is to closely control the availability of IGF2 to the functional receptors.

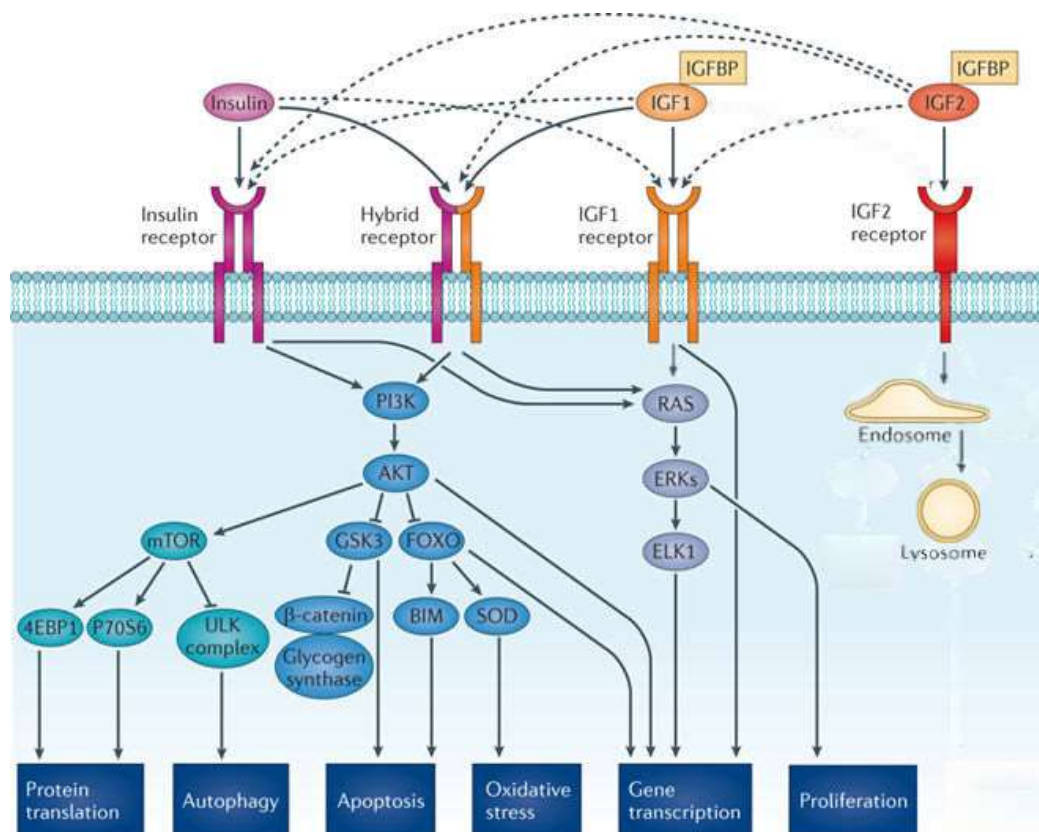


Figure 4 Insulin family signalling pathways (Image adapted from Fernandez and Torres-Alemán, 2012).

The ligands: insulin, IGF1 and IGF2 can bind the receptor tyrosine kinases IR, IGF1R or the hybrid receptor. Canonical IGF1R signalling proceeds through the MAPK/ERK and the PI3K/AKT cascades. IGF2R binds IGF2 and directs it for lysosomal degradation. IGF1 and IGF2 are bound to IGFBPs in the extracellular space to closely regulate the local ligand bioavailability.

2.1.2 Smart Growth Factors

Natural growth factors have certain fundamental characteristics that limit their use as therapeutics. There are native enzyme and binding proteins that limit their half-life and tissue accumulation. There is also widespread expression of their cognate receptor resulting in adverse off-target effects. Silver Creek Pharmaceuticals have developed a novel technology platform to engineer Smart Growth Factors™ (SGFs) that address these limitations and specifically target and prolong growth factor signalling to promote repair in cell types/tissues of interest. Given the growing body of evidence demonstrating that intranasal delivery of growth factors has a therapeutic effect in multiple animal models of brain injury by activating SVZ neurogenesis, there is reason to consider developing SGFs for neuroregeneration.

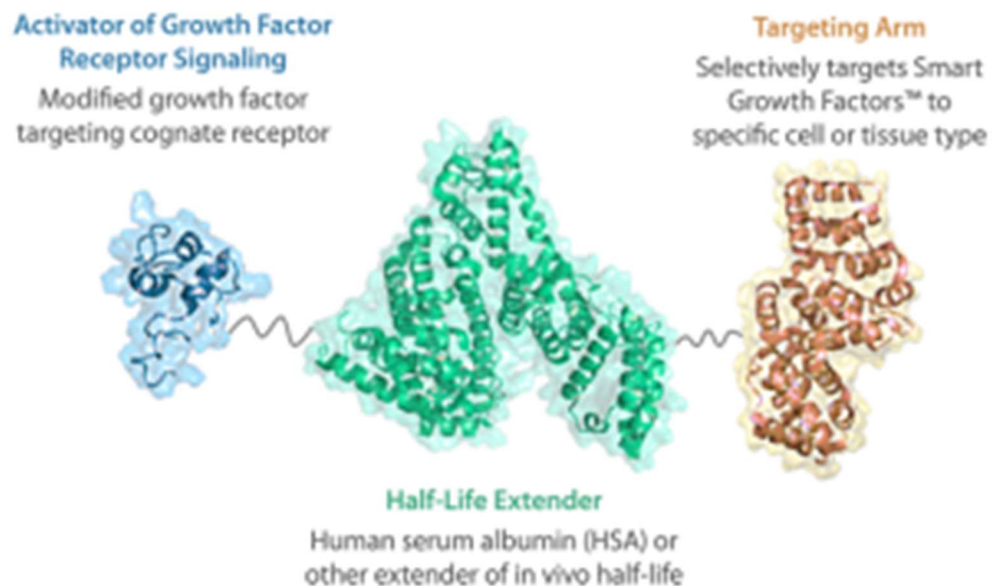


Figure 5 Smart Growth Factor diagram (<https://www.silvercreekpharma.com/>)

2.2 Aims

The goals of this chapter were to characterise the diversity of response of SVZ neuroblasts to growth factors previously indicated to modulate migration and to identify factors that controlled the magnitude of this response. The aim was to generate a simple model by which to compare RTK signalling such that we could identify potential strategies for the development of Smart Growth Factors to stimulate neuroregeneration.

2.3 Methods

2.3.1 Animals Used

CD1 Mice were obtained from Charles River. GAD65-GFP breeder mice were obtained from Enrico Mugnaini (Northwestern University) (De Marchis *et al.*, 2004).

2.3.2 Murine Dissection and stem cell harvesting

Hypothermia was induced in four or five day old postnatal (P4 or P5) mice followed by decapitation. Intact brains were carefully removed and placed in Hanks Balanced Salt Solution (HBSS) before being sliced into 50µm coronal sections using a McIlwain tissue chopper. The SVZ were microdissected from coronal sections using 1ml syringes and 27G needles under a dissecting microscope. SVZ/RMS tissue was pooled in a chilled Eppendorf tube. The tissue was centrifuged for 15 seconds to pellet the tissue, the excess supernatant removed and then chopped with fine iris scissors. The tissue was incubated with 1ml Accutase (Sigma A6964-100ML) at room temperature for 15 minutes. The cell pellet was fully resuspended with a P200 pipette to assist mechanical separation and washed three times with 2.5ml NB-A+ media (Neurobasal-A (Gibco 21103) supplemented with 1% (v/v) B27 (Gibco 17504), 2% (v/v), 1Xv/v Glutamax-1 (Gibco 35050-038) and 1% (v/v) 50U/ml Penicillin/Streptomycin (Gibco 15070-063)) and centrifuged for five minutes at 280xg at 4°C. The washed cell pellet was resuspended in 1ml NB-A+GF media (NB-A+ media supplemented with 20ng/ml EGF (Sigma E9644-2MG) and 20ng/ml FGF-2 (R&D Systems 233-FBI) and counted using a haemocytometer and 0.4% Trypan Blue (Sigma T8154). Cells were diluted to a density of 1×10^5 cells ml⁻¹ in NB-A+GF media and 2ml of cell solution was transferred to wells of a 6-well non treated culture plate (BD Falcon 351146). Cells were left to incubate at 37°C and 5% CO₂ in a humidifying incubator.

2.3.3 Subventricular Zone Explant Migration Assay

P2-P6 mouse pups were culled, brains removed and 300µm section made as per the above murine dissection protocol. The RMS is dissected from the olfactory bulb sections and can be identified as the translucent triangle region in the centre. Only the dorsolateral SVZ was dissected as this is the region consisting of the most progenitors. Using a syringe and 27G needles the dissected region was cut into small equally sized cubes ~200µm³. Explants were set in a 10ul

drop of Growth Factor Reduced Matrigel (Corning: #356231) at a concentration of 6mg/mL at the centre of each well in a 24 well culture plate. Explants were cultured in NB-A+ media [Neurobasal-A (Gibco 10888-022) supplemented with 1% Glutamax (Gibco 35050-038) and 2% B27 minus insulin (Gibco A1895601)] supplemented with 20ng/ml EGF (Sigma E9644-2MG), FGF-2 (R&D Systems 233-FBI), Heregulin (Sigma, SRP3055-50UG), IGF-1 (Sigma, I8779-50UG), PDGF-BB (Sigma SRP3229-10UG), VEGF165 (Sigma, SRP4364-10UG), NGF-B (Sigma. SRP4304-20UG) and GDNF (Sigma, SRP3200-10UG). Images were taken of explants after 24 and 48 hours on a Motic AE2000 inverted microscope with Moticam 580 digital camera (Motic). They were then analysed using ImageJ by measuring the 10 maximally migrated cells from each explant and averaging the top 8. Statistical analysis was performed using GraphPad Prism. Two-tailed, unpaired t-tests were performed to test for differences between cells treated with growth factors and controls, with significance at $p < 0.05$. In figures $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

2.3.4 Quantitative Real Time Polymerase Chain Reaction

Total RNA was obtained from the sample with RNeasy Mini Kit following the instructions. DNA was removed with RNase-Free DNase Set (Qiagen 79254) and reverse transcription was performed by SuperScript III RT kit (Invitrogen 18080). For first-strand cDNA synthesis reaction, 1µg RNA was mixed with 1µl 250ng/µl random primers, 1µl 10mM dNTP and RNase free H₂O, and the mixture was incubated at 65°C for 5min and then on ice for 1min. The following were added into the reaction mixture: 4µl 5x First-Strand Buffer, 1µl 0.1M DTT, 1µl RNaseOUT Recombinant RNase Inhibitor and 1µl SuperScript III RT and then the 20µl final mixture was incubated at 25°C for 5min, 55°C for 1hr, and 70°C for 15min. The cDNA was subjected to realtime quantitative PCR with SYBR Green PCR Master Mix. The target mRNAs were normalized relative to 18S ribosomal RNA. Primers for RT-PCR are listed below in Table 4.

Table 4 Primers used for RT-PCR of IGF genes

Gene	Forward Sequence	Reverse Sequence	Source
<i>IGF1</i>	GACAAACAAGAAAACGAAGC	ATTTGGTAGGTGTTTCGATG	Sigma
<i>IGF1R</i>	AGAACCGAATCATCATAACG	TTTAAATGGTGCCTCCTTG	Sigma
<i>IGF2</i>	GTA CTTC CGGACGACTTC	CTGA ACTCTTTGAGCTCTTTG	Sigma
<i>IGF2R</i>	G TAGAGACATAGACTCCCTTC	TTACAGAAGTCTGGCTTTTC	Sigma
<i>INSR</i>	AAGACCTTG GTTACCTTCTC	GGATTAGTGGCATCTGTTTG	Sigma
<i>IGFBP1</i>	ACCTCAAGAAATGGAAGGAG	TCCATTCTTGTTGCAGTTTG	Sigma
<i>IGFBP2</i>	CTTAAGCAGTGCAAGATGTC	GTTTACTGCACACTTTGGG	Sigma
<i>IGFBP3</i>	CTGAATCATCTGAAGTTCCTC	GGCACTGCTTCTTCTTATAG	Sigma
<i>IGFBP4</i>	AGACCTCTGACAAGGATG	TGCTCCGATCTCTAATTTG	Sigma
<i>IGFBP5</i>	CCTGAGATGAGACAGGAATC	GCTTACACTGCTTTCTCTTG	Sigma
<i>IGFBP6</i>	GAGGAAGACTACAAAGGAGAG	CCATCTCACTATCTTGAACAG	Sigma
<i>IGFBPL1</i>	GAACAGATACAAAAGCCTCTAC	ATTCTTGATCATCATCGTC	Sigma
<i>18S</i>	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG	Sigma
<i>GAPDH</i>	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA	Life Technologies

2.3.5 Growth Factor Stimulation Enzyme-linked Immunosorbent Assay (ELISA)

96-well non-treated flat-bottomed plates were incubated with 50µl of 200µg/ml Poly-D-Lysine (Sigma P6403) per well overnight at 4°C. Poly-D-Lysine was aspirated and then 30µl of 10µg/ml Laminin (Gibco 23017-015) was added. The Laminin was incubated for one hour at 37°C before being aspirated carefully to leave a Poly-D-Lysine/Laminin cell adhesion matrix. Tertiary neurospheres were collected, dissociated in 1ml Accutase (Sigma A6964-100ML), washed in NB-A+ media, counted using a haemocytometer and 0.4% Trypan Blue (Sigma T8154), and diluted to 300,000 cells/ml in NB-A+*. 100µl of cell suspension was then added to the entire wells of the 96well plate and returned to the incubator for 6hours to serum starve. 5X growth factor dilutions were prepared in NB-A+* equivalent to a 1X concentration of 100 and 20 ng/ml in 96 deep well block. The growth factors used are EGF (Sigma E9644-2MG), FGF-2 (R&D Systems 233-FBI), Heregulin (Sigma, SRP3055-50UG), IGF-1 (Sigma, I8779-50UG), PDGF-BB (Sigma SRP3229-10UG), VEGF165 (Sigma, SRP4364-10UG), NGF-B (Sigma. SRP4304-20UG) and GDNF (Sigma, SRP3200-10UG). 25ul of 5X growth factor dilution was added to each well of cell mixture and returned to the incubator for 15 minutes. Media was dumped out of the wells and cells washed with 200ul of ice cold PBS to stop the reaction. Cells were lysed immediately with 25ul of R&D lysis buffer #6, sealed with a foil plate sealer and stored at -80oc. Cell lysates were analysed using a modified human/mouse phospho-Akt (R&D DYK2289C) and human/mouse phospho-ERK1/ERK2 (DYK1018B) sandwich ELISA kits. White, clear bottomed, high binding 384 wells

were coated with either the pAkt (4ul/ml in PBS) or pERK (8ul/ml in PBS) capture antibody overnight. Plates were washed three times in wash buffer (0.05% Tween 20 in PBS) and blocked with block buffer (1% BSA in PBS) for 2 hours. Protein standards were prepared as per manufacturer's instructions. Plates were washed three times in wash buffer and 20ul of standard or undiluted sample was added and left to incubate at room temperature for 2 hours. The plates were washed three times in wash buffer, 20ul of either pAkt (100ng/ml) or pERK (400ng/ml) detection antibody was added and left to incubate at room temperature for 2 hours. The plates were washed, 20ul of streptavidin-HRP added and incubated for 20 minutes. The plates are washed, 20ul of substrate solution (R&D DY999) added and incubated for 20 minutes. 10ul of stop solution (2N H₂SO₄) and optical density determined immediately using a BMG PHERAstar FS plate reader.

2.4 Results

2.4.1 Insulin-Like Growth Factors 1 and 2 stimulate the migration of Subventricular Zone neuroblasts *in vitro*

There have been a number of different extracellular factors identified that have modulating effects on neuroblast migration (Sun, Kim and Moon, 2010). In this chapter, we wanted to test the extent to which each of these growth factors modulated migration and to explore potential downstream signalling pathways that could be used as nodes to instruct future systems modelling of neuroblast migration. We performed an *in vitro* explant migration assay, in which we cultured small SVZ explants embedded in Matrigel. The standard culture media used for *in vitro* neural stem cell experiments has super physiological insulin concentrations (25µg/ml) so to enable study of the insulin like growth factors the standard culture media was replaced with a low insulin media. The Matrigel used was the low growth factor variety, which has an initial concentration of 5ng/ml IGF1 and 1.7ng/ml TGFβ. The Matrigel makes up <1% of the final culture volume so, although it is impossible to discount them completely, the basal growth factor concentration in the culture was considered zero. The explants were stimulated with each growth factor at a concentration of 20ng/ml to best represent literature physiological growth factor concentrations (Alagappan *et al.*, 2014). Migration was determined by measuring the distance migrated by the 8 cells surrounding each explant that had migrated the greatest distance. Interestingly, out of the panel tested only

IGF1 and IGF2 significantly altered the migration of the neuroblasts from the explants, as shown in Figure 6 ($p < 0.001$).

RTKs can stimulate various signalling pathways, a key one being the PI3K/Akt pathway. Since RTKs are known to be important regulators of cell migration we also investigated the extent each of the growth factors tested stimulated the PI3K/Akt pathway. Activation of PI3K results in a series of protein interactions that ultimately phosphorylate Akt at two residues Thr308 and Ser473. We measured the concentration of the pAkt(Ser473) in SVZ cells stimulated with each growth factor for 15 minutes. Only EGF, IGF1 and IGF2 elevated pAkt levels significantly above control levels, as shown in Figure 6. Notably EGF stimulated the phosphorylation of Akt substantially more than IGF1 and IGF2 ($P < 0.01$). Interestingly the many growth factors that have been demonstrated in the literature to stimulate these cells failed to elicit a response in this assay.

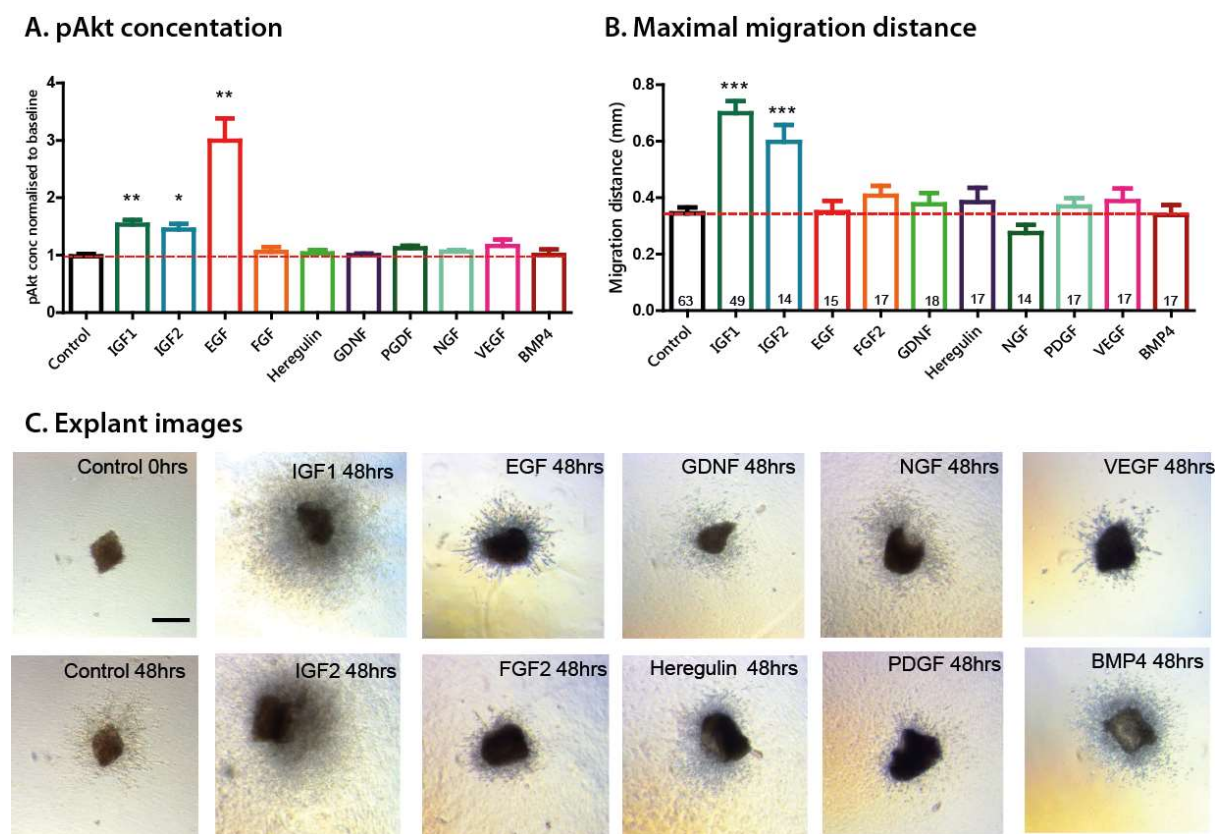


Figure 6. IGF1 and IGF2 significantly increase migration of neuroblasts
(A) pAkt concentration following growth factor stimulation in SVZ neurospheres. Representative data from a single biological replicate. Bar represent SEM of 4 technical replicates. **(B)** Maximal migration distance of explants stimulated by 10 different growth factors *in vitro* after 48 hours stimulation. Pooled data from all experiments. Total number of explants per condition embedded in graphs. **(C)** Representative images of explant migration. One-way ANOVAs were performed between cells treated with growth factors and controls; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. Scale bar = 500 μm.

In a subsequent experiment we investigated whether there were any combinatorial effects of co-stimulating with both IGF1 and IGF2 (Figure 7). Explants cultured in IGF1 and IGF2 had significantly increased migration from the control neuroblasts ($p < 0.001$) but did not show a significantly enhanced migration distance to culture with IGF1 or IGF2 exposure alone ($p < 0.001$). When combined the maximal migration distance increased further to 0.81mm, which was significantly different from IGF2 exposure alone ($P < 0.05$).

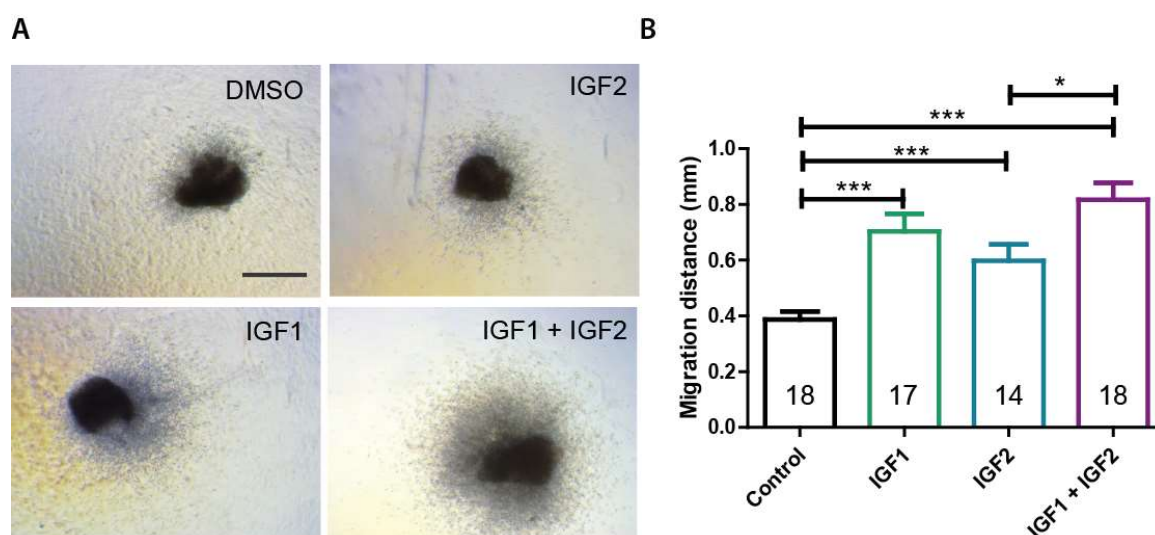


Figure 7. Migration distance of explants exposed to IGF1, IGF2, and in combination.

(A) Panel images show migration of neuroblasts after 48 hours with DMSO (control), IGF1, IGF2, or both IGF1 and IGF2 in the cell culture medium. Scale bar = 500 μ m. **(B)** The bar chart shows the migration distance of the cells with greatest migration. Pooled data from three biological replicates. Total number of explants analysed inset into graphs, with error bars representing SEM. Multi-comparison ANOVA were performed followed by Tukey Test for each variable pair; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

2.4.2 Inhibition of IGF1R and the Akt/PI3K pathway block the IGF1 induced stimulation of SVZ neuroblast migration

IGF1 can bind and activate either the IR or the IGF1R. To investigate the receptor through which IGF1 increases neuroblast migration in our assay we utilised the cyclolignan Picropodophyllin (PPP), which has been shown to specifically inhibit IGF1R kinase activity (Girnita *et al.*, 2004)). When activated, RTKs can stimulate multiple signalling pathways. We also investigated two of the major signalling pathways: MAPK/ERK, inhibited by U0126, and PI3K/AKT, inhibited by LY294,002. We used these small molecule inhibitors for IGF1R and the downstream pathways and repeated the explant assay as described previously in this chapter.

Figure 8 shows how these small molecule inhibitors affected migration of neuroblasts. None of the inhibitors alone significantly affected neuroblast migration. Co-culturing explants with IGF and PPP blocked the increase in neuroblast migration indicating that the pro-migratory effect of IGF1 is directed via the IGF1R. Inhibition of the MAPK/ERK pathway by U0126 did not significantly block the stimulation of migration by IGF1. LY294,002, inhibiting the PI3K/AKT pathway, also did not restore migration levels back to control levels, but did significantly reduce migration distances from IGF1 treatment alone. These results suggest that the PI3K/Akt pathway is involved in mediating the role of IGFR1 in inducing migration.

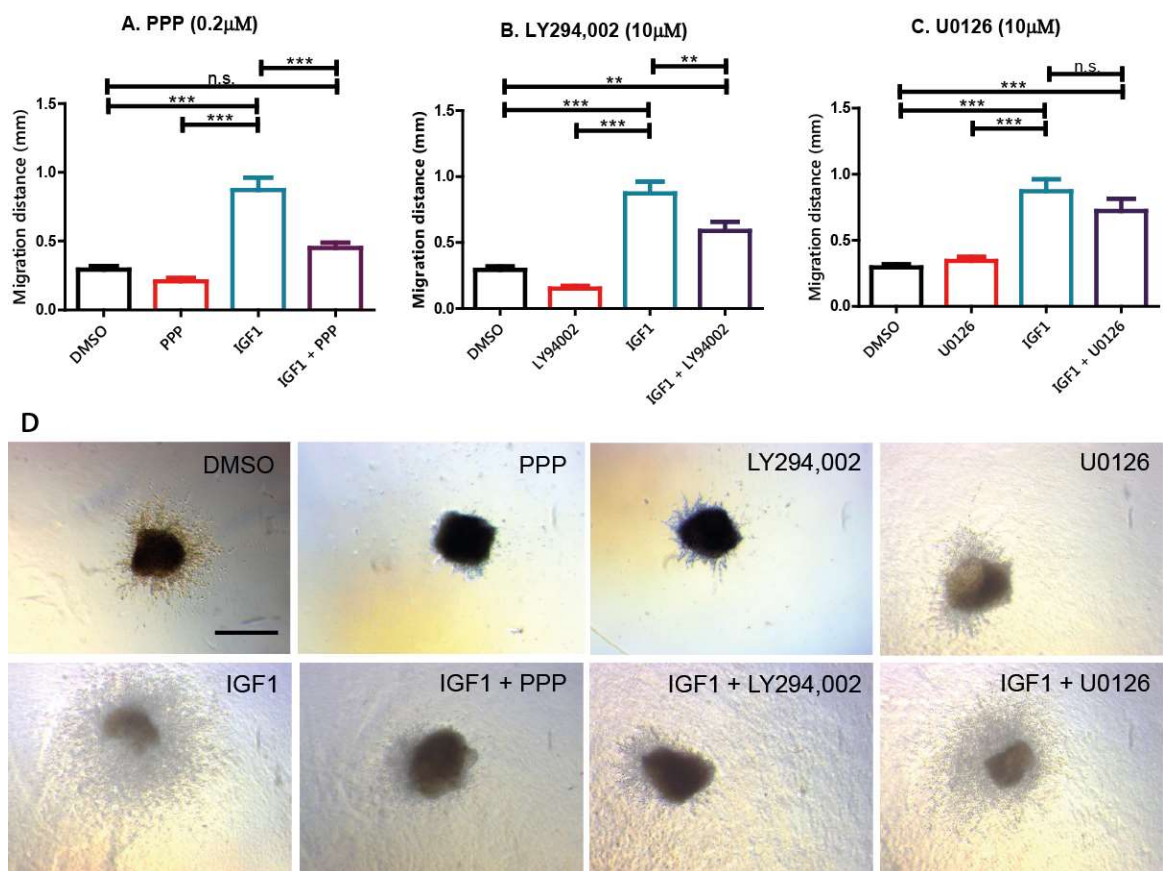


Figure 8. Migration distance of explants exposed to IGF1 and inhibitors of the IGF1 pathway.

Panel images show migration of neuroblasts after 48 hours with DMSO (control), IGF1, LY294002, U0126, and PPP in the cell culture medium. The bar chart shows the migration distance of the cells with greatest migration. Representative data from single biological replicate of 6 technical replicates per condition. Error bars equal to SEM. Multi-comparison ANOVA were performed followed by Tukey Test for each variable pair; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. Scale bar = 500μm.

2.4.3 Co-stimulation with EGF blocks the IGF1 induced increases in SVZ neuroblast migration

Finding that EGF stimulated the PI3K/Akt pathway substantially more than IGF1 and IGF2 (Figure 1A), we decided to investigate whether the combination of EGF and IGF1 may increase migration and the stimulation of the PI3K/Akt pathway above the levels of either growth factor alone. We repeated the RMS explant assays with either no growth factors, IGF1 only, EGF only, or EGF and IGF1, and then measured migration after 24 and 48hrs.

After 24 hrs the combination of EGF and IGF1 prevented the stimulation of neuroblast migration (Figure 9A). This suggests that stimulation with EGF blocks the effect of IGF1 and, therefore cross talk between these two signalling pathways. Interestingly, however, at 48hours the suppression of IGF1 induced migration by EGF was no longer significant. To investigate this in more detail we used a transgenic mouse strain which expresses GFP under the neuroblast specific promoter: GAD65. The explants stimulated with EGF had a substantially reduced green intensity compared to IGF1 indicating that there is a shift in the fate of these cells (Figure 9B). The majority of the cells migrating from the explants stimulated by the EGF do not express GFP and have a characteristically glial morphology, suggesting that these cells are not neuroblasts, and that stimulation with EGF suppresses neuroblast fate. This is compatible with the previous finding from the Szele Lab that activation of the EGFR drives transit amplifying cells and neuroblasts back towards a more stem cell like state (Kim *et al.*, 2009).

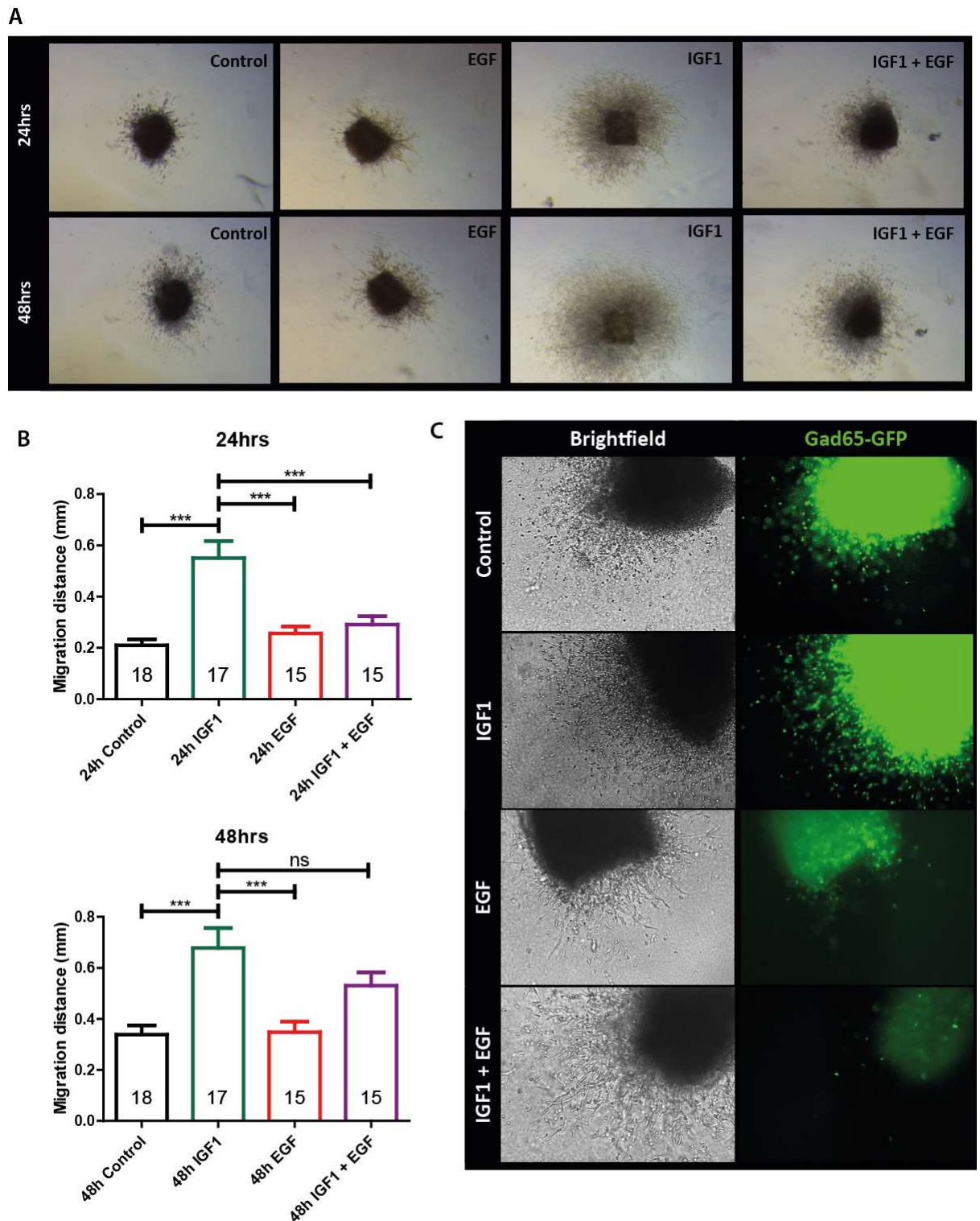


Figure 9. Migration of neuroblasts stimulated with IGF1, EGF, and both IGF1 and EGF for 24 and 48 hours.

(A) Image panel shows neuroblast migration after 24 or 48 hours in the presence of growth factors and controls. **(B)** Bar charts show the migration distance of the cells with greatest migration. Pooled data from three biological replicates. Total number of explants analysed inset into graphs, with error bars representing SEM. Multi-comparison ANOVA were performed followed by Tukey Test for each variable pair; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. **(C)** Image panel shows GAD65-GFP labelled neuroblasts in the presence of IGF1, EGF, and IGF1 and EGF.

2.4.4 Expression of IGF related genes is differentially regulated in the SVZ vs the RMS

A large family of different proteins conducts IGF signalling. Signalling acts through at least three receptors and the ligands IGF1, IGF2, and insulin, which all have a series of metabolites. There are also various binding proteins to these ligands, which can both enhance or repress insulin signalling in a context dependent manner. To gain a better understanding of how IGF1 appears to drive the migration of neuroblasts specifically, we first completed an analysis of the IGF family genes in the Allen Brain Atlas (<http://mouse.brain-map.org/>). Both ligands IGF1 and IGF2 show relatively low expression apart from in the choroid plexus, which is consistent with literature evidence that these proteins are paracrine signalling molecules (Ferron *et al.*, 2016, Zeigler *et al.*, 2014). The IGF1R and IGF2R have high expression in the anterior SVZ and hippocampus and modest expression in the cortex and olfactory bulb. Expression of the insulin receptor is undetectable in most regions of the brain apart from low expression in the hippocampus and dorsal SVZ. Expression of the IGFBPs is highly regionally specific. IGFBP4, IGFBP5 and IGFBP1 all show very high expression in the SVZ and RMS. While IGFBP2 and IGFBP7 are restricted to high expression in the choroid plexus. In contrast IGFBP8 and IGFBP9 appear to delineate the RMS from the surrounding tissue.

To further characterise the IGF family we completed gene expression analysis using qPCR and the primers detailed in Table 4 (page 42). For the first experiment we cultured tertiary neurospheres and plated them down as a monolayer to differentiate for 6 days prior to RNA collection. The aim here was to understand which genes are upregulated in neurospheres compared to differentiated cells and therefore potentially most important in self-renewal, proliferation, and the inhibition of migration. In the second experiment tissue was dissected from both the SVZ and the RMS for RNA isolation. The aim here was to identify genes that may be involved in controlling the initiation and migration of neuroblasts.

Interestingly IGF1 was not expressed very highly in any of the tissue samples analysed. The CT values from qPCR were typically in the region of 29-31 compared to controls of around 19-20. We did not measure the efficiency of the primers so we cannot directly compare gene expression, however, this is an indication that IGF1 may act primarily as a paracrine signalling molecule in the migration process.

Figure 10 shows the change in expression levels of genes in this pathway. IGF1R expression was not significantly increased in differentiated cells compared to the neurospheres *in vitro*. However, in the tissue section *in vivo* IGF1R was upregulated almost two-fold in the RMS compared to expression levels in the SVZ ($p<0.05$), suggesting that IGF1R may be most important in the migration of neuroblasts rather than proliferation and differentiation. IGF2R was upregulated in cells differentiated for 6 days compared to the neurosphere stage (2.6 fold; $p<0.05$) and showed a trend to be downregulated in the RMS compared to the SVZ (not significant).

IGFBPs also showed altered gene expression in this experiment. IGFBP1 and IGFBP11 had different gene expression patterns in both the *in vitro* neurosphere cultures and the tissue samples. IGFBP1 was downregulated in differentiated cells compared to in proliferating neurospheres and also in the RMS compared to in the SVZ (although both changes were not significant). IGFBP11 however, was upregulated in differentiated cells (non-significant) and in the RMS (4.3 fold; $p<0.05$). IGFBP2 had expression changes in both experiments: it was downregulated in differentiated neurons, and upregulated in the RMS ($p<0.05$), suggesting it may be an important gene to investigate further due to its multiple roles in these processes. IGFBP5 showed significantly upregulated expression in differentiated cells and IGFBP6 showed significantly downregulated expression in differentiated cells compared to neurospheres ($p<0.05$). In the RMS IGFBP3 was significantly downregulated ($p<0.05$) compared to expression levels in the SVZ.

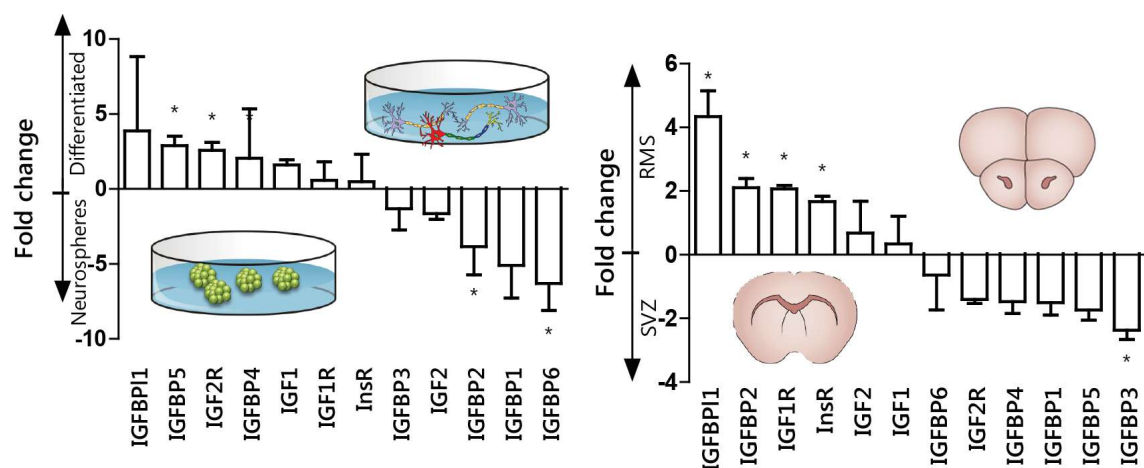


Figure 10 Analysis of IGF family genes expression in the SVZ and RMS. Bar charts show gene expression changes in differentiating neurospheres *in vitro* and in the RMS compared to the SVZ from *in vivo* tissue sections. Data represent average of 3 biological replicates with error bars equal to SEM.

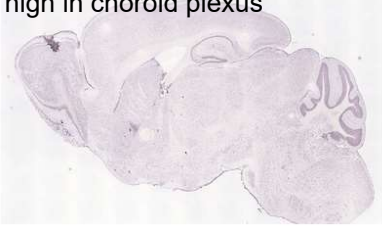
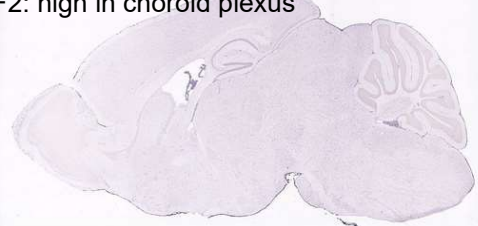
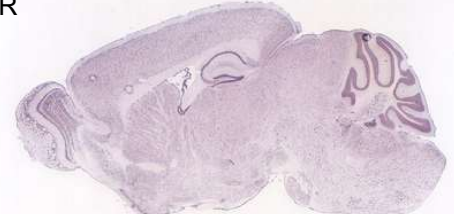
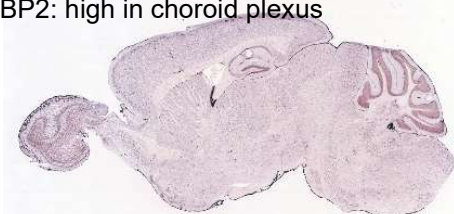
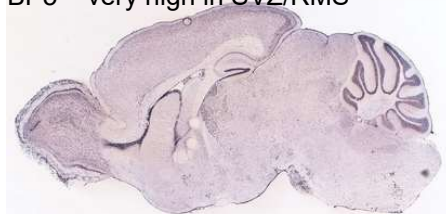
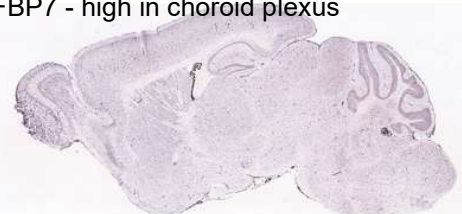
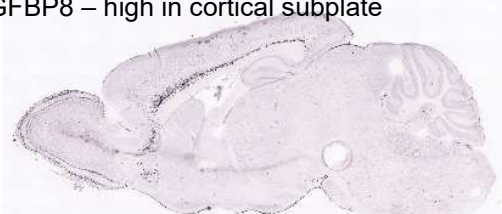
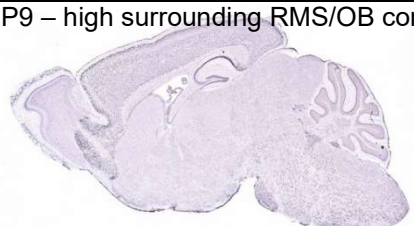
Ligands	
IGF1: high in choroid plexus 	IGF2: high in choroid plexus 
Receptors	
IGF1R – high in SVZ 	IGF2R 
Binding Proteins	
IGFBP1 	IGFBP2: high in choroid plexus 
IGFBP3 	IGFBP4 – high expression in SVZ/RMS 
IGFBP5 – very high in SVZ/RMS 	IGFBP6 
IGFBP7 - high in choroid plexus 	IGFBP8 – high in cortical subplate 
IGFBP9 – high surrounding RMS/OB corridor 	IGFBLP1 – very high in SVZ/RMS 

Figure 11. Expression of IGF related genes in the adult mouse brain. Image panels show in situ hybridization of IGF related genes in sagittal section of adult mouse brain (taken from Allen Brain Atlas). The IGF1R, IGFBP4, IGFBP5 and IGFBLP1 all display high expression in the SVZ/RMS.

2.4.5 Co-stimulation with IGFBP5 does not modulate neuroblast migration *in vitro*.

Unpublished transcriptomic data from the Szele Lab has also previously indicated that IGFBP5 may have a functional role in the SVZ (Sun, 2016; unpublished). We therefore investigated whether or not IGFBP5 had a regulatory function in neuroblast migration. To do this we completed another series of explant assays comparing the extent of migration in the presence of (20ng/ml) IGF1 and (200ng/ml) IGFBP5. The concentration of 200ng/ml for IGFBP5 was decided upon because at this concentration IGFBP5 has been shown to have several significant effects in various other *in vitro* assays (Kim *et al.*, 2007; Pellegrinelli *et al.*, 2015).

We did not find IGFBP5 to have an effect on the migration distance of neuroblasts from the explants (Figure 12). IGFBP5 also did not have any effect on the increased migration of neuroblasts induced by IGF1 stimulation, and migration distance in the presence of IGF1 and IGFBP5 together was similar to the distance with IGF1 alone.

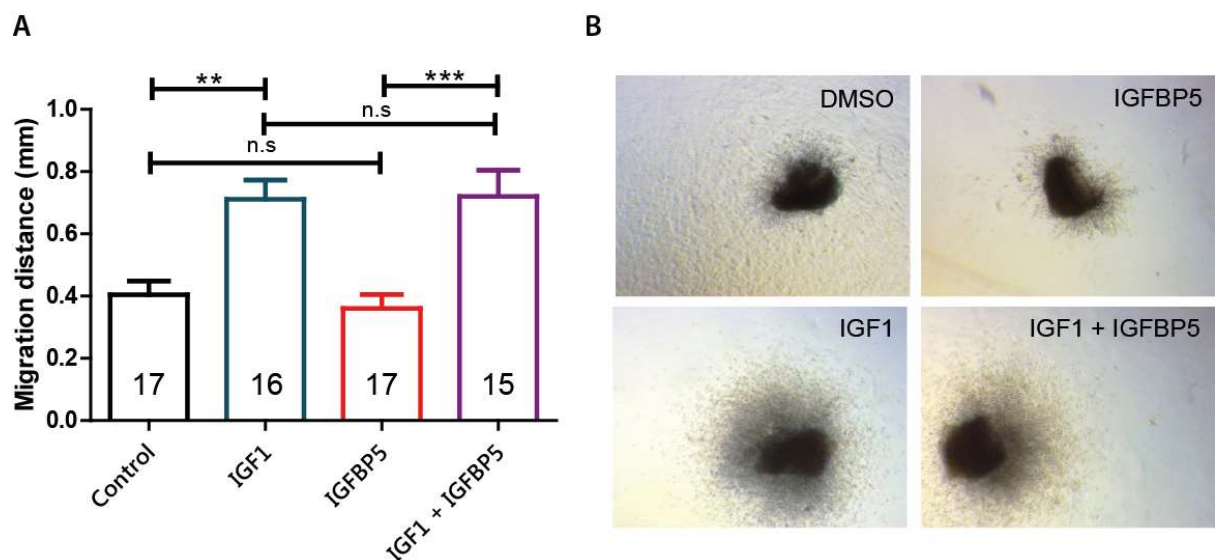


Figure 12. Migration of neuroblasts co-stimulated by IGF1 and IGFBP5. Panel images show migration of neuroblasts after 48 hours with DMSO (control), IGF1, and IGFBP5 in the cell culture medium. The bar chart shows the migration distance of the cells with greatest migration. Pooled data from three biological replicates. Total number of explants analysed inset into graphs, with error bars representing SEM. Multi-comparison ANOVA were performed followed by Tukey Test for each variable pair; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

2.5 Discussion

2.5.1 IGF1 increases migration of SVZ neuroblasts *in vitro*

Several growth factor and neurotrophic factors have previously been suggested as modulators of neuroblast migration. To investigate the mechanism of action and explore the potential of these proteins as neuroregenerative therapeutics, a panel of growth factors were tested for their effect on the migration of neuroblasts from SVZ explants. Stimulation with both IGF1 and IGF2 significantly increased the migration of neuroblasts, and the combination did not increase migration above that of IGF1 alone. Interestingly the many other growth factors that have been demonstrated in the literature to stimulate these migratory cells failed to elicit a response in this assay. One reason for this could be that growth factors act via another cell type *in vivo*, as it is known that many cell types are important in the successful migration via the RMS, whereas this *in vitro* assay investigates only the signalling pathways in the neuroblasts themselves. It is also possible that insulin signalling is required as a competence factor for these signalling pathways.

This is not the first time IGF signalling has been implicated in the initiation of neuroblast migration. In IGF1 knockout mice, neuroblasts accumulate in the SVZ and display impaired radial migration in the olfactory bulb indicating that IGF signalling is necessary for the exit of neuroblasts from the SVZ (Hurtado-Chong *et al.*, 2009). The migration from RMS explants made from the knockout mice was indistinguishable from wild type but both demonstrated a significant increase in migration when stimulated with IGF1 indicating that IGF1 is a paracrine signalling molecule in the SVZ. In contrast, conditional knockout of IGF1R in adult born NSCs and their progeny reduced the long term decline of migrating neuroblasts in the RMS (control: -52%, KO -34%), which the authors concluded was due to reduced depletion of the neuroblast pool in the SVZ (Chaker *et al.*, 2015). In this study there was no difference in the number of Mash1+ cells in the RMS indicating that IGF1R selectively targets the neuroblasts. Ectopic expression of IGF1 by lentivirus injection in the striatum induced a significant increase in both the number and distance of neuroblast recruitment into the striatum (Maucksch *et al.*, 2013). Transwell migration assays confirmed this was due to both directional chemotaxis and increased motility. IGF1 has also been shown to increase neuronal migration from the zebra finch neural crest and subependymal zone (Jiang *et*

al., 1998), mouse dorsal root ganglion neurons (Xiang *et al.*, 2011) and underlie the light-dark cycle velocity changes in postnatal cerebellar granule cell migration (Y. Li *et al.*, 2012). What currently is still not clear is what aspect of SVZ neuroblast migration IGF1 acts on. Migration can be broken down into 5 key stages: 1) Stimulation, 2) Invasive protrusion, 3) adhesion, 4) contractile tension and 5) tail retraction. Different mechanisms are at play at each stage and it is unlikely IGF1 interacts with all equally.

2.5.2 IGF1-driven increases in migration signal via the Akt/PI3K pathway

To determine how IGFs increase neuroblast migration from explants cells we manipulated the IGF1R and its major downstream pathways: MAPK/ERK and PI3K/AKT. Inhibiting the IGF1R with the small molecule PPP blocked the observed migration increase in the presence of IGF1, supporting the importance of IGF1R signalling in this IGF1-stimulated migration. PPP has also been shown to disrupt microtubule dynamics via an IGF1R independent mechanism, so this should be taken with caution (Waraky *et al.*, 2014). Stimulation with IGF1, IGF2, and EGF were all found to increase pAKT concentration in SVZ cells, suggesting they activate the PI3K/Akt pathway. Additionally, the small molecule LY294,002, which inhibits the catalytic subunit of PI3K, blocked the IGF1-induced stimulation of SVZ neuroblast migration. This suggests that neuroblast migration is driven by the IGF1R/PI3K/Akt pathway. It is worth noting that although LY294,002 did not significantly inhibit migration in the absence of IGF1 stimulation there was still a negative trend. It could simply be that IGF1 stimulation results in a proportional increase in motility irrespective of PI3K signalling in the specific culture conditions. Interestingly, despite EGF increasing pAKT levels to a greater degree than IGF1, this growth factor did not significantly increase neuroblast migration. Moreover, co-stimulation of neuroblasts with EGF in addition to IGF1 blocked the IGF1 induced increases in SVZ neuroblast migration. Initiation of migration is thus more complex than the sole activation of the Akt/PI3K pathway, and that other pathways activated by EGF are likely have a role in migration of neuroblasts.

Selective inhibition of Src family kinase and PI3K have previously been shown to significantly reduce the pro-migratory effect of IGF1 on olfactory bulb explants (Hurtado-Chong *et al.*, 2009). Genes related to PI3K/Akt (Aktq, PIK3r1, Hdac2 and Hsbp1) are upregulated in the RMS

compared to the SVZ and shRNA silencing of these genes reduced the number of cells that migrated to the OB (Khodosevic *et al.*, 2009). Interestingly in this study, genes related to MAPK/ERK had no effect on migration, while silencing of PKC ζ caused neuroblasts to change their migratory route suggesting a role in cell polarization (Khodosevich *et al.*, 2009). Contrary to this, another group found that PI3K/Akt signalling is switched off as transit amplifying cells give rise to neuroblasts and is not reactivated until they reach their destination in the olfactory bulb. Genetic knockdown of PTEN, a major negative regulator of the PI3K/Akt pathway, in the postnatal SVZ did not alter normal directional migration but resulted in premature termination of migration and differentiation of a subset of neuroblasts in the RMS (Zhu *et al.*, 2012). Another study combining CRISPR/Cas9 and the piggyBac transposon to knockout PTEN expression in the SVZ also did not detect any migration deficits but did note that ~10% of transfected cells were ectopically located in the white matter (Chen *et al.*, 2015).

Precisely what mechanism downstream of PI3K/Akt is modulated to drive migration has not been investigated in the SVZ but borrowing ideas from other systems may be instructive. Dynamic and cooperative signalling between the IGF1R and B-integrin is necessary for migration of many normal and cancerous cell types, including fibroblast, vascular smooth muscle cells, colon cancer and prostate cancer. The mechanism is suggested to involve the IGF1R being recruited into a signalling complex with β 1 integrin, RACK1, focal adhesion kinase (FAK) and key IGF/PI3K/Akt signalling components IRS1, IRS2, Shc, Src, PP2A, Shp-2, and c-Abl. Given Rack1 is very highly expressed in the RMS and B-integrins are known to be essential for the correct chain formation of migrating neuroblasts, it would be interesting to see whether a comparable interaction is active in the SVZ (Mobley *et al.*, 2011, Belvindrah *et al.*, 2007). Similarly IGF1R can associate with E-cadherin and induce EMT in mammary epithelial cells by the rapid internalisation of E-cadherin and subsequent activation of the nuclear factor kB (NF-kB)-Snail axis (Kim *et al.*, 2007). It is postulated that the degree to which IGF1R contributes to migration may be influenced by proteins key to the engagement with the extracellular matrix. One such example is PDLIM2, which promotes both cell polarity and the stability of NFkB, STATs, and beta catenin. It is directly regulated by IGF1R signalling and, according to the Allen Brain Atlas, is almost exclusively restricted to the SVZ in the brain. Cross talk between the discoid domain receptor 1 (DDR1) and IGF1R has also been described to contribute to tumour progression (Malaguarnera *et al.*, 2015; Matà *et al.*, 2016). An unanswered question, even in the cancer field, is whether IGF1R and IR-A

function differently in cooperation with adhesion signalling. IGF1 has also been shown to enhance the migratory response of fetal derived neural progenitors and mesenchymal stem cells to SDF-1 by upregulating its receptor CXCR4 (Li *et al.*, 2007; Zhu *et al.*, 2015). IGF1 potentiates SDF-1 mediated migration of myeloma cells by cooperative activation of p-21 activated kinase (Rø *et al.*, 2013). It has also been shown to transactivate CXCR4 in MDA-MB-231 breast cancer cells, independent of SDF-1 (Akekawatchai *et al.*, 2005).

2.5.3 IGF related genes are differentially expressed in the SVZ and the RMS.

The IGF family includes ligands, receptors and IGFBPs. Classically, there are three ligands: Insulin, IGF1 and IGF2, three cell surface receptors: insulin receptor (IR), IGF1R and IGF2R, and at least seven IGFBPs. We found a number of genes in this family to be differentially regulated in the RMS compared to the SVZ. Stem cells in the SVZ are proliferative until they begin their migratory route via the RMS. Therefore, we predict the differentially regulated genes in the RMS compared to the SVZ tissue to have a role in the migration process, while being conscious that the key initiation of migration occurs in the SVZ (Altman, 1969). The IGF1R, IR, IGFBP1, and IGFBP2 were upregulated in the RMS, whereas IGFBP3 was suppressed. In general, this expression matches what would be expected. Both the SVZ and RMS contain a myriad of different cell types so the distinction between migratory and non-migratory cells is complex. Nevertheless, these genes could be further investigated to determine how they influence the migration switch following neurogenesis.

2.5.4 Challenges and limitations.

The explant assay utilised in this chapter is a useful tool to study neuroblast migration *in vitro*. The complex cellular architecture of the SVZ and RMS are retained and the cells migrate out radially closely replicating the *in vivo* dynamics. It is not however, ideal for screening multiple conditions when the response may be subtle. Each explant is manually dissected and embedded, which introduces two unavoidable elements of variability 1) differences in explant dimensions due to experimental practice, and 2) differences in cellular composition due to origin of tissue. The result of this is that, even when the experimenter is very experienced at the dissection procedure, at least 6 replicates per condition to confidently complete an experiment. It is typical to expect to

dissect 6 explants per animal dissected, hence the scope for scaling the assay is fairly limited. Related to this is the challenge of quantification. For all the experiments in this chapter I used a relatively simple, manual quantification method, measured as the average distance travelled of the top 8 migrated cells. The reason for this is that many explants do not migrate out perfectly radially. About 20% migrate out of one edge only, likely representing a slight error in dissection and in about 30% only very few cells migrate out at all, likely representing a region with fewer starting neuroblasts. The maximal distance migrated is less dependent on these variations, but underlying variations in the assay preclude more sophisticated, automatable analysis methods.

Another consideration with *in vitro* assays that rely on media supplementation with exogenous recombinant proteins is how physiologically relevant they are. In this study only one concentration was used for all growth factors that was set based on previous literature examples. It is also not known by how much interactions with the Matrigel ECM components limit diffusion to the explant. The relative exposure *in vitro* is unknown so there may be a response at much higher concentrations. Nor is it known whether these growth factors signal in an autocrine or paracrine way. For instance, although unlikely, endogenously produced VEGF may saturate all VEGFRs on the cells so no amount of exogenous recombinant protein would elicit a phenotype. This is particularly true for proteins that have known cytoplasmic and nuclear functions. For example, IGFBP5 has been found to have IGF1 dependent and independent effects on the migration of various cell types via interactions with cytoplasmic and nuclear proteins (see Güllü *et al.*, (2012) for review). We did not find an effect from recombinant IGFBP5 on neuroblast migration in the current study however this does not confirm no function. The IGFBPs are notoriously difficult to study genetically due to functional redundancy. However, a genetic over expression and knockdown study in neuroblasts is advised.

The promiscuity of small molecule kinase inhibitors weakens the conclusions drawn about the signalling pathways through which IGF1 directs neuroblast migration. As mentioned previously PPP has been shown to disrupt microtubule dynamics via an IGF1R independent mechanism, so the inhibition of migration should be taken with caution (Waraky *et al.*, 2014). Similarly, LY294002 is widely described, and sold, as a selective inhibitor of the catalytic subunits of PI3K (Vlahos *et al.*, 1994). However, it is known that it also inhibits several other proteins at the concentrations

used to inhibit PI3K (Arrowsmith *et al.*, 2016). Interestingly a Google Scholar search for 'LY294002 & PI3K' in 2016–2017 returned over 5000 articles so clearly this compound continues to be misused despite several specific compounds now being available.

An initial objective of this chapter, and indeed the entire thesis, was to establish a reciprocal *in vitro* <-> *in silico* model to study the activation of SVZ neurogenesis and predict molecular interventions that would have therapeutic benefit. The premise of this was based on the assertion that there was substantial prior data on growth factor signalling in the SVZ. The error in this assertion was revealed fairly early in the project upon interrogating the literature, however we persevered using the Niepel *et al.*, (2014) ELISA based RTK systems analysis framework as a guide. The aim was to measure pAkt and pERK activation by a panel of growth factors and correlate that with measurable cellular processes (proliferation, lineage progression, migration). Establishing the ELISA protocol turned out to be extremely challenging, only ever worked sufficiently well to measure pAkt activation and revealed that only EGF and IGF1 substantially activate this signalling pathway in neurospheres. The project subsequently evolved, and we stopped investigating growth factors, however, should we wish to explore this in the future we could utilise the spheroid migration assay developed in chapter 5. This is a more robust *in vitro* assay to study neuroblast migration and should facilitate more consistent screening. This could be via ELISAs, as before, however high content imaging of spheroids stimulated, fixed and post-stained with antibodies against the phosphorylated proteins of interest may be a better option. In this way the multiplexed signalling of several kinases could be quantified in individual cells.

2.5.5 Future work and unanswered questions

2.5.5.1 How does IGF signalling direct neuroblast migration?

This study has successfully shown that IGF1 and IGF2 stimulate neuroblast migration *in vitro*; confirming the findings from previous groups (Maucksch *et al* 2013; Hurtado-Chong *et al.*, 2009). It is therefore appealing to investigate a genetic manipulation of this system *in vivo*. Ablation of the IGF1R has been studied previously in the SVZ (Hurtado-Chong *et al.*, 2009, Chaker *et al.*, 2015). However, the complexity of the IGF signalling family makes the results of genetic

knockdown studies both challenging to design and not necessarily the best to interpret. For instance, the IGF1R is known to have several IGF1 and kinase independent functions via interactions with scaffolding proteins and nuclear translocation (Janku *et al.*, 2013; Solomon-Zemler, Sarfstein and Werner, 2017). It is therefore important to study a genetic manipulation that precisely modulates the interaction between the ligands and the receptors. In the past few years studies have increasingly recognised the role of IGF2 in SVZ neurogenesis, however its physiological role in neuroblast migration remain poorly understood (Ziegler *et al.*, 2014). In the following chapter we will investigate a novel mouse model that has a single point mutation on the IGF2R to eliminate IGF2 bindings. This enables the precise study of the function of IGF2:IGF2R binding in the developing brain and has the potential to be used as a pseudo IGF2 overexpression model due to the reduced ligand clearance.

Further investigation of the PI3K/Akt pathway in SVZ neuroblast migration is needed. However, simply using small molecule inhibitors will not be sufficient for many reasons. Protein kinases can come in multiple isoforms that have specific functions, pharmacological inhibitors are rarely specific and can have multiple unknown off target effects. In addition, inhibiting a downstream node in a signalling pathway doesn't guarantee that the phenotype blocked is instructed from a specific upstream node. For instance, LY294,002 may be inhibiting migration in the explant assay through a mechanism entirely independent of IGF1. A more refined approach would be to knockout IGF1R genetically and then express constitutively active forms of downstream signalling nodes, such as PI3K, Ak1, Rac1 or ERK5, to try and rescue the phenotype (Konno *et al.*, 2005; Khodosevich and Monyer, 2011; Guil *et al.*, 2017)

During the course of this thesis it was not possible to thoroughly investigate the dynamics of neuroblast migration. We did complete some pilot studies using two-photon imaging of neuroblasts migrating down the RMS. However, for technical reasons these were prematurely halted. This is still an opportunity in need of further study, however, a very recent paper by Guil *et al.* (2017) has provided some insights. They used Golgi and actin specific stains to study the effect of IGF1R knockdown on the migration of embryonic cortical neuroblasts in organotypic slices. They show that IGF1R signalling is necessary for correct polarity, orientation and axon formation; and that this is dependent on PI3K. An equivalent study could be designed to examine the same mechanisms in postnatal RMS neuroblasts.

2.5.5.2 Convergence of EGF and IGF signalling in the SVZ.

Two of the most remarkable findings in this study were that in this model of neuroblast migration from SVZ explants 1) only IGF1 and IGF2 that significantly increased migration, and 2) co-stimulation with EGF and IGF1 inhibited the increase in migration. *In vivo* it is very unlikely that any growth factor signals independently and recently more significance has been given to the cross talk between the different signalling pathways. For example, IGF1R signalling has been shown to be necessary for EGF mediated proliferation of SVZ derived neurospheres (Alagappan *et al.*, 2014). Analyses of cell cycle progression revealed that IGF1R activation together with EGFR co-signalling decreased the percentage of cells in G1, enhanced cell progression into S and G2 and increased levels of phosphohistone 3 and cyclin D. Similarly, IGF1 and FGF2 signalling converge on cyclin D to have additive effects on proliferation of NSCs (Adepoju *et al.*, 2014). Another very smart study immobilised different growth factors, alone and in combination, on which the authors plated NPCs derived from the embryonic rat striatum. Alone IGF1 stimulated neuronal differentiation and did not affect glial differentiation or stem cell proliferation. In combination with EGF, IGF1 increased stem cell proliferation and the percentage of Nestin+ progenitors. IGF1 decreased the astrocytic differentiation mediated by CNTF. Most interestingly, both IGF1 and BDNF alone promote neuronal differentiation, but in combination resulted in fewer mature neurons (Konagaya *et al.*, 2011). I purposely set up the screen on explant migration in a way to limit potential cross talk between the growth factors tested. The development of the spheroid assay (see Chapter 5) provides the perfect opportunity to investigate further combinatorial effects to try and elucidate the downstream convergence points.

2.5.5.3 Future Smart Growth Factor Development

It is important to note here that the collaboration with Silver Creek Pharmaceuticals was halted due to commercial interests of the company. For this reason, development of a predictive mathematical signalling model of motility was also not pursued. Should using targeted growth factor therapeutics for activating SVZ neuroblast migration be re-examined in future, I offer the following recommendations:

Bioactive Signalling Arm. The results from this chapter indicate that IGF1 and IGF2 stimulate neuroblast migration. I would, therefore, propose that future research efforts are focused on these two signalling molecules. The result that IGF1R inhibitor PPP blocks migration combined with literature evidence (Hurtado-Chong *et al.*, 2009; Maucksch *et al.*, 2013), indicates that it is the IGF1R not IR that drives migration. It is therefore recommended that modifications are made to the structure of IGF1 to increase selectivity to IGF1R. Besides these archetypal IGF ligands other potential members have been identified, which may be considered as starting points. IGF1 is believed to undergo post-translational N-terminal cleavage by a specific protease into des-N-(1–3) IGF1, which may be the dominant form in the brain (Yamamoto and Murphy, 1995). Des-N-(1–3) IGF1 has lower affinity for IGFBPs caused by the absence of glutamate at position 3 (Guan *et al.*, 2015), and thus, relatively higher local concentration of its free form. *In vitro* culture of the postnatal rat olfactory bulb, des-N-(1–3) IGF1 is shown to be potent supporter of viability, cell survival and differentiated cell growth (Russo *et al.*, 2005). The N-terminal gly-pro-glu tripeptide fragment (GPE tripeptide), generated along with des-N-(1–3) IGF1 during proteolytic cleavage of IGF1, is also capable of mediating neuroprotective effects both *in vivo* and *in vitro* (Shapira *et al.*, 2009), although it remains to be determined whether GPE actions depend on IGF1R. Activation of the PI3K/Akt pathway may be used a proxy node for modelling purposes.

Targeting Arm. To accumulate the active ligand in the SVZ I propose exploring the following extracellular binding partners.

X-IGF1 fusion protein	Biomarker	Selectivity & Notes	References
P-Selectin	CD24	SVZ Ependymal cells Neuroblasts	Nieoullon <i>et al.</i> , 2005; Buono <i>et al.</i> , 2015
LQNAPRS (LS-7 peptide)	CD133	Neural stem cells Transit amplifying cells	Sun <i>et al.</i> , 2012; Buono <i>et al.</i> , 2015
KKKKKKRFSFKKSLSGFSF KKNK (MARCKS-ED peptide)	PSA-NCAM	Neuroblasts	Theis <i>et al.</i> , 2013; Buono <i>et al.</i> , 2015
TAASGVRSMH or LTLRWVGLMS	NG2	Transit amplifying cells Oligodendrocyte Precursors	Burg <i>et al.</i> , 1999; Nishiyama <i>et al.</i> , 2014
Galectin-3-binding protein	Galectin-3	SVZ/RMS ependymal cell SVZ/RMS astrocytes.	Yan <i>et al.</i> , 2009; Comte <i>et al.</i> , 2011

Chapter 3 Maternal transmission of an IGF2 binding mutation in the IGF2R results in perinatal lethality and increased proliferation in the neocortical subventricular zone.

3.1 Introduction

3.1.1 Insulin-like growth factor 2

The role of IGF1 and insulin in the brain have been studied extensively, however in the past few years researchers have increasingly recognised the role of IGF2 in postnatal SVZ neurogenesis. IGF2 is the most abundantly expressed IGF in the brain and it promotes the maintenance, expansion and self-renewal of SVZ neural stem cells (Ziegler *et al.*, 2014). The IGF2 gene is paternally imprinted in most human and mouse tissues. However, it is specifically biallelically expressed in the choroid plexus (Ferrón *et al.*, 2015). The stem cells in the SVZ niche do not produce IGF2, like in the hippocampus. Instead high quantities are produced in the neighbouring choroid plexus and leptomeninges that signal to the neural stem cells in a paracrine manner (Ferron *et al.*, 2015). The first evidence for this was from siRNA mediated knockdown of IGF2, which results in decreased proliferation and reduced PI3K/Akt signalling in the DG both *in vivo* and in DG derived neurospheres but no response in the SVZ or SVZ derived neurospheres (Bracko *et al.*, 2012). This precise regulation of its dosage and allelic expression has led us to hypothesize that IGF2 may be more critical to adult neurogenesis than initially thought.

Whether the response to IGF2 in the SVZ is directed via the IR-A or IGF1R is disputed. IGF1R was shown to govern IGF2 effects on NSC proliferation (Lehtinen *et al.*, 2011). This is supported by a reduction in the pro-proliferative effect of IGF2 *in vitro* by an IGF1R function blocking antibody (Ferron *et al.*, 2015). However, a smart study using IGF2 analogues engineered to have different selectivity to the different receptors showed that IGF2 promotes stemness through the IR-A receptor. This is supported by a study on neurospheres generated from rat embryonic striatal precursors, in which IGF1 treatment did not show increased proliferation unless co-treated with insulin suggesting that the pro-proliferative effects are mediated by the IR-A (Arsenijevic, Weiss and Tnn, 1998). A working model, proposed by the Levison Lab, is that the IGF2 stimulates

B1 Cells via IR-A and lineage restricted progenitors, such as neuroblasts via IGF1R (Ziegler *et al*, 2014). The specific role of IGF2 in lineage progression in the SVZ has not been studied

3.1.2 The insulin-like growth factor 2 receptor

The IGF2R, also known as the cation independent mannose 6-phosphate receptor, is a multifunctional receptor that interacts with a diverse group of ligands. They include numerous lysosomal enzymes, transforming growth factor- β (TGF- β) precursor, leukemia inhibitory factor (LIF), proliferin, thyroglobulin and retinoic acid (see Hawkes & Kar, 2004 for review). IGF2 binds to the IGF2R with high affinity via the hydrophobic residue on domain 11 of the IGFR2. No intrinsic enzymatic activity has been observed for the intracellular domain of the IGF2R. There is some suggestion that IGF2R may signal via the MAPK/ERK pathway, however, its main function in the IGF signalling pathway is to regulate the binding of IGF2 to the functional receptors. Genetic disruption of the IGF2R gene results in increased serum and tissue levels of Igf2, brain overgrowth and perinatal lethality. Rescue of the knockout lethality and over-growth phenotype occurs when Igf2, IGF1R or IGFBP5 are also genetically co-disrupted (Lau, Stewart and Liu, 1994; Ludwig *et al.*, 1996; Tripathi *et al.*, 2009). It remains unknown, however, if the phenotype following loss of function of Igf2r is either due to increased IGF2 alone, or to a combination of altered ligand supply, including M6P-lysosomal proteases.

The IGF2R can be identified at the two-cell stage of the mouse embryo whereas the IR and IGF1R are detected only from the eight-cell embryo—thus making the IGF2R the first receptor of the IGF family to appear during development (Latham, Doherty and Schultz, 1994). Like IGF2, the mammalian Igf2r is also imprinted, with expression of the maternal allele up-regulated relative to the paternal allele. Interestingly, Igf2r is not imprinted in humans and both alleles are expressed potentially. The developmental significance of this has not been fully characterised but it is conceivable that it may underlie the reduced neurogenesis observed in adult humans. Differential allele expression is dependent on methylation of the maternal promoter of an anti-sense long non-coding RNA (lncRNA) termed Airn, located within intron 2 of Igf2r (Figure 12). Disruption of either the Airn promoter or deletion and truncation of Airn transcripts, results in establishment of increased paternal allele Igf2r expression relative to the maternal allele, and bi-allelic expression.

Unlike the IGF1R, very little is currently known as to the physiological significance of the IGF2R in the functioning of the CNS and it has not been specifically studied in the SVZ. The IGF2R is widely distributed in the brain with relatively high levels expressed in the striatum, deeper layers (layers IV and V) of the cortex, pyramidal and granule cell layers of the hippocampus, selected thalamic nuclei, Purkinje cells of the cerebellum, pontine nucleus and motoneurons of the brainstem (Hawkes & Kar, 2003). Data from a FACS/Microarray study by Ramos *et al.*, (2013), indicates that IGF2R expression is lower (0.34x) in neuroblasts and higher in transit amplifying cells (1.6x) than neural stem cells, and in Chapter 2 of this thesis, qPCR analysis showed that the IGF2R is significantly more expressed in differentiated cells compared to neurosphere cells. When combined these results indicate that the relative expression of IGF2R may be important in titrating the dose of IGF2 during neurogenesis. Interestingly this postulate is reinforced by the identification of M6P/IGF2R as the first putative “IQ gene” in humans (Chorney *et al.*, 1998).

3.1.3 The Igf2rI1565A mouse model

Mouse genetic data suggest that gene dosage of Igf2r is able to titrate the supply of Igf2 to regulate mammalian growth, but do not directly attribute the Igf2r loss of function phenotype to IGF2 alone. Genetic targeting for both the Igf2r and Igf2 locus is further complicated by associated disruption of co-expressed miRNAs. For example, disruption of the Igf2 locus can result in loss and gain of both miR-483 and miR-675. MiR-675 can negatively regulate IGF1R translation, and so may alter the sensitivity of tissues to IGF2 ligand activation (Smits *et al.*, 2008; Venkatraman *et al.*, 2013). Moreover, Igf2r 3' UTR appears a specific target for several miRNA (195 and 211) (Dror *et al.*, 2016; Zhang *et al.*, 2017). To overcome this the Hassan Lab have previously determined the structural and functional basis of the mechanism of IGF2 binding to evolutionary forms of domain 11 of IGF2R and identified the evolutionary changes in binding domain loops (Frago *et al.*, 2016). They have also recently introduced a single alanine point mutation into the codon of an important CD loop hydrophobic residue (I1571 in human, I1656 in mouse) in domain 11 of Igf2r, to generate a specific loss of IGF2 binding allele of Igf2r. This mutation results in loss of IGF2 binding capacity by at least 100-fold. The I1572A mutation significantly reduced IGF2 binding by 100-fold, yet the 1-15 protein domains bound M6P and plasminogen with similar affinities, (Figure 13). Thus, the Igf2rI1656A allele enables the study of

reduced clearance of IGF2 without the previous confounding factors and could therefore be a useful tool to study IGF2 signalling in the SVZ.

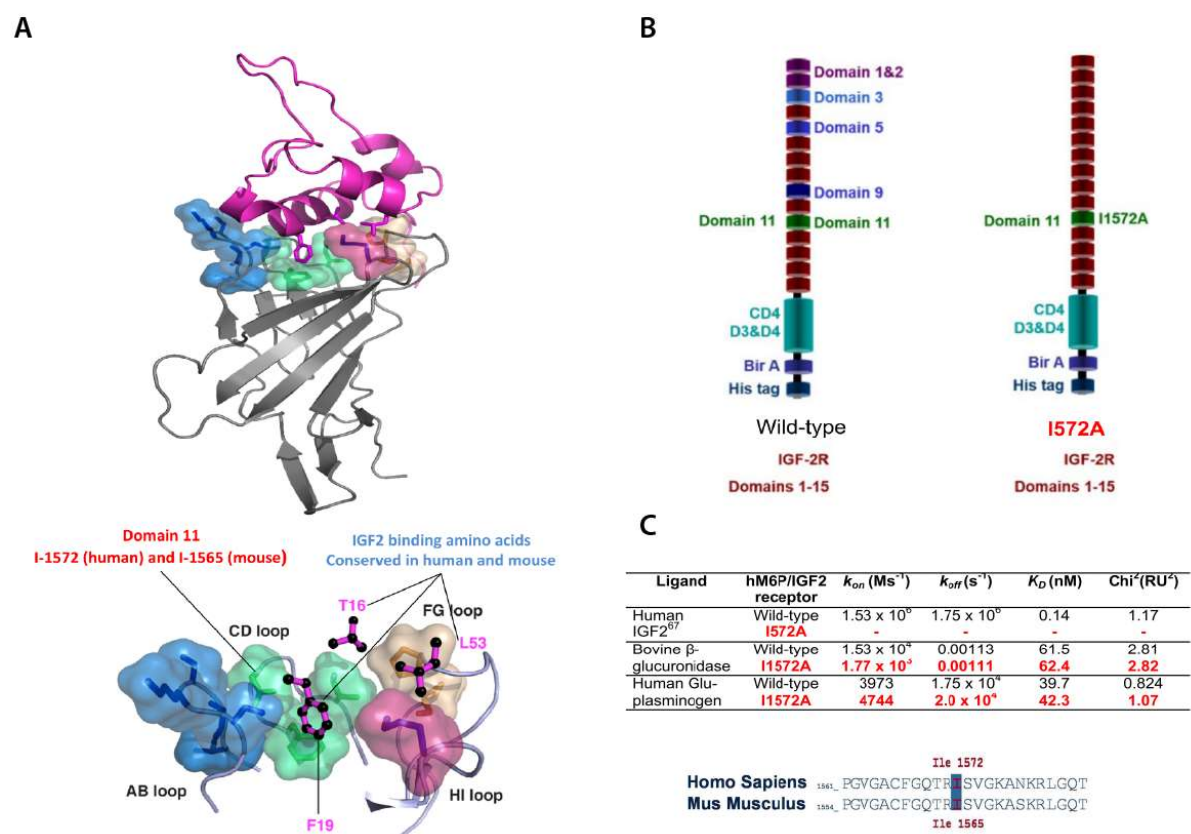


Figure 13. The structural and molecular basis for the Igf2r11565A allele

(A) Structural configuration of human IGF2 binding to domain 11 of IGF2R (upper panel), including key interacting residues of IGF2 with CD, AB, FG and HI loops of domain 11 (lower panel). **(B)** Graphic comparison of the Wild-type and Igf2r11565A receptors showing the location of domain 11. **(C)** Association rates, dissociation rates and affinity constants for wild-type and I1572A interaction with ligands, including Chi2 values of their respective fits. Line up of 'TRIS' CD loop sequence and designation of I1572 (human) and I1565 (mouse).

3.2 Aims

The aim of this chapter was to investigate the SVZ phenotype of the Igf2r11565A mouse model and to assess it's potential to study the role of IGF2 signalling in neuroblast migration.

3.3 Methods

3.3.1 Generation of the *Igf2r*^{l1565A} mouse model.

All animal work was approved by University of Oxford animal ethics committee, and was performed under a UK Home Office Project Licence in the lab of Professor Bass Hassan. Genetic targeting of *Igf2r* to insert a point mutation (C57Bl/6NTac-*Igf2r*^{tm2760(l1565A)Arte}) was generated in collaboration with Artemis Taconic (Cologne, Germany, Figure 2). Viable mice were generated bearing the knock-in allele on the paternal allele *Igf2r*^{+/m/l1565A} (Please see Hughes *et al.*, in press, for further details). Briefly, the pIgf2r-targeting vector was constructed using a PCR based cloning strategy using BAC clones from the C57BL/6J RPCI-731 BAC library. The flanking short arm (6.3Kb) contained exons 28-31, followed by a positive PuroR (Puromycin resistance) selection marker flanked by Frt sites into intron 31, the mutation in exon 34 (ATC to GCC to generate l1565A) and the long arm (10.3Kb) that contained exons 35-42 followed by the thymidine kinase (Tk) negative selection marker. The final constitutive expressing allele was generated followed breeding with Flp deleter mice (*Igf2r*^{+/m/l1565A}), to remove the PuroR, but retaining a single Frt site, used then for genotyping using flanking PCR primers to generate a larger product (302bp) compared to the wild-type allele (227bp). Primers used were O3: GGTGCAGGTGTTATGACTGG and O4:AGTACATCAGCCTGTATCCTGG flanking the Frt site.

3.3.2 Measurement of size and weight of tissue samples.

Body and brain weight of the embryos were measured at the point of dissection, prior to fixations using analytical laboratory scales. Excess fluid was absorbed using tissue paper, but the samples were not dried. Images were also taken with a phone camera next to a ruler for scale. These were later analysed in ImageJ.

3.3.3 Immunohistochemistry.

Embryo brains were dissected and kept in 4% PFA at 4°C overnight. On the second day, brains were transferred into 30% sucrose in PB and kept at 4°C for 48 hrs. Brains were frozen down on dry ice and cut into 50µm sections using a Leica sliding microtome and stored in a cryoprotectant solution.

Brain sections were washed 3x10min in PBS (pH 7.4), 1x15min in 50mM glycine in PBS, 3x10min in PBS and then blocked in PBS+(10% donkey serum, 0.1% Triton-X in PBS) for 1hr. Sections were then incubated in primary antibodies at 4°C overnight. The next day, sections were washed with PBS 3x10min, and incubated in secondary antibodies (1:500 in PBS+) for 1hr in dark and washed again 3x10min in PBS. Then brain sections were treated with 4',6-diamidino-2-phenylindole (DAPI, 1:1000) for 15min and washed with 0.1M PB for 3x10min. Sections were mounted on SuperFrost Plus microscope slides with FluorSave Reagent. Table 5 contains a list of all primary antibodies used for immunohistochemistry and their dilution.

Table 5 Primary antibodies used for immunohistochemistry of *Igf2r*^{H1565A/+p} embryos

Antigen	Dilution	Host	Manufacturer
PHi3	1:1000	Mouse	Abcam - ab14955
PAX6	1:200	Rabbit	Abcam - ab5790
Tbr2	1:1000	Chick	Millipore - AB15894
CUX1	1:200	Goat	Santa Cruz - sc-6327
OTX1	1:1000	Rabbit	G. Corte - n/a
Ctip2	1:400	Rat	Abcam - ab18465

3.3.4 Confocal microscopy.

Slides were imaged using a Zeiss 710 laser scanning confocal microscope with a 20X (Zeiss Plan-Neofluar 20X/0.50) objective. To excite DAPI, Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 fluorophore, excitation wavelengths with 405nm, 488nm, 568nm and 647nm were used respectively. 2µm interval was used for Z-stack and tile scans were taken to capture the whole ventricular region. Confocal images were analysed in LSM Image Browser and ImageJ.

3.4 Results

3.4.1 Maternal transmission *Igf2r*^{H1565A} results in perinatal lethality and increased body and brain weight.

The Hassan Group have recently generated the *Igf2r*^{H1565A} knockin mouse model, which specifically disrupts the binding of the IGF2R to its ligand Igf2. Previously reported IGF2R total gene knockout studies show that heterozygous offspring inheriting the *Igf2r*-null allele from their fathers (*Igf2r*^{+m/-p}) are viable, reach adulthood, and are fertile. In contrast, the majority of offspring inheriting the mutated allele from their mothers (*Igf2r*^{-m/p}) die at or near the time of birth (Lau *et al.*, 1994).

Evaluation of 20 litters and 182 pups at birth, showed that maternal transmission of the *Igf2r*^{1565A/+p} allele resulted in normal Mendelian segregation, yet following homozygote breeding, perinatal *Igf2r*^{1565A/1565A} numbers of pups were significantly depleted (P=0.0006). Subsequent survival of neonatal animals revealed significantly decreased survival for heterozygotes *Igf2r*^{1565A/+p} (40%), and significantly worse survival for *Igf2r*^{1565A/1565A} homozygotes, with all pups failing to reach weaning (p<0.01). Whole body weight of the surviving heterozygote pups was slightly higher than wild-type littermate controls, but with a similar trajectory during development.

To eliminate complications resulting from the low viability of the *Igf2r*^{1565A/+p} pups all subsequent work was carried out on E18.5 embryos; the latest stage of development prior to birth. Despite the gross anatomy of the brain appearing similar at E18.5 there are some notable differences between embryonic and postnatal/adult neurogenesis. During embryonic development the primary neurogenic niche is in the VZ/SVZ of the neocortex, which would be referred to as the dorsal wall of the adult ventricle. This contains radial glia cells that give rise to the main projection neurons of the cortex. The embryonic lateral wall comprises the LGE and MGE, which at E18.5 will generate interneurons destined for the olfactory bulb and neocortex. A single timed mating was set up and whole bodies and brain weight were measured at E18.5. Both the whole body and brain weight of heterozygotes *Igf2r*^{1565A/+p} was significantly greater than wild-type littermates. The ratio of brain/body weight was proportional (Figure 14).

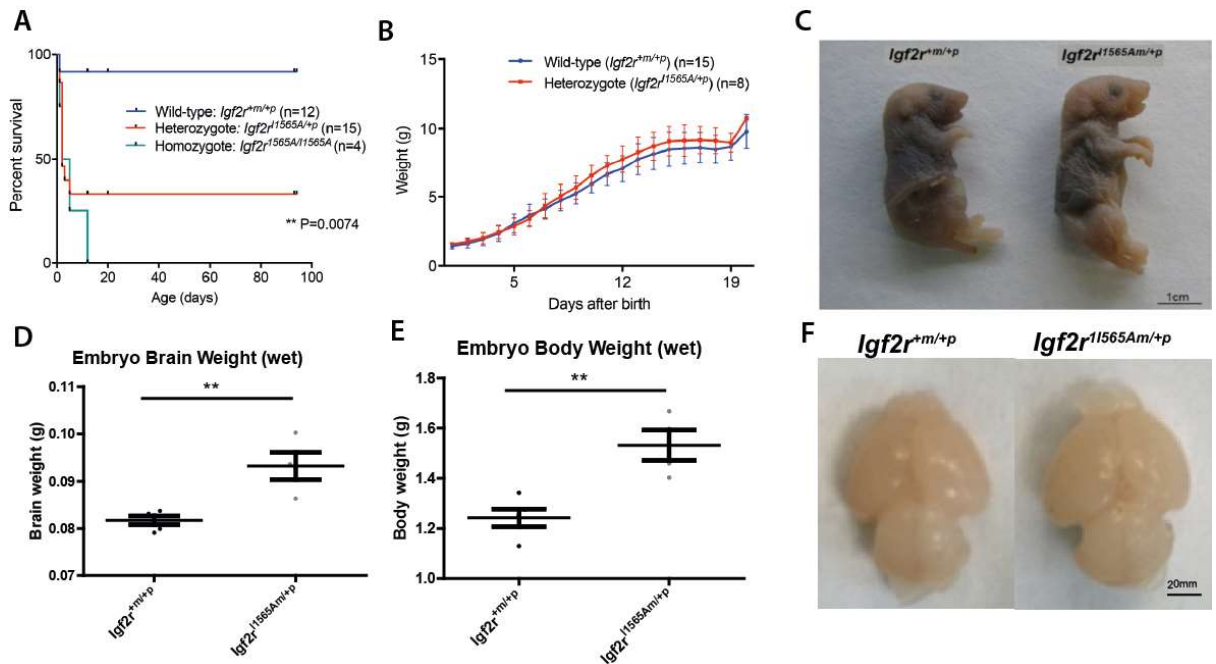


Figure 14 The effects of maternal transmission of *Igf2r*^{1565A/+p} on embryo and brain growth.

(A) Kaplan-Meier survival plots following maternal transmission of *Igf2r*^{1565A/+p} $p = 0.0074$, Log-rank (Mantel-Cox) test, (B) Postnatal weights of surviving littermates ($\pm$ S.D., non-significant) from birth to 21 days, (C) Gross appearances of neonatal wild-type and heterozygote *Igf2r*^{1565A/+p} littermates, (D) Brain weights at E18.5 are significantly increased in heterozygote *Igf2r*^{1565A/+p} ($n=9$) compared to wild-type littermates ($n=8$). (E) Body weights at E18.5 are significantly increased in heterozygote *Igf2r*^{1565A/+p} compared to wild-type littermates. (F) Example pictures of heterozygote *Igf2r*^{1565A/+p} and wild-type brains; no gross anatomical abnormalities were detected. Un-paired two-way t-test with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Table 6 Summary of breeding outcomes at birth

<i>Igf2r</i> ^{1565A} allele transmission	Cross	Genotypes	Observed	Expected	
Maternal	<i>Igf2r</i> ^{+/m/1565A} ♀ x <i>Igf2r</i> ^{+/m/+p} ♂ (11 litters, 5 pairs)	<i>Igf2r</i> ^{+/m/+p}	39	34.5	P=NS
		<i>Igf2r</i> ^{1565A/+p}	30	34.5	
Paternal	<i>Igf2r</i> ^{+/m/+p} ♀ x <i>Igf2r</i> ^{+/m/1565A} ♂ (10 litters, 3 pairs)	<i>Igf2r</i> ^{+/m/+p}	25	27	P=NS
		<i>Igf2r</i> ^{+/m/1565A}	29	27	
Homozygote	<i>Igf2r</i> ^{+/m/1565A} ♀ x <i>Igf2r</i> ^{+/m/1565A} ♂ (9 litters, 3 pairs)	<i>Igf2r</i> ^{+/m/+p}	25	14.75	P=0.0006
		<i>Igf2r</i> ^{+/m/1565A} or <i>Igf2r</i> ^{1565A/+p}	30	29.5	
		<i>Igf2r</i> ^{1565A/1565A}	4	14.75	

3.4.2 Maternal transmission *Igf2r*^{1565A} results increased proliferation in the embryonic subventricular zone and in neural stem cells *in vitro*.

The greater brain weight in the *Igf2r*^{1565Am/+p} embryos and the knowledge that IGF2 is a significant driver of neural stem cell proliferation (Lehitinen *et al.*, 2011, Ferron *et al.*, 2015) indicated to us that there may be increased production of neurons in the *Igf2r*^{1565Am/+p} brain.

To confirm our hypothesis of increased neurogenesis we investigated the expression of the proliferation marker phospho-histone H3 (pHH3). The phosphorylation of histone H3 plays an important role in chromatin remodelling during metaphase of the cell cycle, thus making it a useful marker for cells undergoing mitosis. Two further timed matings were set up between an *Igf2r*^{+m/1565Ap} female and an *Igf2r*^{+m/+p} male, which yielded nine mutant *Igf2r*^{1565Am/+p} embryos and eight *Igf2r*^{+m/+p} embryos. As before, the embryos were culled at E18.5 embryos to remove any possible confounding factors related to poor postnatal survival rates in the *Igf2r*^{1565Am/+p} pups. Brains were fixed, cut into 50µm sections and stained for pHH3. The number of pHH3 cells was then quantified for the cortical VZ/SVZ and subpallial VZ/SVZ. The whole lateral wall was quantified as one due to the challenges in segmenting the LGE and MGE at E18.5. The VZ and SVZ were counted as two separate regions. The VZ contains mainly radial glia, whereas the SVZ is primarily intermediate progenitor cells. Counting them as separate regions therefore provides insight into cell type specific effects. The VZ was estimated to be any cell within two cell diameters from the ventricle wall and the SVZ was estimated to be all other cells in the region of hyper density determined by the DAPI co-stain.

We found that in the cortical VZ and SVZ there was significantly more pHH3+ cells in the *Igf2r*^{1565Am/+p} embryos ($p < 0.05$) (Figure 15). However, in the subpallial VZ and SVZ there was no difference between *Igf2r*^{1565Am/+p} and wild-type littermates controls (Figure 16).

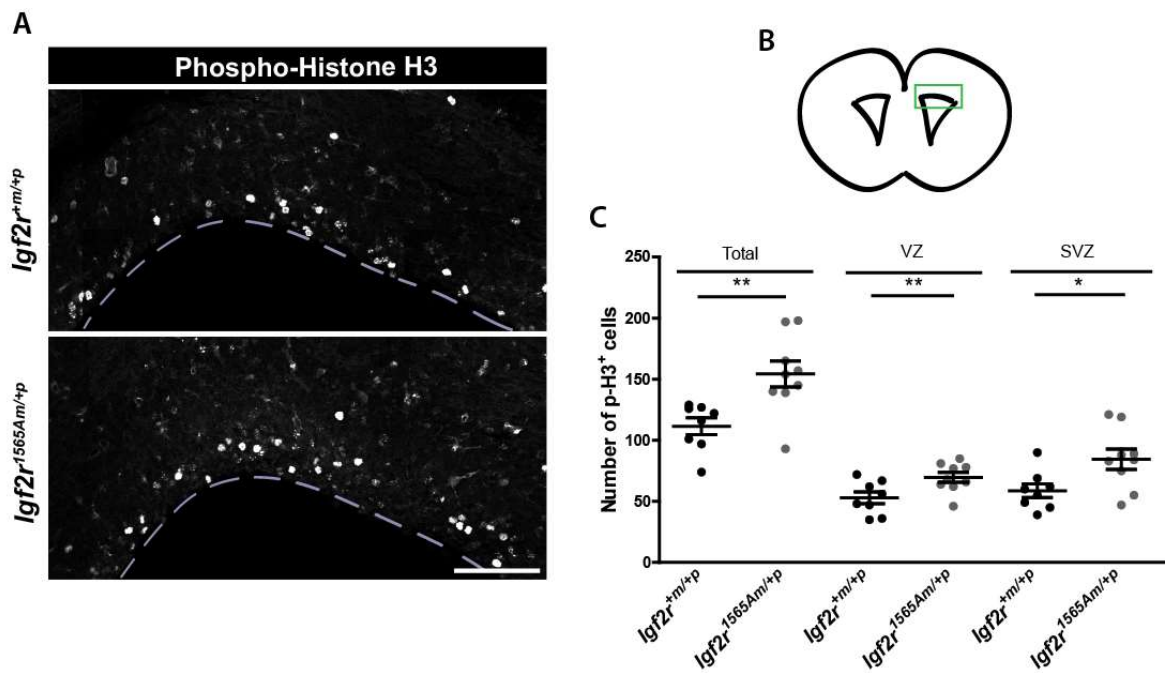


Figure 15 The effects of maternal transmission of *Igf2r*^{1565A/+p} on the cortical VZ/SVZ

(A) Immuno-localisation in 50µm coronal brain sections of phospho-histone H3 at E18.5 in wild-type and *Igf2r*^{1565A/+p} heterozygous littermates. (B) Graphical representation of region analysed, (C) Quantification of pH3 positive cell numbers obtained from confocal images (z-stacks) of the ventricular zone (VZ), subventricular zone (SVZ) and in both of these regions (Total) from single brain sections of wild-type (n=8) and *Igf2r*^{1565A/+p} (n=9) littermates. Proliferation as judged by this biomarker appears increased in both the VZ and SVZ in heterozygote *Igf2r*^{1565A/+p} neocortex. Un-paired two-way t-test with *** p<0.001, ** p<0.01 and * p<0.05.

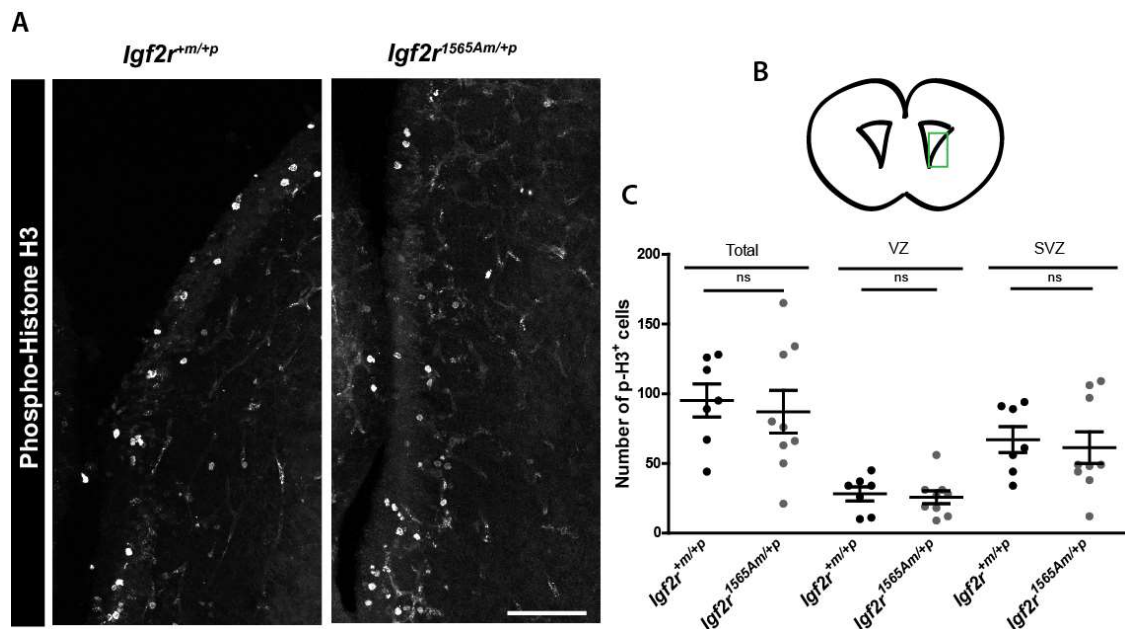


Figure 16 The effects of maternal transmission of *Igf2r*^{1565A/+p} on the subpallial VZ/SVZ

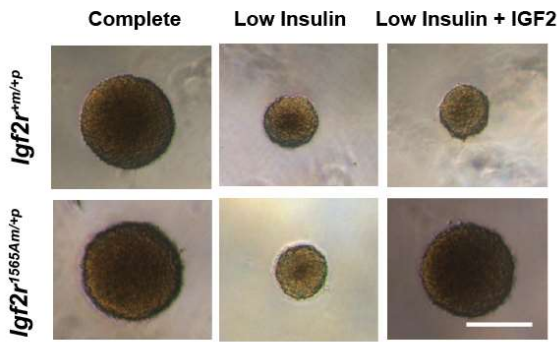
(A) Immuno-localisation in 50µm coronal brain sections of phospho-histone H3 at E18.5 in the subpallium of wild-type and *Igf2r*^{1565A/+p} heterozygous littermates. (B) Graphical representation of region analysed, (C) Quantification of pH3 positive cell numbers obtained from confocal images (z-stacks) of the ventricular zone (VZ), subventricular zone (SVZ) and in both of these regions (Total) from single brain sections of wild-type

(n=8) and *Igf2^{r1565A/+p}* (n=9) littermates. Proliferation as judged by this biomarker appears unaltered in the LGE.

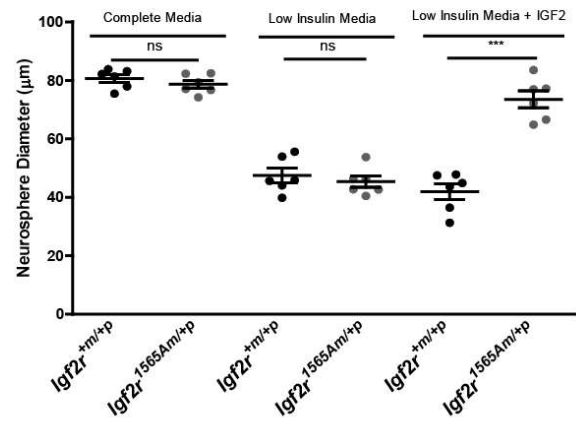
To further validate this result we decided to study the proliferation of the two different populations of cells *in vitro*. Another timed mating was set up and at E18.5 the embryos were culled and the cortical VZ/SVZ and subpallial VZ/SVZ carefully dissected for *in vitro* neurosphere culture. Following two passages the cells were split across three different growth conditions: 1) a low insulin media (5nM), 2) a low insulin media supplemented with IGF2 (15nM); and 3) control basal media (5μM insulin). The rationale for this came from work from the Levison Lab that has previously demonstrated that reducing the media insulin concentration down to 5nM abrogates the activation of IGF1R and results in a great reduction in neurosphere size and number. Supplementation with 15nM IGF2 was shown to modestly rescue neurosphere growth and supplementation with 30nM IGF2 was shown to fully restore neurosphere growth (Ziegler *et al.*, 2014). Hence, the hypothesis was that the reduced binding of IGF2 to the mutant *Igf2^{r1565A}* receptor would increase the bioavailability of IGF2 to the IGF-1R and IR, reproducing the differential rescues observed between the 15nM and 30nM IGF2 concentrations used by the Levison Lab.

As expected, no difference was observed in either cell type when cultured in the control basal media and in both cell types the reduced insulin concentration resulted in a marked reduction in neurosphere diameter (Figure 17). In neurospheres cultured from the neocortical VZ/VZ, which we have previously observed an increased rate of proliferation *in vivo*, no difference in neurosphere diameter was observed between *Igf2^{r1565Am/+p}* and *Igf2^{r+m/+p}* cells cultured in the low insulin media. However, supplementation of 15nM IGF2 resulted in a complete rescue in *Igf2^{r1565Am/+p}* cells but not *Igf2^{r+m/+p}* cells. The results from the subpallial SVZ neurospheres were not as expected but do provide clues as to why we observe differences between the two regions *in vivo*. Interestingly, when cultured in a low insulin media both wild type and mutant cells types had begun to adhere. Nevertheless there was a significant increase in neurosphere diameter for the cells cultured from the *Igf2^{r1565Am/+p}* embryos. Similarly, when supplemented with 15nM IGF2 the *Igf2^{r1565Am/+p}* subpallial SVZ neurospheres were significantly larger than the *Igf2^{r+m/+p}* neurospheres. In addition there was a comparable increase in neurosphere diameter for both compared to the low insulin condition, indicative of a dose response.

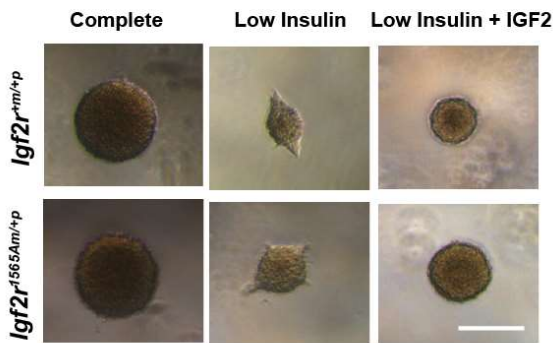
A. Example Neurospheres - Cortical SVZ



B. Neurosphere Diameter - Cortical SVZ



C. Example Neurospheres - Subpallial SVZ



D. Neurosphere Diameter - Subpallial SVZ

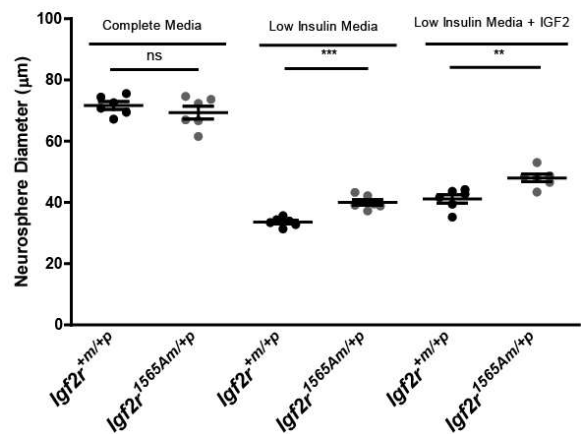


Figure 17 The effects of maternal transmission of *Igf2r*^{1565A/+p} on *in vitro* neurosphere growth.

(A) Example neurospheres grown from the neocortical VZ/SVZ of *Igf2r*^{1565A/+p} and wild type littermates, (B) Maternal transmission of *Igf2r*^{1565A/+p} significantly increased the size of neocortical neurospheres grown in the presence of IGF2 when compared to wild type controls. (C) Example neurospheres grown from the subpallial VZ/SVZ of *Igf2r*^{1565A/+p} and wild type littermates, (D) Maternal transmission of *Igf2r*^{1565A/+p} significantly increased the size of LGE neurospheres grown in the presence of IGF2 when compared to wild type controls Un-paired two-way t-test with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. Scale bars = 50 μm.

3.4.3 Maternal transmission of *Igf2r*1565A does not change the thickness of the embryonic subventricular zone.

We next investigated whether the increased cell proliferation resulted in an expanded VZ/SVZ region. The developing neocortex contains two types of progenitor cells. The first type, the radial glia, divide at the ventricular surface and express Pax6, a homeodomain transcription factor. The second type, intermediate progenitor cells, derive from radial glia but divide away from the surface in the SVZ. The transition from radial glia to intermediate progenitors is associated with a downregulation of Pax6 and upregulation of Tbr2, a T-domain transcription factor (Englund, 2005).

We therefore, completed an immunostain for PAX6 and TBR2 on the cortical VZ/SVZ sections from the embryo brains. The expectation was that there would be a clear lineation between the PAX6+ radial glia and TBR2+ intermediate progenitors with only a small region of overlap. The staining, however, revealed a much larger region of coexpression than expected in both sample sets. This precluded much of the finer quantification we wished to complete. Nevertheless, we could estimate the thickness of the VZ/SVZ from the thickness of the central intense region of the PAX6+ band and the VZ from the region of exclusion between the ventricle surface and the start of the TBR2+ band (Figure 18A).

The average thickness of the PAX6+ band in the wild type embryo neocortex was 70.8µm and the thickness of the region between the ventricle surface and start of the TBR2 band was 30.8µm (Figure 18C). There was no statistical difference detected in the *Igf2r*^{1565Am/+p} embryos for either region.

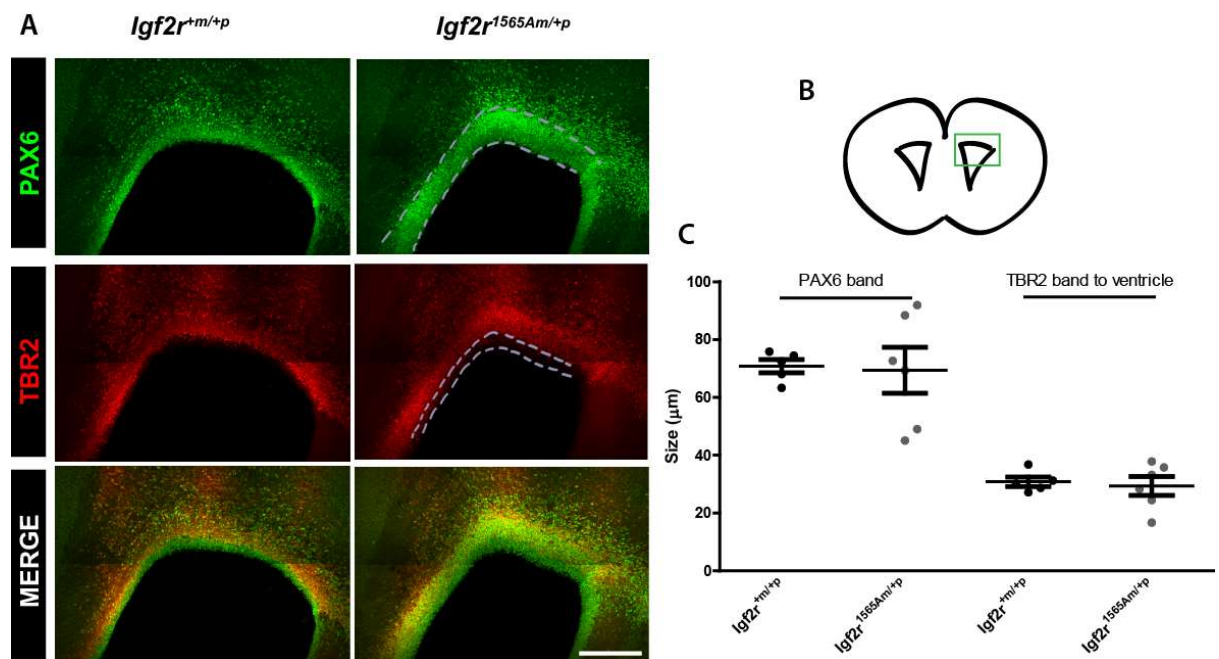


Figure 18 The effects of maternal transmission of *Igf2r*^{1565A/+p} on the neocortical VZ/SVZ.

(A) Immuno-localisation in 50µm coronal brain sections of VZ marker, PAX6, and SVZ marker, TBR2, at E18.5 in wild-type and *Igf2r*^{1565A/+p} heterozygous littermates. Scale bars = 100µm. **(B)** Graphical representation of region of interest. **(C)** Quantification of the PAX6 and TBR2 band width in wild-type (n=8) and *Igf2r*^{1565A/+p} heterozygous (n=9) littermates. There was no significant difference between genotypes. Unpaired two-way t-test with *** p<0.001, ** p<0.01 and * p<0.05.

3.4.4 Maternal transmission *Igf2r*^{1565A} does not change the thickness of layer V/VI cortical neurons.

Given the greater overall brain weight and increased proliferation found in VZ/SVZ of the *Igf2r*^{1565Am/+p} mice we hypothesised that there is an increase in neuron production in the *Igf2r*^{1565Am/+p} embryos. Neural stem cells of the embryonic VZ/SVZ produce several kinds of progenitor cells. The earliest neurons arising from these precursors come to reside within a structure known as the preplate that emerges by about embryonic day 10.5 in the mouse. About a day later the cortical plate begins within the prelate, eventually yielding the 6 layers of the neocortex. The formation of the neocortex follows a strict sequence over a period of about 6 days in mice with the deepest neurons in layer VI being formed first. Each neocortical layer has characteristic neuronal types with specific transcription factor markers. For example, neurons residing in layers V/VI express the marker CTIP2 and layers II/III express CUX1.

We therefore predicted that the increased VZ/SVZ proliferation would lead to an increased thickness of neocortical layers. We were also expectant that differential thicknesses between cortical layers may provide insights into which stage of neocortical development the *Igf2r*^{1565A} mutation was having its greatest effect and whether or not the mutation caused an altered development time course.

To investigate this, we completed an immunostain for the layer V/VI neocortical marker CTIP2 on brain sections from E18.5 mouse embryo brains. Immunostains for other neocortical markers: OTX1, BRN2 and CUX1 were also attempted but these experiments were unsuccessful. Images were obtained via confocal microscopy and maximum intensity z-projections generated for analysis. Three measurements were taken: 1) the thickness of the CTIP2 band, which is equivalent to layers V/VI; 2) the distance from the band to the ventricle, which includes the VZ/SVZ, intermediate zone and subplate; and 3) the distance from the band to the dorsal surface, which is roughly equivalent to cortical layers I-IV (Figure 19A). The average of 3 points across an equivalent region of neocortex was measured for each embryo. In the wild type embryos, the CTIP2 band was 240µm, the distance to the ventricle was 253µm and the distance to the dorsal surface was 86µm (Figure 19C). No statistical difference was observed in the *Igf2r*^{1565Am/+p} embryos for any of the measures.

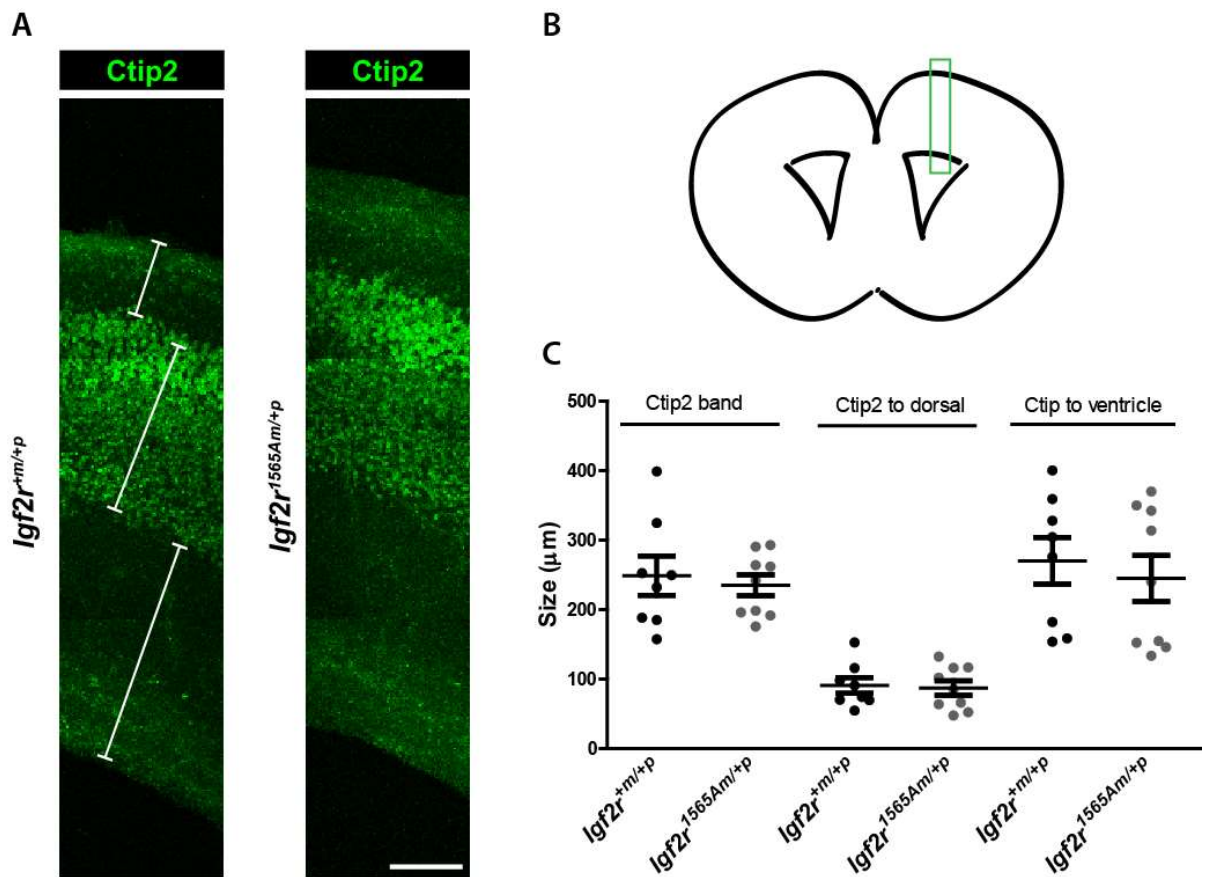


Figure 19 The effects of maternal transmission of *Igf2r*^{1565A/+p} on neocortical layering.

(A) Immuno-localisation in 50μm coronal brain sections of cortical layer marker, CTip2, at E18.5 in wild-type and *Igf2r*^{1565A/+p} heterozygous littermates. Scale bars = 100μm. (B) Graphical representation of region of interest. (C) Quantification of the relative size and position of the Ctip2 positive band in wild-type (n=8) and *Igf2r*^{1565A/+p} heterozygous (n=9) littermates.

3.5 Discussion

3.5.1 Maternal transmission of *Igf2r*1565A results in perinatal lethality and increased proliferation in the Subventricular Zone.

To study the function of Igf2 binding the IGF2R in the brain we investigated the neural phenotype of *Igf2r*1565A mouse model, in which a single knockin point mutation results in a loss of Igf2 binding without disruption to other binding and catalytic functions.

Maternal allele transmission of *Igf2r*^{1565A/+p} resulted in partial neonatal lethality (~60%), indicating an essential role during development, although this phenotype is less severe than previously reported complete loss of function *Igf2r* alleles. This suggests that the contribution of other IGF2 independent functions of *Igf2r* underlie the more severe phenotype associated with complete *Igf2r* allele disruption previously reported (Lau, Stewart and Liu, 1994; Ludwig *et al.*, 1996; Tripathi *et*

et al., 2009). These additional functions of IGF2R are known to include binding retinoic acid, the proteolytic activation of Transforming Growth Factor β (TGF β), the clearance of Leukemia Inhibitory Factor (LIF), and cleavage of IGFBPs by enzymes such as the PAPP-A protease; all of which have established regulatory roles on the SVZ (Blanchard *et al.*, 1999; Ghahary *et al.*, 1999; Kang *et al.*, 1999; Lemamy, Ndeboko and Thierry, 2016). Homozygotes (*Igf2r*^{1656A/1656A}) developed perinatal mortality (>90%), suggesting that paternal allele expression may attenuate the heterozygote phenotype. Paternal allele transmission of *Igf2r*^{+m/1656A} survival is indistinguishable from wild type, indicating the allele is normally regulated and that the mutation did not alter imprinting control.

The perinatal lethality of *Igf2r* allele disruption appears to be a result from major cardiac abnormalities, including disproportionate overgrowth of the ventricular myocardium and septal and valvular defects (Lau, Stewart and Liu, 1994). Maternal allele transmission of *Igf2r*^{1656A/+p} resulted in embryo brain and body overgrowth. However, the ratio of brain/body was not significantly different indicating that brain growth was not disproportionate to overall body growth. Increased proliferation was detected in both the VZ and SVZ of the cerebral cortex of *Igf2r*^{1656A/+p} embryos, consistent with stimulation of cell proliferation of radial glial stem cells and intermediate progenitors, respectively. No significant difference was detected in the proliferation of progenitors in the LGE of *Igf2r*^{1656A/+p} embryos indicating that the regulation of IGF2R and IGF2 signalling may differ between these two structures. One possibility could be that the relative expression of IGF2R and Igf2 in the two regions is different. We examined the expression of radial glia marker, PAX6, intermediate progenitor marker, TBR2 and cortical layer V/VI marker, CTIP2, and did not observe changes in position or thickness of CTIP2⁺ cells, suggesting migration was unaltered.

3.5.2 Challenges and limitations

In writing this thesis I have discovered some prior research that may require us to further investigate the gene expression of the *Igf2r*^{1656Am/+p} samples used. Our study design was reliant on the premise that the IGF2R gene is imprinted in all tissues from E6.5 (Lerchner *et al.*, 1997). Thus, the *Igf2r*^{1656Am/+p} embryo brains are considered to be a loss of function because there is no functional IGF2R expressed. However, this was not confirmed, and literature evidence suggests that the premise may be wrong. *Igf2r* has been shown to escape imprinting in both neonatal and

adult brains. Expression of *Igf2r* is detected from both parental alleles and expression of *Air* is detected only from the paternal allele (Hu *et al.*, 1998; Hu *et al.*, 1999). A subsequent study found that the loss of imprinting begins to occur around E10, coinciding with the start of neurogenesis, and went on to show that the biallelic expression of *Igf2r* is specific to neurons not astrocytes (Yamasaki *et al.*, 2005). This poses the dilemma of how to interpret the data from this thesis. If we accept that a functional *Igf2r* allele is expressed in the SVZ of the E18.5 *Igf2r*^{H1565Am/+p} embryos studied than the increase in brain weight and proliferation is likely down to a general increase in circulating IGF2 in these mice, not a specific loss of local ligand titration. The absence of gross abnormalities in cortical layering fits this interpretation. Another more intriguing scenario is that the imprinting status of *Igf2r* is differently regulated in neural stem cells compared to mature neurons. *Igf2r* expression is elevated as SVZ derived neural stem cell differentiate *in vitro* (see figure 10). mRNA abundance does not infer imprinting status, but, this result points towards lineage dependent regulation of the *Igf2r* gene in the SVZ. The literature evidence that biallelic expression is restricted to neurons and correlates with the onset of cortical neurogenesis further supports this hypothesis (Yamasaki *et al.*, 2005). Neural stem cells are themselves radial glia and by definition can give rise to astrocytes, neurons and oligodendrocytes. Investigations into the imprinting status of IGF2R at different stages of neural lineage progression are needed. These experiments are essential to draw any conclusions from this study and before researching the *Igf2r*^{H1565Am/+p} neural phenotype further.

At the onset of the collaboration between the Szele Lab and the Hassan Lab we intended to use the *Igf2r*H1565A mouse to study *Igf2* signalling in the postnatal SVZ. It was also hoped to cross this strain with the *Igf2*^{flox/flox} to combine loss and gain of-function experiments. This was not possible due to the poor survival of the *Igf2r*H1565A mouse strain and the decision was therefore made study the phenotype in the embryonic SVZ. The age E18.5 was selected because it provided the latest development stage prior to birth and would therefore be the closest to the postnatal SVZ. In retrospect more consideration should have been given to the gestation stage selected. During embryonic development, the SVZ of the lateral ventricle is known as the Lateral Ganglionic Eminence (LGE) and it is not equivalent to the postnatal SVZ. The LGE does give rise to migratory interneurons, however, there is substantial heterogeneity between the gene expression, morphology and migration of embryonic and postnatal interneurons, which precludes

direct comparison (Batista-Brito *et al.*, 2008). E18.5 is also not ideal for studying proliferation during neocortical neurogenesis due to the temporal regulation of neural development. In mice, radial glia begin to proliferate and give rise to neurons from around E10, reaching a peak at E15. At around E17 they begin to release their apical attachment to the ventricle, stop producing neurons and the first astrocytes start to appear. Hence, by E18.5 very little neurogenesis is occurring and it is more likely that the increased proliferation detected in the neocortical SVZ is astrocytogenesis. Further work at subsequent time points is needed to confirm the ultimate fate choice, but given the additional uncertainty over the cell specific IGF2R imprinting status this could be a rewarding line of investigation. It is possible that neural development is protected in the *Igf2r*^{1565Am/+p} due to the expression of the paternal allele but the neurogenesis-gliogenesis transition is disrupted. This model would align with the high lethality in P0-P14 mice, which correlates with the peak phase of astrocytogenesis and oligodendrogenesis (Martynoga *et al.*, 2012). It is tempting to speculate that the increased survival of the *Igf2r*^{1565Am/+p} mice compared to the homozygotes over this period is due to the yet unexplored paternal expression in neurons.

Breeding the *Igf2r*^{1565Am/+p} mice is not straightforward. I was fortunate that much of the difficult husbandry was kindly completed Dr Marvat Surakhy from the Hassan Lab. However, the breeding success rate severely limited tissue and sample supply. During the course of the brain phenotypic study seven timed matings were set up. Of these, only three successfully resulted in embryos that could be studied. The other four failed either because the pregnancy terminated or upon dissection only one embryo was found. The use of embryonic tissue brought other challenges. To retain tissue integrity the brains had to be sectioned in to relatively thick 50µm slices. This meant that there was only sufficient tissue for around 6 rounds of staining. Several combinations of different stains were tried, including for Igf2r, the apoptotic marker cleaved caspase 3 and several cortical markers. However, none were successful enough to image and quantify, thus restricting the breadth of the analysis. The thickness of the sections also made quantification of the markers that did work difficult and precluded some of the marker lineation analysis that was planned. While we did not observe any gross abnormalities in cortical marker expression the staining methodology may be masking subtle differences in differentiation, maturation and integration.

3.5.3 Future directions

Characterising the imprinting status at the different stages of the neural lineage from radial glia through intermediate progenitor, projection neuron, astrocyte and oligodendrocyte is necessary before any conclusions can be affirmatively made from this study. The paternally transmitted heterozygote (*Igf2^{r+m/1656A}*) and homozygote (*Igf2^{r1656A/1656A}*) have not yet been studied in relation to their brain phenotype. Future work should include analysis of their brains if any the glial/neuron imprinting relationship is confirmed.

The fate of the additional cells that result from the increased proliferation is not yet known. We did not observe any gross changes in Layer V/VI thickness, however there may be changes at different stages of development or to different cell types. It may also be that these cells do not incorporate into the brain circuitry and die. In utero electroporation at several gestation stages during neural development may assist with this. This technique enables the fluorescent labelling of a subset of radial glia and their progeny at defined time points, thus permitting greater analysis of neural migration, differentiation and maturation.

Genetic knockout and hypomorphic alleles of *Igf1r* have been most studied in relation to brain aging and have indicated a potentially significant role for the IGFs in neurodegeneration and CNS malignancies (Russo *et al.*, 2005; Chaker *et al.*, 2015; Folch *et al.*, 2018). Studying the function of the *IGF2R* in the postnatal and aged SVZ should therefore still be a target. With the introduction of *in vivo* CRISPR neural editing (Staahl *et al.*, 2017) there is now the possibility of selectively introducing the *Igf2r1565A* mutation into the adult SVZ. This could be combined with selective mutations to the *IGF2* gene known to regulate its relative affinity to the IR-A, *IGF1R* and *IGF2* (Sakano *et al.*, 1991). However, these would be a very challenging series of experiments and could only be justified once the imprinting questions have been resolved and a specific phenotype characterised.

Chapter 4 Development of a new 3D spheroid migration assay to study neuroblast migration

4.1 Introduction

Pharmacologically modulating the migration of neuroblasts has the potential to dramatically improve the prognosis of a plethora of neurological disorders. Research into the process of neuronal migration and the mechanisms regulating this process has evolved due to advances in molecular genetic technologies, allowing for the effects of individual genes on migration to be interrogated. The field is now developing assay platforms to study cell migration in physiologically relevant conditions, with a number of *in vitro* and *ex vivo* assays established, and many superior assays still in development.

High throughput screening (HTS) allows for the effects of large numbers of pharmacological compounds on a biological assay to be tested. For migration researchers these platforms could allow multiple compounds to be tested on a large scale to study compounds that perturb or enhance migration. Existing models for studying neuroblast migration have not been suitable to use on a high throughput scale due to the cost and labour-intensive design. Thus, a new more robust approach is needed. The drug discovery field is transitioning from standard HTS platforms using immortalised cell lines to *in vitro* assays that utilise 3D matrices offering physiologically relevant environments for high content screening (HCS) assays (Hulkower and Herber, 2011). HCS platforms include high power microscopy and analysis software, offering automated drug discovery and characterisation to researchers (L. Li *et al.*, 2017). Phenotypes can be measured and analysed that closely predict *in vivo* cellular mechanisms. In the ten years leading up to 2008 32% of the 183 small molecule therapeutics approved for use were discovered by phenotypic assays (Swinney and Anthony, 2011).

4.1.1 Existing assays available to study SVZ progenitor migration

Several *in vitro* assays have been developed to study neuronal migration. These assays are less technically demanding and inexpensive compared to *in vivo* studies, however, no existing methods are suitable for high throughput phenotypic screening of neuroblast migration.

4.1.1.1 Transwell migration assay

The transwell migration assay is one of the most commonly used assays to assess cell migration *in vitro*. It is based on two medium containing chambers separated by a porous membrane. Primary neuroblasts are dissociated, allowed to adhere to the top of the membrane and a gradient of a soluble substance of interest is created between the two chambers. The number of cells that have transmigrated to the lower side of the membrane at the end of the experiment is quantified to determine the chemoattractant or a chemorepellent response to the substance of interest (Kramer *et al.*, 2013).

While this is a highly characterised assay, with commercially available screening formats (e.g. ChemoTx, Neuro Probe Inc) it has several limitations that restrict its use for screening of compounds that modify the migration of neuroblasts. It is widely accepted that cells growing in a monolayer on plastic dishes have little in common with the complex 3D multicellular organization found in living organisms. For this reason, it is not believed that the form of migration is equivalent to the chain migration typically observed *in vivo*. The assay is designed to study chemotaxis and is not ideal for screening compounds that modulate motility. In addition, tracking cell migration in real time is difficult without sophisticated equipment limiting the possibility to study migration dynamics.

4.1.1.2 Organotypic slice culture

Organotypic slices are brain slices of 100–400µm thickness, which can be kept in culture for several days, thus mimicking *in vivo* conditions. Stoppini *et al.* (1991) developed the technique using a semiporous membrane culture that is still commonly used today. Brain organotypic slices can be used to study various aspects of neuronal development and function, such as neuronal networks formation, synaptic plasticity, electrophysiology, axonal transport, chronic toxicology, gene function and also migration. The use of organotypic slices offers the advantage to live monitor the migration of neurons, which can be labelled with vital dyes or reporter genes. The Szele Lab have previously established such a method using two-photon imaging to track neuroblast migration in real time (Nam *et al.*, 2007). Figure 20 demonstrates examples of neuroblasts migrating in a slice culture from both GAD65-GFP and DCX-GFP reporter mice as well as cells labelled with an *in vivo* electroporation of a GFP expressing plasmid.

Neuroblast migration is usually assessed by measuring the distance travelled or by quantifying the number/proportion of electroporated cells in the RMS. To obtain a reliable quantification many replicates need to be analysed as this technique provides highly variable results. There have been two studies that have successfully used organotypic cultures to screen for compounds that modulate embryonic cortical progenitor migration (Jossin *et al.*, 2003; Zhou, Jossin and Goffinet, 2007). However, the migration of SVZ neuroblasts is a lot more challenging due to their complex migration patterns. Whereas in cortical migration multiple slices can be obtained from a single animal. To ensure the RMS is accurately represented it must be limited to just two slices. For this reason, organotypic slices are not suitable for screening compounds that modulate neuroblast migration.

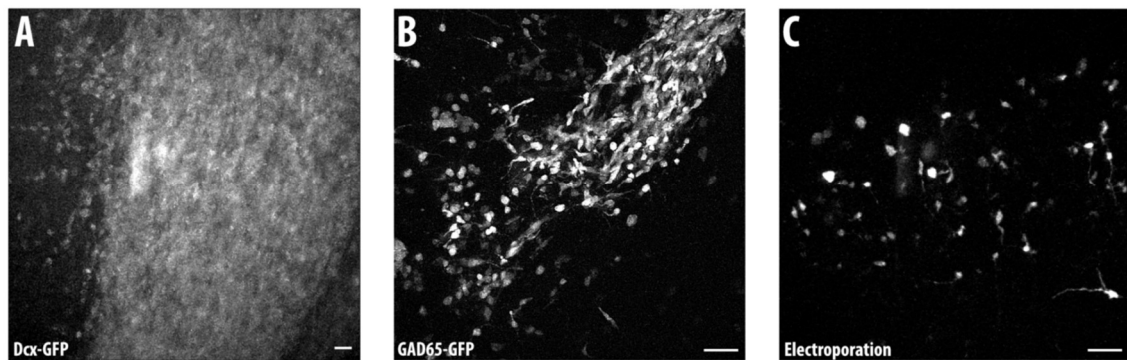


Figure 20: Two-photon excitation microscopy of different cell labelling methods.

(A) Dcx-GFP (P6) - The low signal and high cell density precludes single cell tracking. **(B)** GAD65-GFP (P7) - Very high signal and sparse labelling. **(C)** Electroporated with the pCAGIG plasmid (7DPE) - Labelling is extremely sparse and very few cells identified as neuroblasts. Scale bar = 50 μ m.

4.1.1.3 Ex vivo explant assay

The *ex vivo* explant assay used in Chapter 2 is a more convenient alternative to organotypic slice cultures. In this assay small (<1mm²) chunks of SVZ or RMS tissue are dissected and embedded in an extracellular matrix, such as Matrigel. Neuroblasts contained within the explant are then able to migrate out radially in 3 dimensions enabling the measurement of multiple parameters such as speed and directionality. Explants retain the complex cellular architecture of the brain and recapitulate both single cell and chain migration. Leong *et al.* (2011) utilised this to demonstrate that the ROCK1 inhibitor Y27632 disrupted chain formation. It is possible to culture explants

together with an attractive or repellent source. Alternatively, explants can be cultured above a feeder layer of cortical cells to mimic *in vivo* conditions.

Retaining the cellular architecture also brings about some challenges. Every explant is inherently different and is therefore composed of different starting cells. This leads to an unavoidably high sample to sample variation. As an experimenter, it is also very challenging to precisely dissect out only the desired region without contaminating tissue, which results in about one in every five explants displaying asymmetrical or no migration at all. This inherent variability means that it is essential to have at least 6 replicates per condition. Thus, restricting each assay round to a maximum of around 8 experimental conditions per litter. Another limitation is that because the tissue remains intact, genetic manipulations are almost impossible without the use of prior *in vivo* manipulations.

4.1.1.4 Hanging drop assay

Giovanni Lalli's group used the hanging drop method to overcome many of the limitations of the explant assay (Ward and Rao, 2005; Falenta *et al.*, 2013). In this approach the RMS is carefully dissected, dissociated and reaggregated into homogenous clumps by suspending the cell suspension in a small drop of media. The aggregates can then be embedded in a 3D matrix and migration determined in much the same way as the explant assay. Despite the loss of tissue structure, RMS aggregates display both single cell and chain migration. Being homogenous the aggregates display far less sample to sample variability when compared to explants. Furthermore, when combined with nucleofection this approach permits precise genetic manipulations, which the Lalli group used successfully to demonstrate the regulation of neuroblast migration by Fascin and Debrin (Falenta *et al.*, 2013; Martina Sonogo *et al.*, 2013).

There are some limitations to this method. Firstly, while the cellular composition of the aggregates is homogenous, the individual aggregates vary in size and shape making quantification challenging. Moreover, the preparation disperses the aggregates randomly on culture dishes which limits the use of automated image acquisition. The number of aggregates formed per experiment is limited to ~20 restricting their use in drug screening. Finally, the assay preparation

is even more challenging and laborious than the explant assay meaning that it requires a highly trained individual to complete it.

4.1.2 3D Tumour Spheroid and Cerebral Organoid Assays

In the last few years there has been growing attention to cerebral organoids. These are small 'brains in a dish' that are formed from culturing induced pluripotent stem cells in a 3D environment (Lancaster *et al.*, 2013). It has been shown that these are able to recapitulate much of the complex cellular architecture of the developing brain and have now been used to successfully model many neurodevelopmental disorders (Quadrato, Elnaggar and Giovanni, 2014). The development of 3D culture assays promises a physiologically relevant model system with tissue-like morphology and cell adhesion properties that would occur *in vivo* due to realistic nutrient and oxygen supply conditions (Kramer *et al.*, 2013). In cancer biology studies to investigate tumours have been developed using small clusters, known as spheroids. Vinci *et al.* (2015) published a 3D spheroid assay for using tumour cells, forming spheroids in U-bottomed, ultra-low attachment culture plates. The specifications of these plates allowed for cells from multiple human tumour cell lines to compact and form 3D spheroids, creating a more physiologically relevant model for targeting tumours. The 3D spheroid assay seemed relevant for studying neuroblast migration in the CNS, and the potential for applying this technique to rodent SVZ migration was appealing.

4.2 Aims

The aim of this chapter was to establish a convenient, robust and high throughput phenotypic assay to screen for modulators of SVZ neuroblast migration.

4.3 Methods

4.3.1 Culturing 3D RMS Spheroids.

One litter of P3-P5 CD1 wild type mice pups were culled and their brains removed and sliced into 300µm sections as described in Chapter 2. To dissect out the RMS tissue the olfactory bulb and anterior most 2-3 sections were collected in ice cold HBSS (Gibco 10888-022). Under a dissection microscope and using 27G syringe needles the RMS was carefully dissected and transferred into an 2ml Eppendorf tube on ice. The RMS can be identified as the translucent, triangular region at the centre of the olfactory bulb sections (Figure 21). As the RMS traces back into the more posterior sections it elongated into a tear drop shape, this can also be collected until the ventricle begins to open up. Do not collect tissue from the ventricle region as this will contain ciliated ependymal cells, which can prevent correct spheroid formation.

The dissected RMS tissue was spun down briefly using a bench top centrifuge and the HBSS carefully removed. The tissue was then resuspended in 2ml Accutase (ThermoFisher A1110501) and carefully dissociated using a P200 pipette. It is very important that all the cells are dissociated so take time with this step. The Accutase was diluted out by adding the 2ml of cells to 8ml of NB-A+ media [Neurobasal-A (Gibco 10888-022) supplemented with 1% Glutamax (Gibco 35050-038) and 2% B27 (Gibco 17504)]. The cell suspension was transferred through a 40µm cell strainer (Corning 352340) into a 50ml Falcon tube and washed with a further 10ml NB-A+. The cell suspension was spun down at 300g for 5 minutes, resuspended in 1ml NB-A+ media and counted using a haemocytometer. Typically resulting in approximately 500,000 cells per mouse. The cells were diluted to a concentration of 50,000 cells per ml of NB-A+ and 100ul added to each well of a 96 well ultra low adhesion round bottom plate (Greiner 650970). The culture plate was then sealed with a gas permeable seal (4TI 044850) and incubated overnight at 37°C, 5% CO₂ and spheroids formed overnight. It was critical to not spin the plate down as this trapped cell debris in the centre of the spheroid.

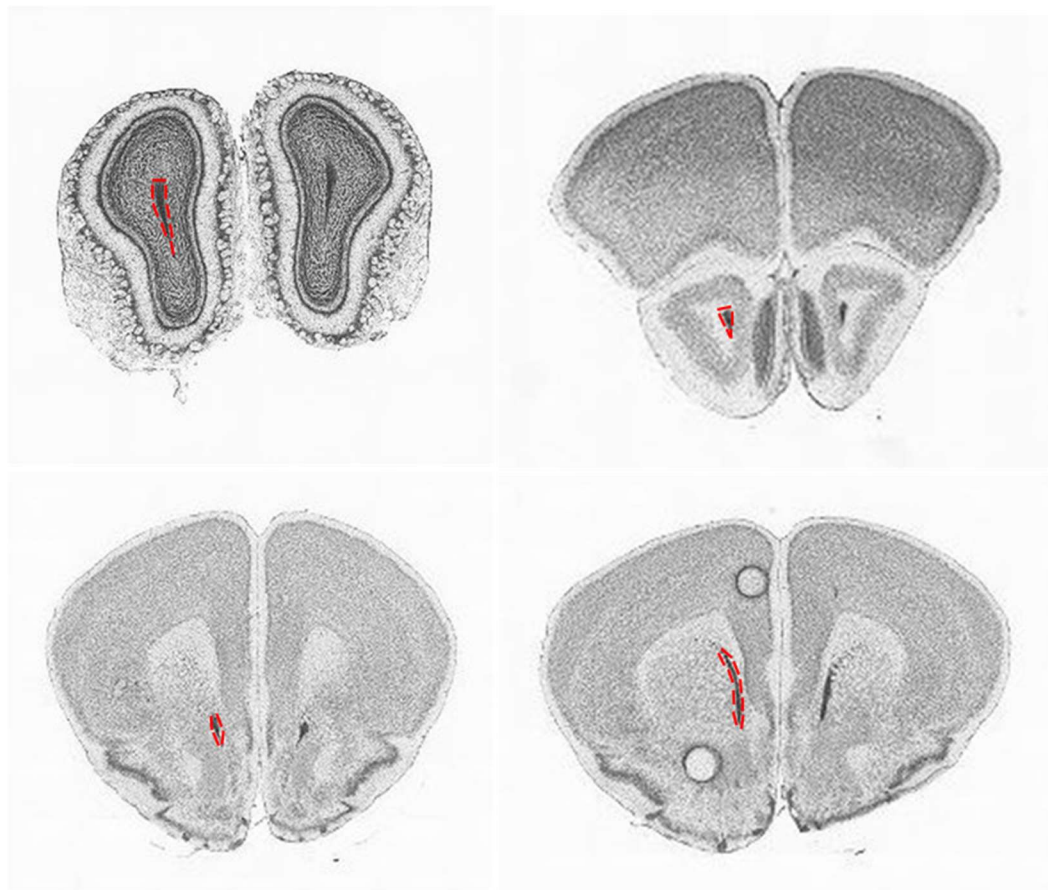


Figure 21: Diagrammatic representation of the regions to be dissected for the RMS spheroid assay. The RMS is 'speckled' in appearance can be traced back from the centre of the olfactory bulb back to the Lateral Ventricle in a tear drop shape. Be careful not to dissect out the anterior commissure which is white in appearance and can be mistaken for the RMS. The rostral half of the olfactory bulb should be excluded as this does not contain many migrating neuroblasts. It is also important not to dissect the SVZ once the ventricle has opened to avoid contamination with ciliated ependymal cells as these may obstruct spheroid formation.

4.3.2 3D RMS Spheroid Migration Assay

RMS spheroids were prepared as described above. The migration was studied by embedding the spheroids in Growth Factor Reduced Matrigel (BD 356231). The spheroid plate was removed from the incubator and left to cool to room temperature. The Matrigel was diluted to 50% in ice cold NB-A+ media. The spheroid plate was then briefly chilled in ice and seal removed. 30µl of Matrigel was then carefully trickled down the side of each well using a multichannel pipette and the plate returned to the ice for the Matrigel to settle to the bottom of the well. The plate was then re-sealed and left for ~3 minutes to warm to room temperature before replacing to the incubator for the Matrigel to set. Images of the spheroid were taken at 2hrs, 24hrs and 48hrs using the InCell high content image system.

4.3.3 Application of vital dyes to 3D RMS Spheroids

To enable analysis of cell viability at the end of the assay I used a terminal stain with Propidium Iodide (Invitrogen P3566) and Hoechst (ThermoFisher 62249). A concentrated stock of Live/Dead reagent was prepared by adding 5µg/ml Propidium Iodide solution and 10µg/ml Hoechst to NB-A+. Two hours prior to the final timepoint of the assay the plates were removed from the incubator, seal carefully removed and 10µl of Live/Dead reagent was added to each well. The plate was returned to the incubator for the remaining time period and assayed as normal. The Hoechst can be imaged using the standard DAPI filter set: 361/497, and Propidium Iodide can be imaged using the standard Texas Red filter set: 535nm/617nm.

4.3.4 Preparation of shRNA plasmid DNA

Plasmid DNA was prepared as per the EndoFree Maxi Kit (Qiagen 12362). In brief, stock cultures from the Dharmacon Mouse shRNA library (Cat# RMM5829) were cultured overnight at 37°C in 3ml LB Broth supplemented with ampicillin. The following day the cell suspension was plated overnight on LB Agarose supplemented with ampicillin. The next day, 3 single colonies were picked per plate and transferred to 3ml of LB Broth supplemented with ampicillin for 4 hours before being transferred to 250ml of LB Broth supplemented with ampicillin and left to shake at 37°C overnight. The following morning the cultures were processed as per the Qiagen kit protocol apart from the following change to the final stage. Once the DNA pellet was precipitated out using isopropanol, DNA was redissolved in 500µl of pure water and transferred to a 2ml Eppendorf tube. 1400µl of 100% ethanol was added, mixed well and spun down at max speed for 5 minutes on a desk top microcentrifuge. Ethanol was removed carefully to allow the DNA pellet to dry completely, and the pellet was resuspended in 50µl pure water and the concentration was measured using a Nanodrop.

4.3.5 Postnatal electroporation

Plasmids used for postnatal electroporation were prepared by Endofree Plasmid Maxi Kit as above. Before use, 1% fast green in PBS (filtered through a 0.22µm filter) was mixed with the plasmid solution as 1:10 for tracing. For the postnatal electroporation procedure, P0-P1 pups were anesthetized by hypothermia. ~2µl of plasmid solution (5µg/µl) was injected into the right

lateral ventricle via a pulled glass capillary. Pups were then subjected to electrical pulses (-100V, 50ms ON with 850ms intervals for 5 cycles) (BTX ECM 830) by tweezer electrodes coated with conductive gel. After surgery, pups were kept in a 37°C warming box for recovery and then returned to their cage. The pups were monitored for the following 4 days to check for adverse reactions to the procedure and on the 5th day the pups were culled and right hemisphere removed for spheroid preparation.

4.3.6 Nucleofection of primary RMS cells

The following describes the method used to successfully nucleofect RMS cells prior to formation of 3D spheroids. Please see text for earlier iterations.

Cells from the RMS were nucleofected via a modified version of LONZA protocol VPG-1001. In brief, the RMS was dissected out as described above, dissociated, counted and split across 2-4 different 15ml Falcon tubes containing 1×10^6 cells and 5ml NB-A+ media. The cells were spun down at 300g for 5 minutes, the supernatant removed and then very carefully resuspended in 50% of the standard nucleofection solution diluted in HBSS minus calcium and magnesium (41µl of Nucleofector Solution + 9µl of supplement + 50µl of HBSS) and 5µg DNA before being transferred into the certified cuvette. Nucleofector program G-13 was used instead of the recommended O-005. 500µl of pre-warmed culture medium was added into the cuvette and the sample was then transferred by the supplied pipette into 1ml medium and centrifuged at 300g for 5min. Cells were then suspended with 4ml fresh medium, plated in a single well per condition in a 6 well plate and left to recover at 37°C. After 4 hours the cells were collected and used as normal for the remainder of the spheroid assay.

4.3.7 Image acquisition

Imaging was primarily completed using the GE InCell 6000 High Content Imager. This system comprises both brightfield and a line scanning laser confocal microscope making it ideal for quick 3D cell imaging, albeit at a slightly poorer resolution than standalone point scanning confocal microscopes. A protocol was established on the InCell software that imaged the centre of each well of the Greiner ULA plate. The starting plane was set by the microscope's in built 'autofind' function and 9 Z slices were imaged at 25µm per well, which amounted to a total section of

200µm. Prior to starting the imaging experiment a trial scan was completed to check the position of the spheroid in each image. Should any spheroid be dramatically off-centre or shifted in the z-plane the acquisition field was manually set for that well. All images were taken with the 10X 0.45NA objective. The InCell has four excitation filters (UV, Blue, Green & Red) and four capture filters (DAPI, GFP, RFP and brightfield). All scans included a brightfield image using filters: Red/brightfield and exposure 0.2s. When fluorescence was used the exposure was set based on the individual signal level of that experiment. To scan each plate it took around 15 minutes on brightfield only and around 25 minutes when using fluorescence.

Imaging was also completed on three other systems: 1) a Motic AE2000 inverted microscope with Moticam 580 digital camera, 2) an Essen Bioscience IncuCyte, or 3) an EVOS FLauto. For the Essen Bioscience IncuCyte the 10X objective was used, imaging in the centre of the plate with the software's inbuilt autofocus. Images were taken every 30 minutes for 24 hours.

4.3.8 Manual image analysis

To manually quantify the spheroid migration the individual brightfield image stacks were loaded into ImageJ and a maximum intensity projection created. Unlike explants and hanging drop aggregates the migration from each spheroid is relatively symmetrical. This property was exploited to provide a quick and simple quantification method. An ellipse was drawn around the spheroid such that it satisfied the following criteria: 1) it was centred on the spheroid, 2) it cut through at least 6 neuroblast deemed to have migrated from the spheroid, 3) it was round (Circularity > 0.8). When there are a few single cells that had migrated far from the spheroid individually these were ignored so not to skew the analysis. The area was then calculated and saved as a "Region of Interest". In some instances, the "Find Edges" function was applied to assist in visualising individual cells.

Manual analysis of fluorescent nucleofected spheroids was completed by analysis of the average of the 8 maximally migrated cells. The fluorescent channel image stacks were loaded into ImageJ and a maximum intensity projection created. The line function was then used to measure the distance from the spheroid edge to the 10 cells that appeared furthest away making sure to save each as a 'Region of Interest'. Only the top 8 of these measures were used for quantification.

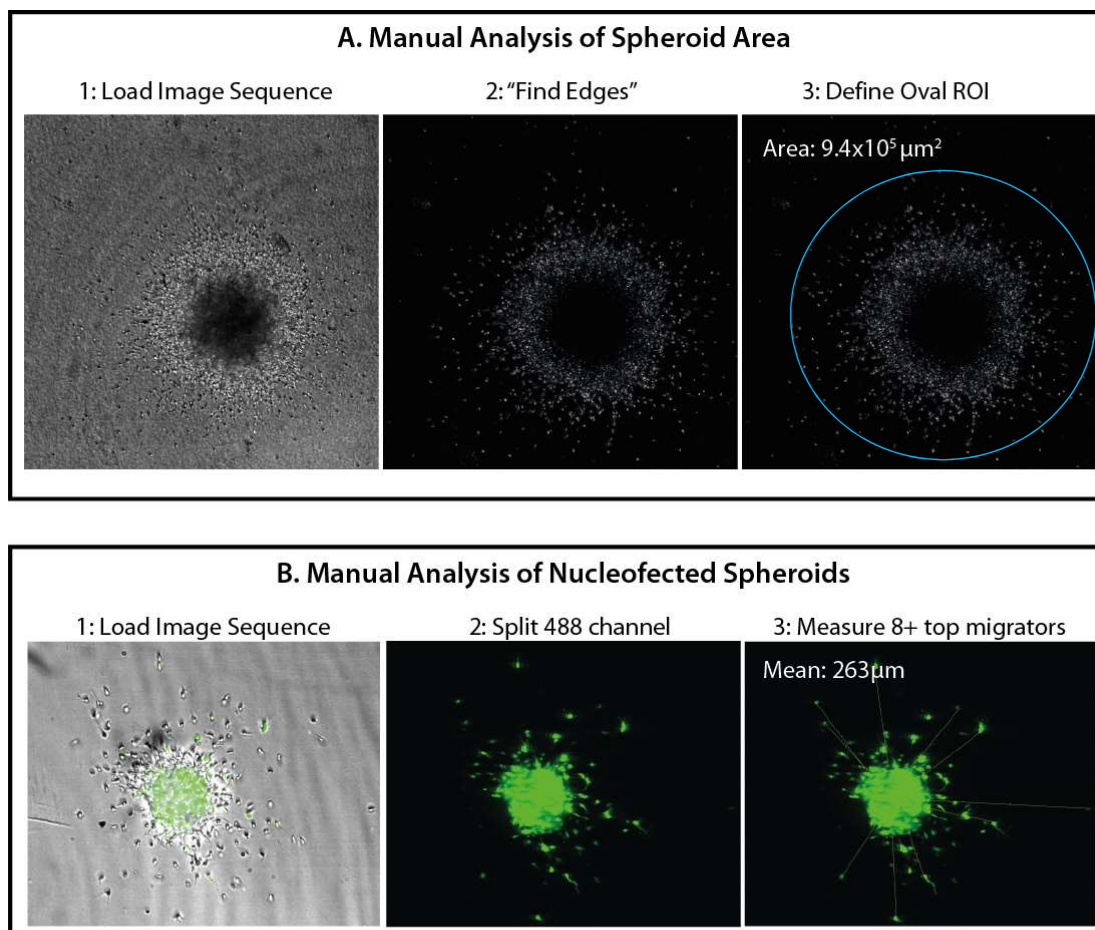


Figure 22: Images showing the workflow for manually quantifying spheroid images.

(A) To analyse bright field images, a maximum intensity projection is created and the area of an ellipse encircling the migration is calculated. The "Find Edges" function may be used to improve the signal to noise ratio. **(B)** For manual analysis of fluorescent nucleofected cell the average of the 8 maximally migrated cells is taken.

4.3.9 Automated image analysis

Automated image analysis was all carried out using the GE InCell Analyzer Workstation. To analyse the collective migration from the spheroid, extended focus images were created from each brightfield image stack. A Macro was written to apply the following functions to each image: *Smoothen > Find Edges > Dilation > Fill Holes > Erosion > Segment > Sieve*. This resulted in segmentation of the spheroid and halo of migrated cells ready for quantification. To analyse the number and location of individual cells the fluorescent channels (Hoechst / Propidium Iodide) we used. First extended focus projections were created for each image stack and the contrast enhanced. A Macro was written to apply the following functions to each image: *Segment > Fill Holes > Sieve*. The Hoechst and Propidium Iodide images were then compared to identify live cells.

4.4 Results

4.4.1 Development of 3D RMS spheroids invasion assay.

Inspired by the hanging drop assay and cerebral organoids, a novel 3D RMS spheroid invasion assay was developed. The protocol was optimised for size of spheroid, plate brand, amount of debris in cultures, and the specifications of the microscope.

RMS dissection, dissociation and reaggregation utilizing the hanging drop technique has previously been described (Ward and Rao, 2005; Falenta *et al.*, 2013) whereby cells are suspended in droplets of medium such that they come together via gravity. Recently many manufacturers have released culture plates with a round well-bottom design, and an Ultra-Low Attachment (ULA) surface coating. These mimic the meniscus of a hanging drop and enable the formation and growth of a single spheroid per well with reproducible size. The rapid growth of organoid research has meant that many different variations of this design exist, many of which are considerably expensive (see table 7). The Greiner ULA 96 well plates have not been specifically developed for spheroid assays but were selected because they are by far the most cost-effective option.

Table 7 Examples of Ultra Low Adhesion round bottomed plated currently available.

Microplate	Company	References	Format	Surface	Cost
CELLSTAR® Cell-Repellent Surface	Greiner Bio-One	650970	96	Grafted cell-repelling polymer	£25 per 6
Corning® Ultra-Low Attachment (ULA)	Corning Life Sciences	4520, 3830	96, 384	Grafted hydrogel	£185 per 5
GravityTRAP™ ULA	InSphero	ISP-09-001	96, 384	Non-adhesive coating (NAC)	£190 per 10
CellCarrier® ULA	PerkinElmer	6055330,	96	NAC	£144 per 10
Nexcelom3D ULA	Nexcelom Bioscience	ULA-96U, ULA-384U	96, 384	NAC	£225 per 10

To confirm that the Greiner ULA plate system could be applied to RMS cells I set up a pilot experiment in which the RMS was dissected from GAD65-GFP mice. This is a convenient mouse strain for studying neuroblasts because the majority of the cells in the RMS express GFP making dissecting and subsequent imaging relatively straightforward. Two P4 mice pups were used for this pilot experiment, which comfortably provided sufficient cells for culturing 5000 cells in each of the middle 20 wells of a 96 well plate. The cells naturally come together overnight and reaggregate into tight uniform spheroids. In the pilot experiment one well had to be excluded due to debris, nevertheless 19 uniform spheroids with an average 2D surface area of 15,603µm² and

standard deviation $214\mu\text{m}^2$ were formed (Figure 23). Reliable phenotypic screening requires that initial test conditions are comparable. This means that spheroids should present the same or very similar initial size. Spheroid size is expected to be normally distributed and the variances in initial sizes are expected to arise only due to statistical fluctuation. To confirm this assumption a larger sample of 384 spheroids (4 x 96 well plates) were formed, their areas measured and a frequency analysis, followed by a D'Agostino & Pearson omnibus normality test, was conducted. Analysis of the frequency distribution indicated a Gaussian distribution, which was supported by a passed D'Agostino & Pearson omnibus normality test ($P=0.1813$). The mean spheroid size was $27,437\mu\text{m}^2$ and standard deviation $1073\mu\text{m}^2$ confirming the Greiner ULA system can produce large quantities of reproducible RMS spheroids. Moreover, excellent optical properties make them compatible with various types of readout devices, such as standard and lens-free microscopes, high-content imaging systems, and microplate readers.

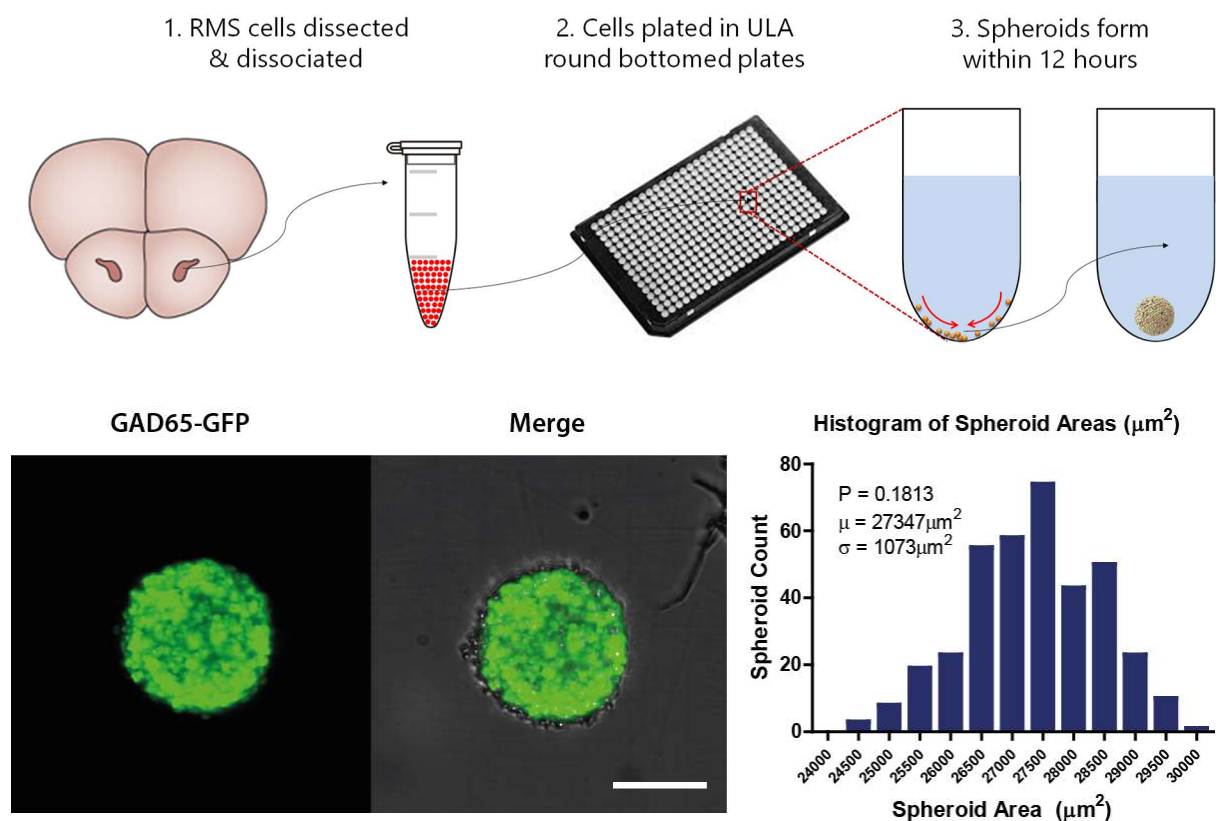


Figure 23. Workflow of the 3D SVZ spheroid assay. The workflow demonstrates cells isolated from the SVZ forming spheroids in round bottomed wells. Spheroid formation should occur within 24 hours and spheroid shape and size can be determined by imaging GFP-labelled cells once transferred onto Matrigel. Spheroid area can vary between 24500 and $30000\mu\text{m}^2$, with an average spheroid area of $27347 \pm 1073\mu\text{m}^2$.

The RMS spheroids remain as stable floating colonies when left in media. To induce migration we next had to introduce a method of embedding the spheroids in an extracellular matrix. With the *ex vivo* explant and hanging drop assays the sample tissue are mixed with an ECM matrix then manually positioned as a drop in the culture dish. It is possible to do the same with the spheroids, however this method does not lend itself to medium to high throughput screening due both to costs and time. After some tinkering with different options an ideal method was determined that requires just one additional step in the protocol, robustly positions the spheroids in the centre of each well and is cost effective. Growth Factor Reduced Matrigel™ was used because it has previously been demonstrated to support the migration of neuroblasts *in vitro* (Azzarelli *et al.*, 2017). Matrigel is a soluble basement membrane biomaterial extracted from Engelbreth-Holm-Swarm mouse tumours; it contains several molecules including laminin, collagen IV, heparan sulfate proteoglycans, and entactin/nidogen-1, 2. It is liquid below 4°C but sets to a gel above 10°C. Importantly Matrigel™ is viscous; it has a protein concentration of 8-12mg/ml and will sink in water. This property was exploited for the spheroid invasion assay (see Figure 24). A plate of 80 spheroids from wild type CD1 pups were formed and 50µL of 50% Matrigel was carefully pipetted down the side of each well so that it sank to the bottom of the well and encased the spheroid. Returning the plate to the incubator set the Matrigel™ plug and induced neuroblast migration from the spheroid. Migration area was determined manually by drawing an ellipse around the radiation and measuring the area. Analysis of the frequency distribution indicated a Gaussian distribution, which was supported by a passed D'Agostino & Pearson omnibus normality test ($P=0.7114$). The mean radiation area was $365,598\mu\text{m}^2$ with standard deviation $35,642\mu\text{m}^2$

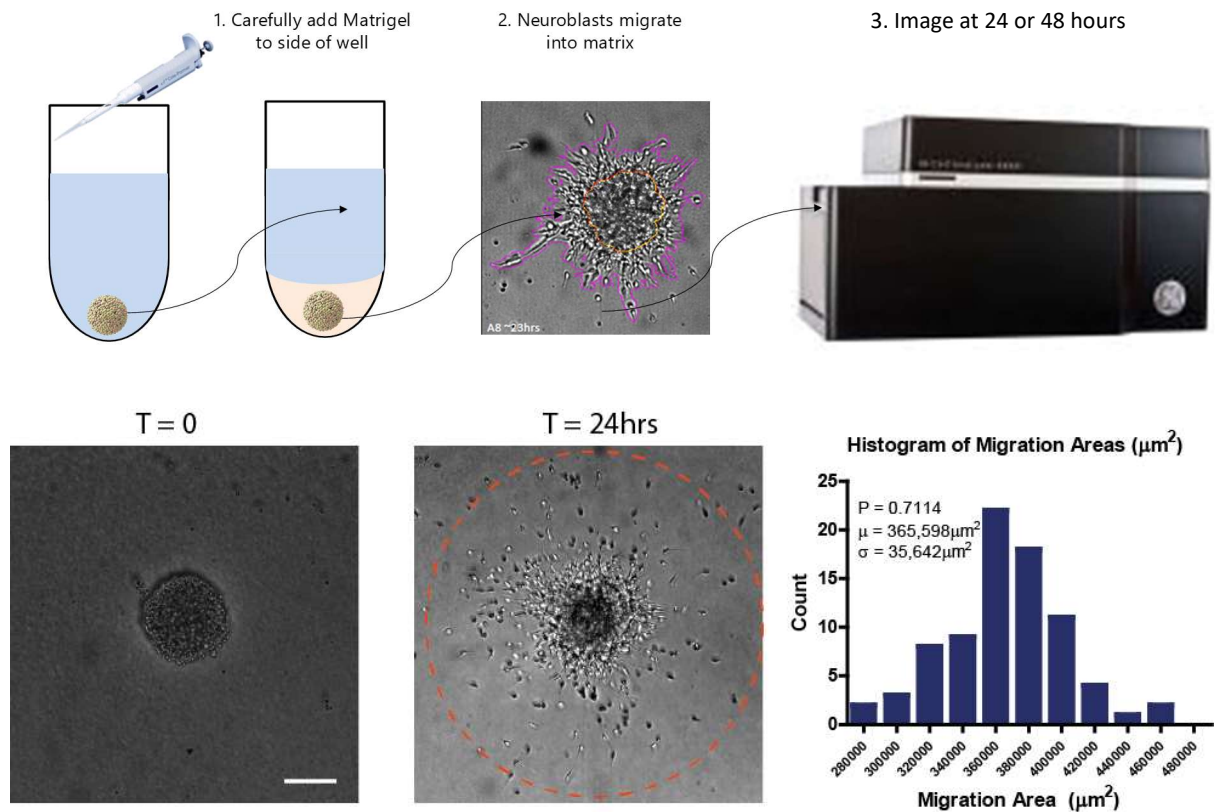


Figure 24. Assay development for neuroblast migration. Spheroids are adhered in Matrigel and distance of neuroblast migration is imaged and measured at 24 hours. The images show the spheroid at 0 hours in Matrigel and at 24 hours with the size of the migration diameter manually marked on to determine radiation area. The maximum migration area was over 460,000 μm^2 and the mean radiation area was 365,598 μm^2 .

4.4.2 Neuroblast migration from 3D RMS spheroids closely recapitulates *in vivo* migration.

A critical feature in developing a neuroblast migration assay was to recapitulate the neuroblast chain migration that occurs *in vivo*. It is argued that chain migration is orchestrated by the glial tube, however, as Figure 25 illustrates, the neuroblasts radiating out from the spheroids are able to self-organise into chains. It is important to emphasize here that the 3D RMS spheroids are distinct from the SVZ neurospheres discussed in previous chapters. As evidenced by the GAD65-GFP expression in Figure 23, RMS spheroids are predominantly neuroblasts. Neurospheres are clonal colonies of neural stem cells used to study stem cell behaviours, such as self-renewal, proliferation and fate choice. Each colony has a heterogeneous cell composition that varies depending on age, seeding density, culture conditions, and passage number. Nevertheless, the predominant cell type is generally considered to be the rapidly dividing transit amplifying cells (Gil-

Perotín *et al.*, 2013). DCX+ neuroblasts are only found in very few numbers. A study that did not find PSA-NCAM expression in the neurosphere, argued against the presence of neuroblasts capable of migration (Parmar *et al.*, 2003). This concurs with the consistent finding from the Szele Lab that the radial migration from neurospheres is almost entirely glial cells. Moreover, the migration is amoebic in nature and does not display the mixture of chain and single cell migration evident in both the explant assay and now the spheroid assay.

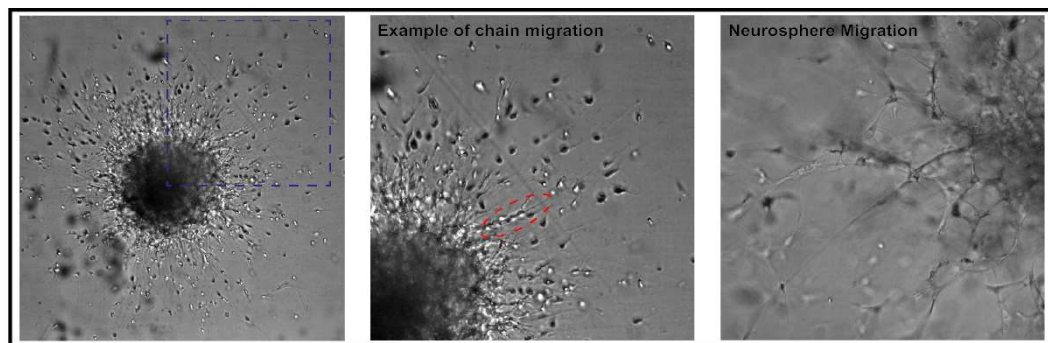


Figure 25. Magnified image of the chain and single cell migration of neuroblasts from an RMS spheroid. Left and central image show cells migrating out as chains from an RMS spheroid. Neuroblasts self-organise into chains to radiate out from the spheroid before continuing as single cells. The right image shows the amoebic glial migration that radiates from a neurosphere as a comparison.

4.4.3 Assay Optimisation

Once it had been determined that RMS spheroids formation was robust it was important to optimise a few criteria. The first of these was spheroid size or cell number per well. The greater the cell number the more continuous the radiating cell halo becomes, making image quantification more consistent. Moreover, the greater number of cells permits greater complexity in 3D cellular architecture. However, the transfer of nutrients into the spheroid centre gradually decreases with spheroid size, potentially causing variations in cell viability or metabolism. In SVZ neurospheres this is known to lead to the formation of hollow necrotic cores once the diameter exceeds $\sim 150\mu\text{m}$ (Dan Ge, 2012). In addition, monitoring cell migration across by videomicroscopy is limited by the field size and camera aperture, thus smaller spheroids may enable migration to be tracked over longer periods of time. A balance is therefore required to retain physiological relevance, while permitting high content screening. Spheroids can be successfully formed from as little as 150 cell up to 40,000. Figure 26 shows spheroids plated at cell densities between 600 and 10,000 cells per well. Imaging at 24 hours shows that the spheroids with over 10,000 cells at the start of the assay contain a lot of migrating cells, but the cells with highest levels of migration do not remain

within the field of image, and therefore cannot be reliably measured. The largest spheroids also develop central shadowing, indicating necrotic centres due to the lack of nutrients reaching the spheroid core. However, the spheroids tested with less than 2,500 cells do not have sufficiently large numbers of cells to migrate from the spheroid, and therefore do not make a reliable high throughput assay. The best compromise under the experimental conditions and equipment available were spheroids of 5000 cells.

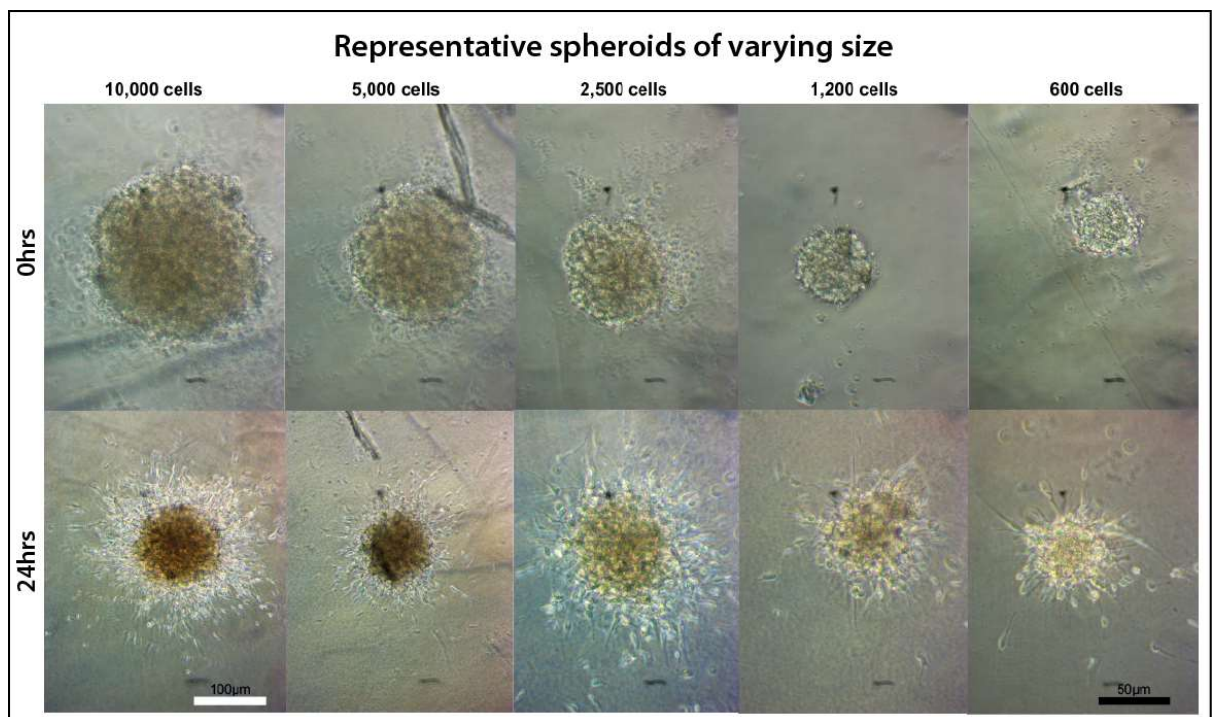


Figure 26. Spheroid size optimisation. Spheroids of different cell number between 600 and 10,000 cells were tested for suitability in the 3D assay with imaging at 0 and 24 hours. Larger spheroids showed more migration after 24 hours, but were too large to measure cells migrating the greatest differences. Therefore, intermediate sized spheroids were selected for high throughput screening. The white (100µm) scale bar reflects a change from a 20x to 10x objective and only applies to the 5000 and 10,000 cell spheroids at the 24 hour time point. The black (50µm) scale bar applies to all other images.

Matrigel™ is a relatively expensive reagent at £250-300 per 10ml bottle. Using 50µl at a 50% dilution per well results in a per plate cost of £75. Therefore, we next sought to determine whether the volume of Matrigel could be reduced. The concentration of Matrigel used is known to strongly influence neuroblast migration so we were reluctant to vary this dramatically (Rao & Ward, 2005). However, experience from explant assays indicates that neuroblasts grow well *in vitro* in drop volumes down to ~10µl. When pipetted carefully with a single tip pipette we confirmed that the volume of Matrigel used could be reduced to 20µl. However, when attempted with a multichannel

this volume proved too restrictive as roughly 10% of the spheres failed to embed correctly. The smaller volume of Matrigel displaced the spheroid from the centre of the well, instead of enveloping it, resulting in the spheroids being embedded off centre. While this may not necessarily effect invasion it hindered both imaging and analysis as steep sides of the U bottom create image aberrations. We hypothesised that this was due to the leading edge of the Matrigel beginning to polymerise as it reaches the bottom of the well. To overcome this the culture plate with the spheroids was cooled on ice prior to embedding and the Matrigel volume was increased to 25 μ l. This resolved the problem of spheroid centring but introduced a second complication. Using ice resulted in watermarks on the bottom of the plate that introduced large artefacts into the brightfield image background. These proved very difficult to prevent via simple cleaning measures. Wiping with alcohol wipes was either too mild to remove the mark or too harsh; either creating scratches on the plate surface or dislodging the Matrigel plug. To cool the spheroid plate we, therefore, now use a large reusable icepack recycled from a consumable delivery covered in a single sheet of tissue paper.

Another strategy considered was miniaturisation to a 384 well format. The smaller volumes and tighter gradient of curvature of the U-bottomed 384 well plates formats offered by Corning allowed the volume of Matrigel per well to be reduced to just 10 μ l. However, because of the increased well curvature, large spherical aberrations are created making them unsuitable for migration assays that require a large image field. This, in addition to the increased plate cost and technical difficulty, confirmed the 96 well Greiner ULA plate with 25 μ l 50% Matrigel as the best format.

The other issues to overcome in the experimental set up are represented in Figure 27. The media composition needed to be optimised to sustain dense spheroid formations while minimising potential variants. Spheroids form in standard neurobasal media supplemented with B27. There is no need for additional supplementation with serum or growth factors. However, medium with low insulin (5nM) did not support spheroid formation. Debris in the cultures was important to minimise as this disrupted spheroid formation and integrity. Debris comes in two forms 1) Contamination, and 2) Cellular debris. This assay will test the experimenter's aseptic technique. Any contamination that falls in the well will be focused into the centre and incorporated in the spheroid.

Arm sleeves are highly recommended to prevent fibre contamination from lab coats. Cellular debris are cells that have not reached the centre of the well and are not incorporated in the spheroid. They are not necessarily dead and can retain the ability to migrate. This does not create a huge obstacle to manual image analysis where the experimenter can be more discerning but for automation it can be a problem. The amount of debris is largely dependent on the accuracy and speed of the dissection. Very little debris is experienced when using a fluorescently labelled mouse line, such as the GAD65-GFP or DCX-GFP strains. However, when using wild type tissue a degree of contamination is unavoidable. In some instances where the SVZ is accidentally dissected ciliated ependymal cells can also disrupt spheroid formation entirely. The best strategy to overcome this is to improve dissection technique. If dissecting more than four brains in one sitting it is also advisable to add in a recovery period after the dissection to let the cells recover and limit the addition of dead or dying cells to the ULA plate. Even with these measures it is impossible to form a spheroid with absolutely no debris. For very high quality images, it is advised to pick the spheroids out of the well and wash prior to embedding.

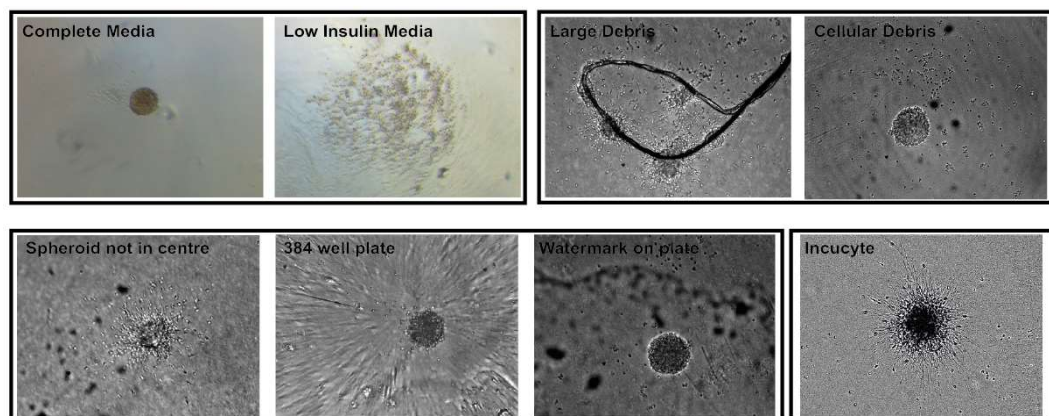


Figure 27. Optimising spheroid assay for media composition, debris levels, spheroid positioning and plate suitability. The success of the spheroid assay was dependent on a number of components to ensure spheroid integrity and imaging resolution. The top left hand image is an 'ideal' example aggregated in standard high insulin media.

4.4.4 Application of vital dyes to improve image quality and provide cell viability information.

An *in vitro* assay spheroid assay allows for vital dyes to be utilised to provide information about the health of spheroid cultures (Figure 28). The combination of Hoechst 33342 and Propidium iodide (PI) was used to assess the viability/cytotoxicity in the spheroid assay. Both of these dyes bind to DNA, but while Hoechst is cell permeable, PI cannot cross the membrane of live cells. Therefore the combination is useful to differentiate apoptotic and healthy cells in a culture. Both dyes worked well in RMS spheroids, however the compact spheroid mass created dye gradients necessitating staining time to be increased from 10 minutes to 60 minutes to reach equilibrium. The response of the spheroid bodies and the migrating cells can be used to distinguish between compounds that inhibit migration vs those that are cytotoxic (see figure 28). However, due to the cytotoxicity of the vital dyes themselves this assay should only be used as an endpoint.

CellTracker Green is a more stable form of the thiol-reactive Fluorescent Probe Calcein-AM that is suitable for long-term cell labelling. It passes freely through cell membranes, but once inside the cell, is transformed into fluorescent cell-impermeant reaction products. To avoid uneven dye transfer into the compact spheroid mass the dye was added to the dissected cells prior to spheroid formation. CellTracker stained all cells in the spheroid with no obvious detriment to spheroid formation or neuroblast migration. Single cells could easily be identified, thus providing the option to use high content analysis software to count and measure the dynamics of single neuroblasts.

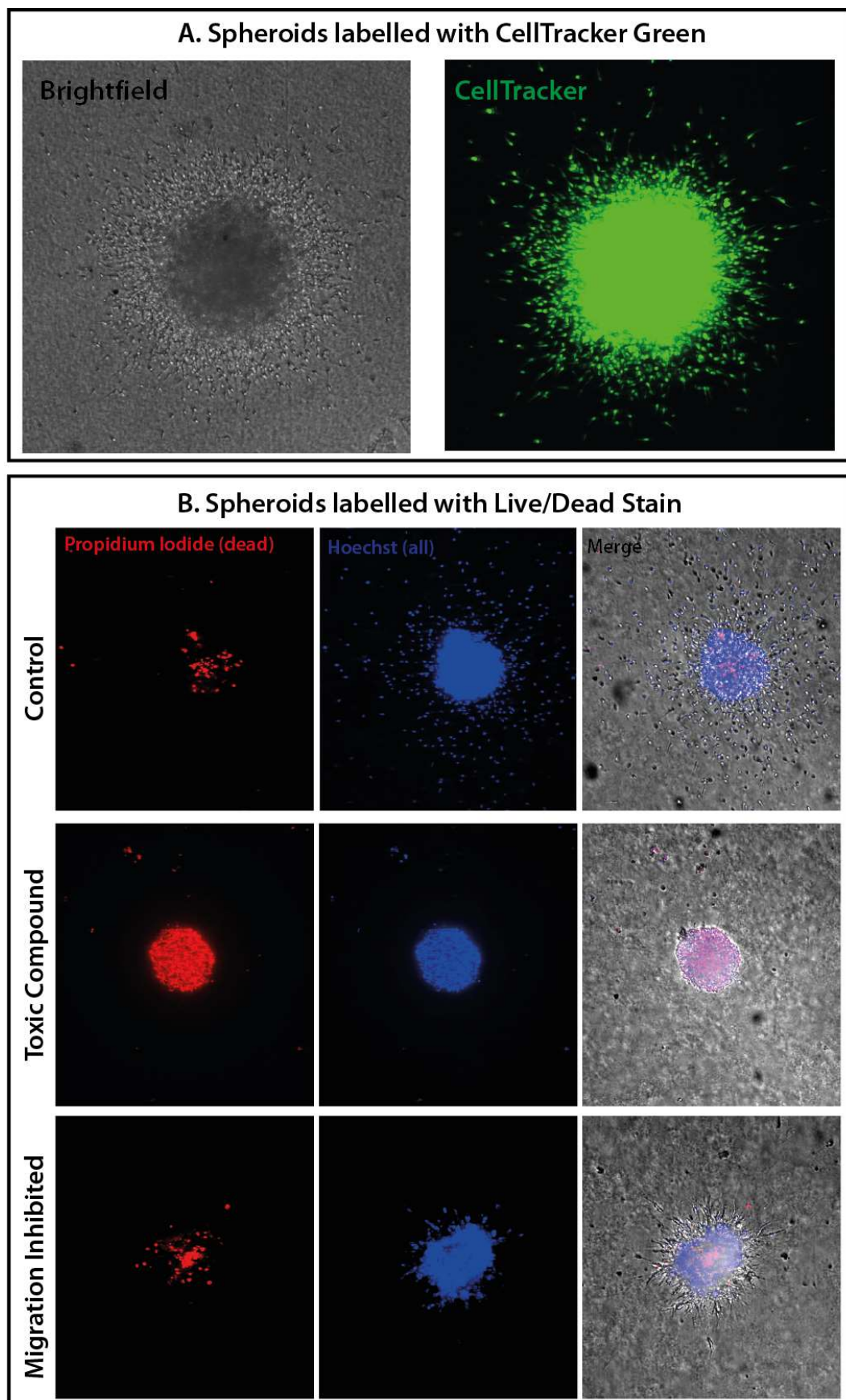


Figure 28. Spheroids labelled with fluorescence dyes. (A) CellTracker green allows for easy visualisation of spheroids and migrating neuroblasts. **(B)** Live/dead dyes Propidium Iodide and Hoechst show response of cells to a toxic compound (GW406731X) and a migration inhibitor (GSK1389063A) compared to DMSO.

4.4.5 Optimising nucleofection protocols to enable genetic manipulation of neuroblasts.

The ability to genetically manipulate cells of interest is an essential part of modern biology. Therefore, in order to demonstrate the wider application of the 3D RMS spheroid assay I set out to confirm that it can be combined with genetic techniques. It has previously been shown that primary mouse neuroblasts can be transfected by the Lonza Nucleofection System (Falenta *et al.*, 2013). To confirm that this technique can also be applied to RMS spheroids the pGIPZ DNA plasmid was nucleofected into dissociated RMS cells prior to spheroid formation. pGIPZ is the control expression plasmid for the Dharmacon Mouse shRNA library and contains the turbo-GFP gene under the CMV promoter. Initially the Nucleofection programme O-005 was used as per the Lonza protocol (VPG-1001). However, this protocol did not yield compact, round spheroids (Figure 29). Instead there was a collection of many small spheroids formed, which all collected at the bottom of each well. A gelatinous substance in the well appeared to prevent complete unification. The same result was also observed when using setting A-33, another programme designed for mouse primary neural cells. The cells were healthy and when washed, dissociated and re-plated could form spheroids. However, when these secondary spheroids were embedded the subsequent migration was limited. Following a conversation with a Lonza representative two modifications were made to the Falenta *et al.*, (2013) protocol. Firstly, the Nucleofection Solution was diluted in Hanks Balanced Saline Solution minus Calcium and Magnesium. The rationale was that the lipophilic nature of the reagent may be disrupting membrane integrity. Secondly the nucleofection programme was changed to G-13, which has a reduced electric pulse power. With these modifications the nucleofection of dissociated neuroblasts did not disrupt spheroid formation and the efficiency was sufficiently high (~50%) to quantify migration.

Another technique used to transfect and study neuroblasts migration is *in vivo* electroporation. In this technique high concentration plasmid dna is injected directly into the lateral ventricle of P0-P2 pups and then a pulse electric current is applied across the skull. This robustly transfects the SVZ stem cells with the plasmid and also therefore their progeny the neuroblasts. It was therefore attempted to electroporate *in vivo* the pGIPZ plasmid 5 days prior to dissection. Using this method, the spheroids formed correctly but the number of GFP+ve cells contained within each spheroid was far too low to quantify.

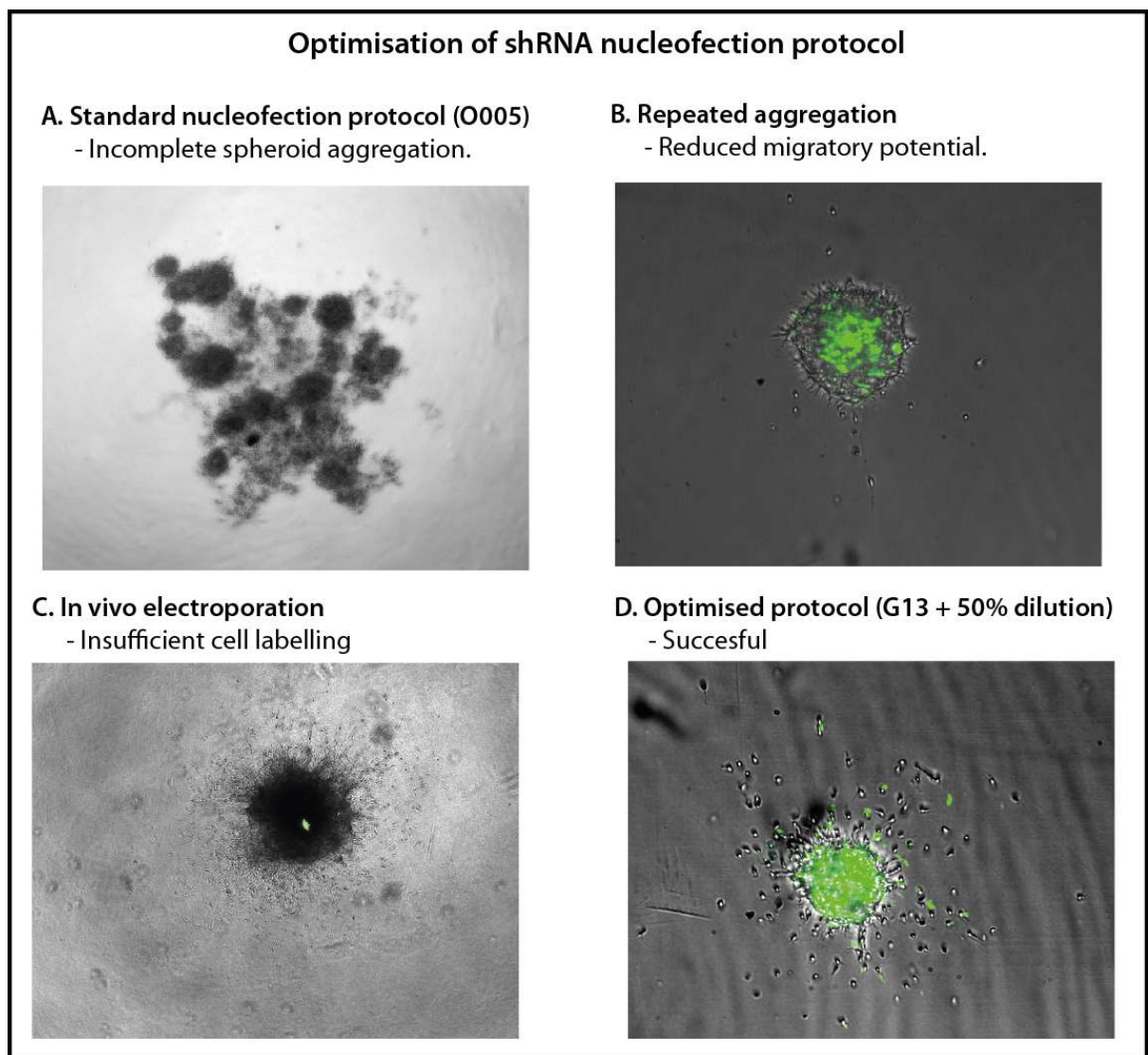


Figure 29 Optimisation of shRNA Nucleofection Protocol. shRNA transfection was optimised for improved viability, aggregation, and transfection efficiency by testing multiple electroporation methods and programmes of different electric pulse powers and different concentrations of electroporation solution.

4.4.6 3D RMS spheroids can be used for time lapse video microscopy.

A major motivation for the development of a more uniform migration assay was the potential to perform time lapse imaging. It is possible to set up time lapse experiments with explants but it can be very challenging in practice due to variability in assay set up and migration potential. Uniform spheroids can be established reproducibly and are robust enough for time lapse microscopy.

To explore the application of time lapse imaging to the spheroid assay a plate of RMS spheroids from wild type CD1 mice was formed, embedded in matrigel and imaged using the GE InCell High Content Image System every 30 minutes for 24 hours. The InCell has an in built incubation lid that can be applied to the culture plate to maintain growth conditions of 37°C and 5% CO₂. Migration is

very rapid being evident within the first 3 hours (as shown in Figure 30). It was observed that neuroblasts routinely changed directions while migrating and alternated between pauses and bursts of fast migration, which is consistent with previously described behaviors of neuroblasts migrating in slices (James *et al.*, 2011). Almost all cells emerge from the spheroid in chains and then continue to migrate as individual cells, often following the path of the neuroblast before.

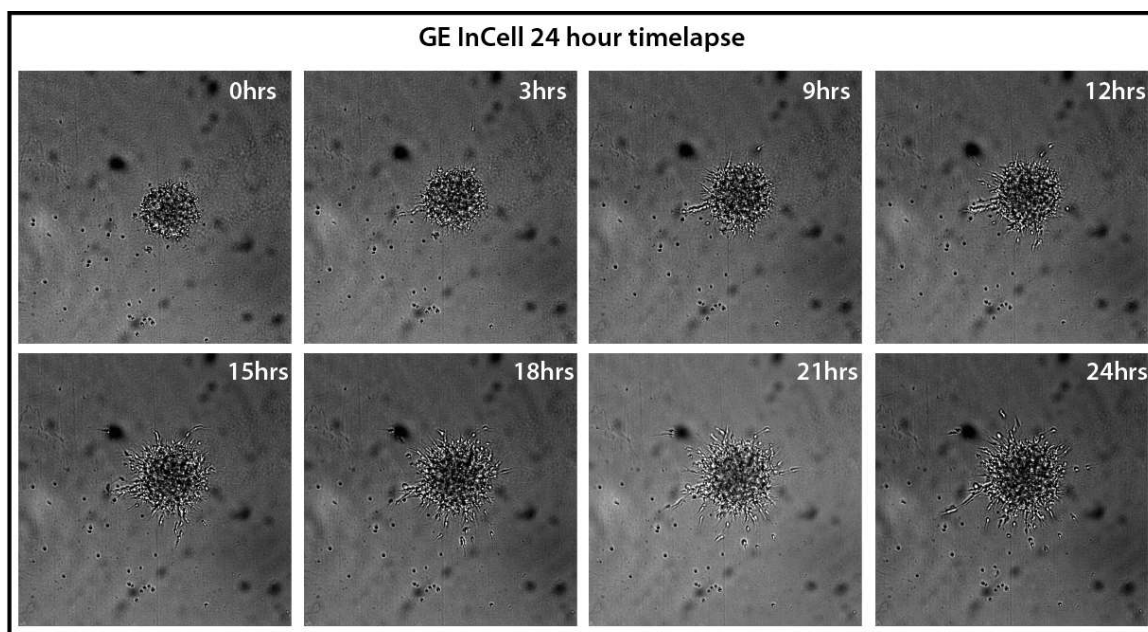


Figure 30. Time-lapse imaging of spheroid migration using the GE InCell. The InCell imaging platform took images over a 24 hour period from plating of the spheroid in Matrigel. Migration of neuroblasts out of the spheroid can be observed at 3 hours and continues throughout the duration of the time-lapse.

Single cell tracking may be an option for future timelapse experiments but as a means of rapid quantification of these images a relatively simple segmentation of the radiation area was quantified using the InCell analysis software. Thresholding enabled the segmentation of both the central spheroid and the halo of cells that has migrated out of the spheroid in chains. This method was not sensitive to intensity variations due to subtle differences in z plane. However, it is not possible to capture single cell migration using this type of segmentation. Ten spheroids were embedded and ten spheroids were left unembedded and the migration area was determined across a 23 hour time course. As evidenced in Figure 31 the area of the migration halo increases at a constant rate for the first 15 hours or so and then begins to plateau. This is consistent with existing evidence that the rate of neuroblast migration *in vitro* declines after the first 24 hours (Falenta *et al.*, 2013).

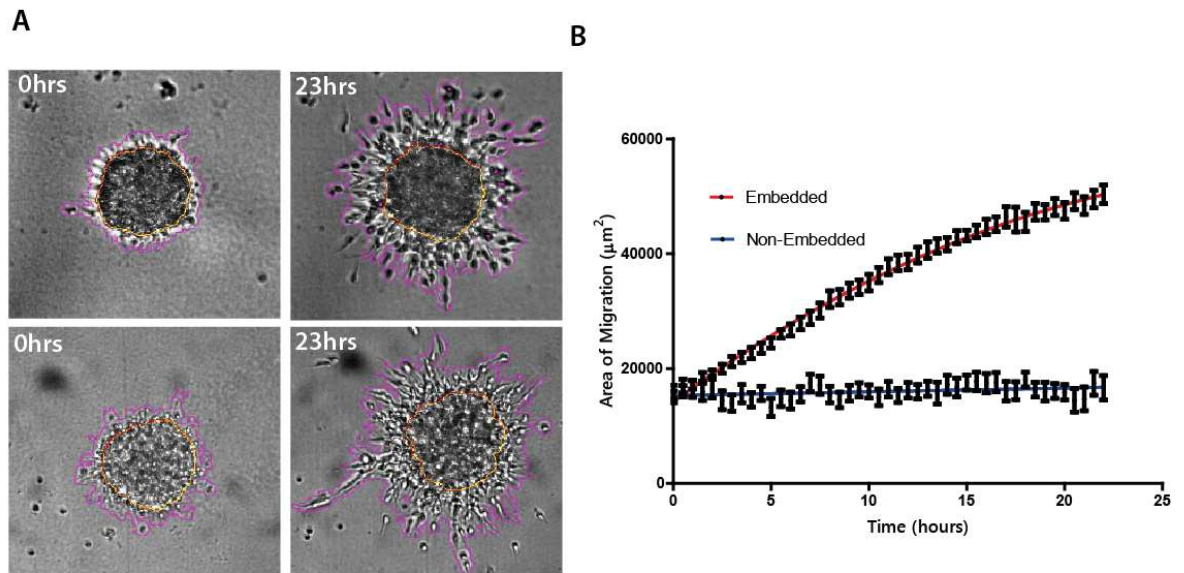


Figure 31: Example images of the segmentation of migration area around the spheroid at 0hrs and 23hrs. The graph shows how the area of the migration halo around 10 spheroids embedded in Matrigel and 10 unembedded spheroids changes over 24 hours. The two separate examples in the left-hand panel demonstrate how the segmentation method is insensitive to variations in illumination intensity.

While the InCell is sufficient for live cell analysis it does have its limitations. Firstly, it can only handle one plate at a time. Given that this system is very expensive to operate (currently £50 per hour) this means that it is not the most cost efficient solution. However, more importantly, the incubation chamber in the microscope is not perfect resulting in the health of the cells declining over the course of the experiment. To try and overcome this the EssenBioscience IncuCyte was trialled. This is a fluorescent microscope that can hold up to 6 culture plates and fits in a standard cell culture incubator. It is therefore potentially very well suited to tracking spheroid migration over longer time periods. However when this system was piloted the results did not support further use. Whilst the health of the cells was improved there were two challenges that limited its use, as evidenced in Figure 32. Firstly there is no option to do z-stacks so a lot of data regarding 3D migration dynamics is not captured. Secondly the software autofocus is reapplied at every timepoint, which often resulted in the focal plane skipping around, thus making reliable automation of quantification very challenging.

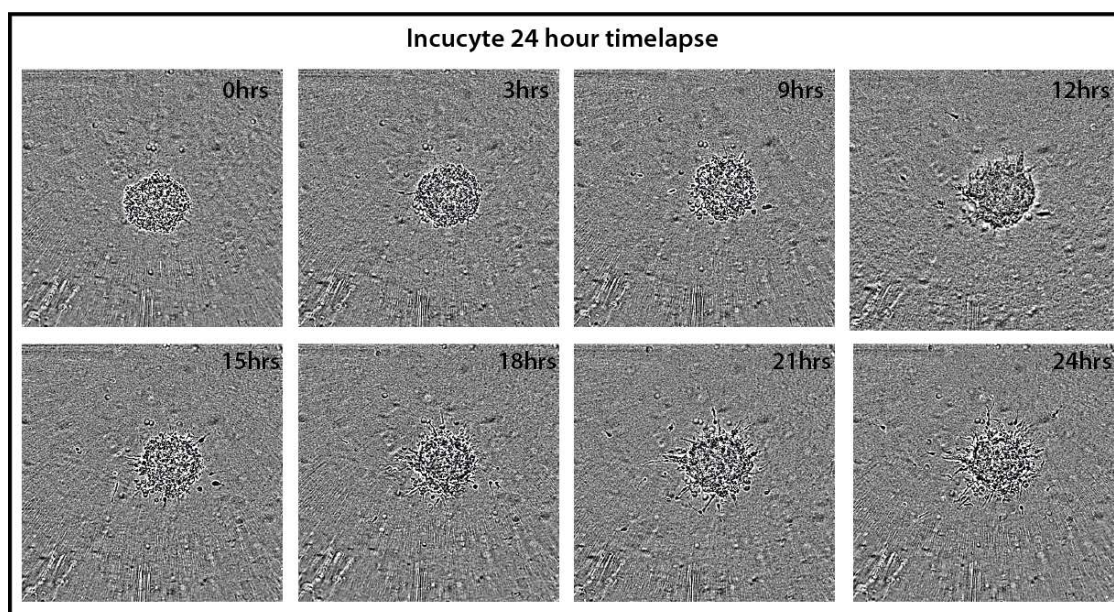


Figure 32. Time lapse imaging of RMS spheroids using the EssenBioscience IncuCyte over 24 hours. The images taken with this system do not remain in the same focal plane making automated image analysis difficult. The standard Incucyte does not allow z-stacks so is not ideal to track cell migration in 3D.

4.4.7 Development of automated image analysis toolset.

To speed up analysis, and remove the potential for human error, an automated toolset for quantifying the migration from the spheroids has been developed. This work was completed in collaboration with Dr Val Millar from the Target Discovery Institute.

Image analysis can be divided into three major steps: image correction, image segmentation, and feature extraction. We have the following inputs for quantification: 1) Brightfield (T=0), Brightfield (T=End), Hoechst (T=End) Propidium Iodide (T=End). Please see Figure 33 for an overview.

The first step was to smooth and enhance the signal in each of the images. Fortunately, there was not too much within plate exposure variations however there was a mottling in the background of the brightfield that had to be removed via a smoothing macro. The contrast was enhanced in the fluorescent channels to capture faint and out of focus cells. Thresholding was then applied to remove line aberrations resulting from the line scanning confocal. A 'Find Particles' macro was applied to the enhanced Hoechst channel to segment all single cells and to the PI channel segment all dead cells. To segment the halo of cells that have radiated out of the spheroid collectively as chains a 'Find edges' macro was applied to the smoothed brightfield image. The image is subsequently segmented and the largest object selected as the contiguous central spheroid and migration halo.

With the central spheroid, halo, and all dead and alive cells segmented it is now possible to extract features and quantify the migration. There are numerous different measurements that could be made based on the goal of the experiment and ultimately single cell tracking of the whole population of neuroblasts will be the target. Until then we have proposed the following which are easily calculated based on the segmentation of the static end points.

The area of the migration halo can be calculated by subtracting the area of the spheroid core from the entire contiguous spheroid area. The halo area is a good measure of the extent of collective or chain migration from the spheroid. However it will not capture single cell migration and, therefore, may underestimate total migration.

The number of single cells can easily be counted for either the entire image or within a given area. To quantify distance of migration it is possible to expand the area of the spheroid by set distances and quantify the number of cells that occupy that area. Repeating this several times, as seen in Figure 33, creates defined zones that can provide data on the frequency distribution of the distance migrated by the neuroblasts. The spacing of these bins can be set depending on the requirements of the experiment. However, it is worth considering how to plan to compare across the sample set. The ratio of the area of the halo to the number or area occupied by single cells may also be used as an indicator of conditions that modulate collective or chain migration.

The viability of the central spheroid is easily extracted by quantifying the intensity of PI within the segmented spheroid area. While this may not be an ideal measure of cytotoxicity it is a good gauge of the health of the spheroid and culture conditions that are definitely toxic can be screened out. The mean PI intensity for control spheroids is typically around 200au with a standard deviation of <50au. Thus, the threshold for toxicity is when spheroid PI intensity exceeds 500au. There are two error checking calculations based on the geometries of the spheroid segment. The first of these is to count the number of segments. Sometimes during spheroid formation two small spheroids may occasionally form due to debris, or when pipetting between wells it is also possible to transfer one spheroid from one well into another. In either of these cases, spheroid count would be more than one and highlight these wells for exclusion. The second error measure is the "Weighted Moment of Inertia". This value represents the torque required to rotate an object and is a good approximation for how round an object is, or in our case if there is non-round debris contaminating the well.

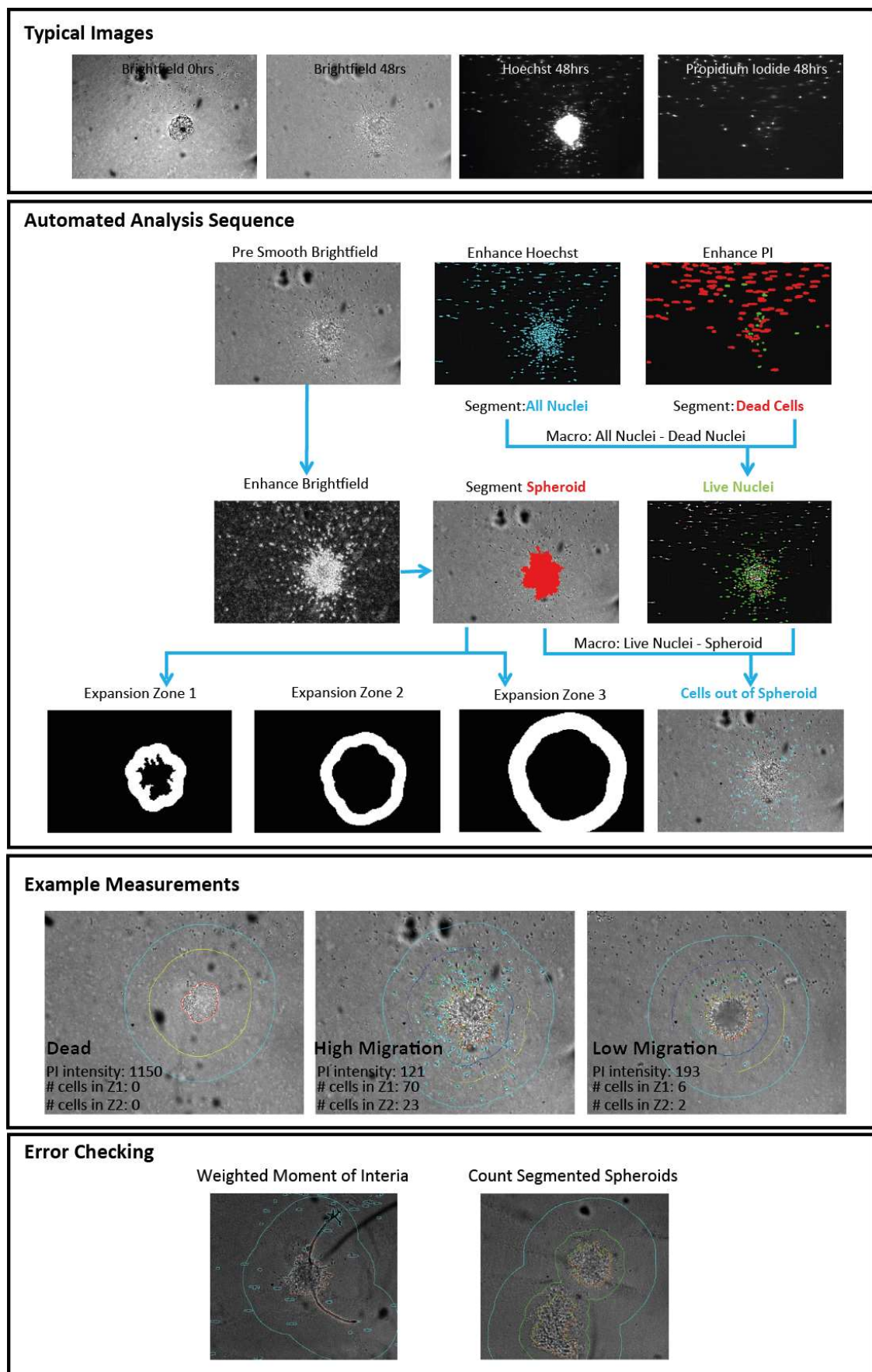


Figure 33 Workflow of automated spheroid image analysis. The automated analysis sequence describes the imaging sequence and the processing done by the analysis software. Neuroblast numbers within increasing radius zones from the spheroid are measured by the software to determine migration levels, and errors affecting the counts are checked automatically.

4.5 Discussion

4.5.1 A novel 3D phenotypic assay to study SVZ neuroblast migration

The results presented in this chapter detail the establishment of a novel *in vitro* phenotypic assay to study the migration of SVZ neuroblasts. An evolution of the hanging drop assay, 3D spheroids are generated from cells dissected from the mouse RMS in a robust and standardised way. The assay has been designed to be easy to follow and cost effective using ULA round bottomed plates. Each preparation of the assay can be conducted by a single experimenter within a 3-day period. Up to six 96 well plates, equating to 576 individual samples, can be tested per dissection of 10 mouse pups using only standard laboratory equipment and reagents.

Spheroids can be successfully formed with as few as 300 and as many as 40,000 cells. The size selected depends on the objective of the assay. The larger spheroids have more cells, which makes quantification and image analysis of migration at a population level easier. However, if tracking individual cells is desired, smaller spheroids may be preferable. Larger spheroids also have more significant molecular gradients that can affect the health and response to drug treatment. The size is also limited by the imaging capacity of the microscope. The best compromise for screening compounds was found to be 5,000 cells per well.

The spheroid assay is compatible with the vital dyes: CellTracker, Propidium Iodide, and Hoechst, and can therefore be used to study other cellular behaviours and mechanisms other than migration. Other colorimetric and chemiluminescence probes, such as MTT tetrazolium reduction metabolic assays can also be considered and, in principle, the more probes used, the more information can be gathered.

4.5.2 Challenges and Limitations

It is important to keep in mind the complexity of the *in vivo* environment dictating neuroblast migration compared with the reductionist approach of *in vitro* assays. *In vivo*, cell migration is dictated by a complex cellular and ECM architecture and a combination of short- as well as long-range guidance molecules. It is impossible to perfectly replicate the complex network of cells, structures and signalling molecules that cells interact with *in vivo*. The spheroids form from the

cellular constituents that make up the RMS and only have limited exposure to *ex vivo* culture conditions. This represents an improvement on neurosphere assays, but the niche architecture is lost. Indeed, although the neuroblasts radiating from the spheroids maintain the ability to migrate in a similar way to their *in vivo* counterparts, displaying chain migration and saltatory dynamics, they lack interactions with other RMS components such as the vasculature. This issue may be addressed in the future by either optimising the matrix and media culture conditions or by developing a fused spheroid coculture. It is conceivable that interactions between RMS spheroids and spheroids of different cellular origin, such as the striatum or cortex, could be studied. A similar approach was recently used to study the interaction of neural progenitors grown in cerebral organoids at different developmental stages (Bagley *et al.*, 2017; Xiang *et al.*, 2017). It is also important to consider that migration is a multistep process. The initiation/termination of motility, directionality, polarity, survival and proliferation can all affect the maximal migration area at the endpoint. Limiting the time series to one cell cycle can to some extent control for proliferation but the results from any screen will need to be further investigated to understand more precisely what underlies the phenotype. Combining the assay with additional live cell or chemotaxis assays should be considered.

Automated image analysis has many advantages. It can substantially cut down analysis time and permit quantification that is technically very difficult to do manually. It can also eliminate the inherent variability of manual scoring because computer measurements are not subject to external factors, such as human fatigue, illumination, or ambient noise. Nevertheless, automated evaluation is also prone to errors due to the inability of the algorithms to deal with non-ideal situations, such as inadequate sample preparation, heterogeneous samples, uneven colour patterns, and cells that appear merged, among others. This is particularly true for the RMS spheroid migration assay. Within the spheroid assays there is an unavoidable amount of cellular debris. This can be controlled for to some extent on small scale experiments but when handling large numbers of plates, the extra time required is detrimental. Simply screening out dead cells in post processing is not sufficient because there is the possibility that the manipulation being tested increases the likelihood of cell death during differentiation. Similarly, there is a high possibility that some cellular debris is alive. This is confounded by the use of different microscopes. For instance, the GE InCell is a line scanning confocal so when imaging in multiple z planes light that is out of

focus is captured as a horizontal band. Layering of these different bands can make it very challenging to precisely identify clustered cells. Widefield and point scanning confocal microscopes will have different but related limitations. In both of these cases migration would be scored incorrectly by an automated algorithm. Human experimenters however, find it much more easy to distinguish between sample and background noise. It is also important to consider the phenotype being tested and whether the automated scoring can detect it. The automated analysis toolset developed in this chapter can quantify multiple parameters and can be optimised to quantify more if needed. However, this provides an equally complex dataset that requires considered post analysis. For instance, when manually scoring it is easy to pick out single cases that look different and may be of interest to follow up. This could be cell morphology, motility (the number of cells that have migrated) and chain vs individual migration, among others. However, when screening for single metrics these may be overlooked. Care is therefore needed, and a combination of manual and automated analysis is recommended.

4.5.3 Future directions

The essential next step for the 3D RMS spheroid migration assay is to validate that it can be used to identify compounds or mechanisms that modulate neuroblast migration. Small molecular probes are the best initial candidates because they can be added to the culture media without considerable adaptation to the standard protocol. In addition, small molecule probes are less likely to be perturbed by the 3D culture than recombinant proteins. In the future it will also be sensible to optimise the genetic approaches for high throughput applications. The nucleofection method described is limited to small validation experiments due to the number of cells required and is comparatively expensive at £10 per condition. There are several alternatives that could be trialled, however the most sensible approach would be to use one of the latest arrayed CRISPR libraries, such as the Dharmacon Edit-R.

Drug discovery relies on high-throughput screening of thousands of chemical and biological entities in highly standardized assay conditions. Therefore, to enable RMS spheroids to be utilised in the discovery of novel neurodegenerative drugs, all variability needs to be minimised. Currently, the biggest source of variation is from the initial dissection. In addition to varying the starting cell population, imperfect dissection can result in increased cell death, and thus smaller,

less healthy spheroids with increased debris. One potential improvement could be to use transgenic mice where the RMS is labelled with a fluorescent protein, such as the DCX-GFP mouse. In fact, the early development of this assay was completed with one such mouse strain, the GAD65-GFP mouse, which made the dissection process far easier, reduced the preparation time, reduced cellular debris, and made imaging and quantification far easier. Returning to use a similar approach would improve the overall performance of the assay. Another consideration will be screening volume. The organization of rat and mouse RMS are remarkably similar and the extent of rat and mouse neuroblast migration *in vitro* is also comparable (Falenta *et al.*, 2013). However, the cell yield from mice pups is considerably less than rats ($2-3 \times 10^5$ cells/brain vs $1-1.5 \times 10^6$ cells/brain). Therefore, adapting to rat pups might represent a convenient neuroblast source to scale the spheroid assay.

The spheroid migration assay has been developed to study neuroblasts. However, there is no reason why it cannot be applied to study different primary cell types. The SVZ also gives rise to oligodendrocyte precursor cells (OPCs), which are another attractive therapeutic target in Multiple Sclerosis. O'Meara *et al.*, (2016) have recently published a protocol to rapidly isolate OPCs from the mouse brain, which may serve as a starting point. Another consideration that needs to be made is whether or not we continue to use primary mouse cells. With the continued debate around how translatable results from mouse experiments are to human biology there is mounting incentive to transition over to human derived cells. At the beginning of my thesis, this was not an option. However, during the course of my project there has been a greatly increased research interest in cerebral organoids, which now present an attractive alternative.

Chapter 5 Screen of kinase inhibitors on the migration of SVZ neuroblasts identifies MUSK and PIK3CB as novel modulators of migration.

5.1 Introduction

5.1.1 Phenotypic drug screening

There is great potential in using phenotypic screening techniques to study the mechanisms regulating neuroblast migration, but to date few successful studies have been completed. Two separate studies employed the same embryonic organotypic brain slice culture method, identifying the Src family kinase inhibitor PP2, the PKC inhibitors BIM1 and Ro318220, and 11 novel nonannotated compounds as disruptors of cortical radial migration (Zhou *et al.*, 2007; Jossin *et al.*, 2003). However, to my knowledge, the search for small molecules that stimulate neural progenitor migration is still an open challenge.

5.1.2 Kinases in neuronal migration

Protein kinases have a vast array of roles in cellular activity, with the human kinome constituting 2% of all genes (over 500 in total) (Manning *et al.*, 2002). It should come as no surprise that many have been strongly implicated in neuroblast migration. Several RTKs are expressed on neuroblasts and have been shown to modulate migration including the IGF1R, Eph receptors EphA4 and EphB1–3, PDGFR and VEGFR (Hurtado-Chong *et al.*, 2009; Kim *et al.*, 2009; Mani *et al.*, 2010; Sato *et al.*, 2016; Todd *et al.*, 2017). The EGFR, or ErbB1, is negatively correlated with neuroblast motility (Kim *et al.*, 2009). Conditional deletion of the related ErbB4 results in aberrant neuroblast chain organization, orientation and migration. The neurotrophin receptors TrkB (Ntrk2) and TrkC (Ntrk3) and ligands BDNF and NT3 are expressed in the VZ/SVZ. Trk inhibitor K252a disrupts axonogenesis and migration in *in vitro* slice cultures (Ip *et al.*, 2011). A large collection of intracellular kinases have also been shown to mediate neuronal migration by phosphorylating the key components of multiple pathways. These include Src, Fyn, PI3K, Cdk5, GSK3 β , MAPK-

upstream protein kinase (MUK), members of c-Jun N-terminal kinase family (JNK), and extracellular signal-regulated kinase (ERK) (Hirai *et al.*, 2002; Konno *et al.*, 2005; Kawauchi *et al.*, 2006; Simó *et al.*, 2007). Cyclin-Dependent Kinase 5 (Cdk5) modulates neuroblast migration through phosphorylation of cytoskeletal proteins. Cdk5 deletion is associated with impairments in chain formation, speed, directionality, and extension of leading processes of neuroblasts (Hirota *et al.*, 2007). The cell-polarity factors GSK3 β and PKC ζ are needed for centrosome reorientation (Lalli, 2014). Inhibition of the PI3K/Akt pathways is known to disrupt neuroblast migration (Kaneko *et al.*, 2017). Despite the roles of a number of protein kinases in migration being well studied, the mechanisms by which each protein kinase is involved in neuronal migration remains far from complete (Ohshima, 2014). Studies have also demonstrated that kinases are key mediators of cell migration and invasion processes in brain cancer, via regulation of mesenchymal genes and rearrangement of cytoskeletal components. Mesenchymal phenotypes, such as upregulated gene expression and increased migration, are regulated by kinases amongst other genes. Therapeutic strategies targeting kinases have been a major focus of drugs for tumorigenesis and metastasis.

5.1.3 The GlaxoSmithKline Published Kinase Inhibitor Sets (PKIS)

The Published Kinase Inhibitor Set (PKIS) is an annotated set of approximately 900 small molecule kinase inhibitors (when PKIS1 and PKIS 2 are combined), developed due to the abundance of uncharacterised kinases (Drewry, Willson and Zuercher, 2014). Developed by GlaxoSmithKline it has been made widely available in the hopes of academic collaborators generating novel targets for disease areas. It is also used as a starting point for medicinal chemists to develop novel probes for orphan kinases.

The PKIS library has been used to investigate the functions of kinases in various cell types and diseases. Hildebrand *et al.* (2014) identified an anti-necroptosis compound that inhibited the pseudokinase MLKL. The library has also been used to study the GRK family kinases, which are implicated in diseases including heart disease and Parkinson's disease, although little is known about their functions. Homan *et al.* (2015) generated ligand bound crystal structures for these kinases using PKIS as a starting point for their medicinal chemistry work. A screen for potency

against the protozoan pathogen that causes sleeping sickness identified 53 PKIS compounds that could have potential for repurposing in this disease (Amata *et al.*, 2016).

PKIS was used in a neuroregeneration high content screen to determine agents that could promote neurite outgrowth in primary neuronal cultures, as a model for axon regrowth after injury to the brain. Al-Ali *et al.* (2015) used the kinase inhibitor annotation together with phenotypic readouts from a drug screen measuring the effect on neurite outgrowth *in vitro* to develop a machine learning algorithm. This machine learning approach produced hypotheses as to how the kinase inhibition profiles of compounds would regulate neurite outgrowth in the neuronal cultures, which could be confirmed by the HCS assay. Kinases that promoted or repressed neurite outgrowth were identified, and a top hit for increasing neurite outgrowth, which showed polypharmacology, making it a strong potential therapeutic compound, was tested *in vivo* in a mouse model of spinal cord injury where the compound promoted growth of corticospinal axons. This study demonstrates the importance of developing physiologically relevant screens for neuroregeneration so that promising compounds *in vitro* can be reliably translated to *in vivo* trials.

5.2 Aims

The aim of this chapter is to use the newly developed *in vitro* SVZ neuroblast spheroid assay, described in Chapter 4, to identify compounds that increase (or decrease) migration of neuroblasts. Compounds that increase migration of neuroblasts could have therapeutic potential in stroke, traumatic brain injury, or neurodegenerative disorders; and compounds that decrease migration may be useful as anti-invasive treatments for brain cancer.

5.3 Methods

5.3.1 Design of screen of kinase inhibitors on migration from 3D RMS spheroids

PKIS1 and PKIS2 are openly available in screening quantities from the Structural Genomics Consortium, based at the University of Oxford. Both libraries can be requested at www.sgc-unc.org and chemical structures and other pharmacologic activity for the compounds can be found at <https://www.ebi.ac.uk/chembl/db/extra/PKIS/compounds.html>. A 1mM solution in DMSO, dispensed in 96 plates is provided. A material transfer agreement was created to ensure that the screening results are made publicly available, with open access being a requirement of the PKIS library.

The PKIS1 is a collection of 367 compounds that are split across 5x96 well plates and the PKIS2 is a collection of 645 compounds that are split across 7x96 well plates. For both the columns 1 and 12 are left blank to be used for controls. It was decided to split the experiment into 4 separate rounds where each round would screen 3 plates in duplicate. A litter of 10x P4 CD1 pups yields ~250,000 cells so the number of cells per spheroid was adjusted down to 4000 to provide sufficient room for dissection error.

5.3.2 Drug treatment of spheroids

The screen was split across four separate experiments with three 96 well plates of compounds tested in duplicate each round. Six 96 well plates of 3D RMS spheroids were prepared overnight as described in Chapter 4. Each spheroid contained 4000 cells and was suspended in 80µl of Neurobasal. The compound libraries were diluted to a 5X (50µM) working concentration in Neurobasal. Columns 1 and 2 were used as DMSO controls. 20µl of working concentration compound was added to each well of the spheroid plates and they were returned to the incubator for 2 hours to incubate. To embed the spheroids in Matrigel the culture plates were cooled on an icepack for ~5 minutes and then 25µl of ice cold 50% Matrigel was carefully added down the side of the well. The plates were then sealed with a gas permeable, optically clear seal (4titude) and returned to the incubator to set for 2 hours. Plates were then imaged at 2 hours (T=0) and 24 hours on brightfield only. 2 hours prior to the 48 hour endpoint the Live/Dead assay was added to each well and the plates were imaged on brightfield, UV and 488.

5.3.3 SVZ neurosphere stem cell spheroids

SVZ neural stem cells were dissected and cultured as neurospheres as per Chapter 2. Tertiary neurospheres were pelleted via centrifugation at 300g for 5 minutes, dissociated in Accutase for 10 minutes and counted using a haemocytometer, as per a normal passage. The cells were resuspended in NB-A+ supplemented with 20ng/ml EGF and 20ng/ml FGF2 to dilution of 100,000 cells per ml and then 100µl of cell suspension added to each well of a 96 well ultra low adhesion round bottom plate (Greiner 650970). Spheroids were left to incubate overnight at 37°C and then the following day carefully collected and washed 3 times in NB-A+ to remove the growth factors. The stem cell spheroids were then carefully picked and transferred back into a new 96 well ultra low adhesion round bottom plate. The remainder of the assay continued as per the RMS spheroid migration assay described in Chapter 4.

5.3.4 Quantitative Polymerase Chain Reaction

5.3.4.1 RNA extraction and quantification

Total RNA was obtained from the sample with RNeasy Mini Kit (Qiagen) following the instructions. The concentration and purity of extracted nucleic acids was measured using the NanoDrop™ 1000 spectrophotometer (ThermoFisher Scientific). The 260/280 and 260/230 absorbance ratios were used to assess the purity of RNA.

5.3.4.2 DNase treatment of RNA

To remove residual genomic DNA contamination, all RNA samples were treated with TURBO DNA-free™ (Invitrogen AM1907). 2µg of extracted RNA was combined with 1µl of TURBO DNase, 2.5µl of TURBO DNase Buffer and made up to 25µl with nuclease-free H₂O. The sample was incubated for 50 min at 37°C. The DNase was subsequently inactivated by incubation with 6µl of DNase Inactivation Reagent at RT for 5 min, vortexing every 30 seconds. The sample was then centrifuged at 13,000 RPM for 90 seconds and the supernatant was transferred to a new eppendorf tube and stored at -80°C.

5.3.4.3 Reverse transcription

Complimentary DNA (cDNA) was synthesised using SuperScript® III Reverse Transcriptase. 1µl of 250ng/µl random hexamers (N8080127) and 1µl 10mM dNTP mix (ThermoFisher R0191) was added to 1µg of DNase-treated RNA, and made up to 13µl with nuclease-free H₂O (Sigma Aldrich,

Gillingham, UK). The mix was incubated at 65°C for 5 min to denature RNA secondary structure and then incubated on ice for 1 minute. The mix was made up to 20µl with 4µl 5x First Strand Buffer (Life Technologies), 5mM Dithithretiol (18080-044), 40 units RNaseOUT™ (10777), 1µl (200 units) SuperScript® III Reverse Transcriptase (Invitrogen 18080-044) and 3µl of nuclease-free H₂O. Samples were incubated in a PCR machine on an RT protocol: 25°C for 5 min, 55°C for 1 hour (optimal temperature for Superscript® III), and 70°C for 15 min (termination of the reaction). Samples were diluted in 100µl nuclease-free H₂O for qPCR.

5.3.4.4 Primer design

PCR primer pairs were designed to amplify an amplicon of approximately 100 base pairs. DNA sequences for genes of interest were obtained from the University of California, Santa Cruz, genome browser (<http://genome.ucsc.edu>) and primers designed using Integrated DNA Technology (IDT) software (<http://www.idtdna.com/primerquest/home/index>). GC content was kept between 40% and 60% and primers were designed with an annealing temperature between 55°C and 65°C with only 1°C between the pair. They were designed to have minimal self-complementarity and no complementarity to the other primer and were tested for single-peak melt curves using uMelt software (<https://www.dna.utah.edu/umelt/umelt.html>). RT-PCR primers were designed to span large exon boundaries to prevent the amplification of any residual genomic DNA. GAPDH primer pair used to test for genomic DNA contamination did not span exon-boundaries.

Oligonucleotides were synthesised by IDT. They were re-suspended in nuclease-free H₂O to a stock concentration of 100µM and used at a working concentration of 2µM.

Table 8 Table of primers used confirmation of shRNA knockdown

Gene	Forward Sequence	Reverse Sequence	Source
PLK1	CTTCGCCAAATGCTTCGAGAT	TAGGCTGCGGTGAATTGAGAT	Life Technologies
RIOK2	TAAGCTGTTCAACAATCCCTCC	GCTGCTTGGTAAACACATTGG	Life Technologies
ABL1	CAGCAGCCTGGAAAAGTTCTT	CCCTGCCCCCTTTGATAAAATGC	Life Technologies
HUNK	GTGCTGACGGGAGAAAAGGTA	GGGTGTGCGATCATCTGCTG	Life Technologies
MERTK	CCTAACCGTACCTGGTCTGAC	GGGAGGGGATTACTTTGATGTTG	Life Technologies
MUSK	TACAGAGGGGAGGTGTGTGAT	TCCCGGTAGGAGGTGTTGAA	Life Technologies
NEK9	TACGAGCGACACTGCGATTCC	ACGCGGATGGGGATGTAGT	Life Technologies
PI3KCB	CTATGGCAGACAACCTTGACAT	CTTCCCGAGGTACTTCCAACCT	Life Technologies

5.3.4.5 RT-PCR protocol

The reaction consisted of 33ng template nucleic acid (4µl), 10µl 2x SYBR® Green PCR Master Mix, 0.2 µM primer mix (3µl), and nuclease-free H₂O to make a total reaction volume of 20µl. All PCR reactions were carried out with one negative control for each primer pair, where H₂O was used as the template. RT-PCR reactions were carried out on a Chromo4™ Real-Time PCR detector (Bio-Rad, Bath, UK) using the following parameters:

	45 cycles		
Initial denaturation	Denaturation	Annealing	Extension
95°C for 15 min	95°C for 30 seconds	60°C for 30 seconds	72°C for 30 seconds

Fluorescence was recorded at the end of each cycle. A melt curve was carried out from 60°C-95°C with a 10 second hold and a plate read at every 1°C increment. Each reaction was carried out with a technical replicate and with 3 biological replicates.

Relative transcript quantification used the cycle threshold (CT) values for the $\Delta\Delta CT$ method to determine relative expression of the target gene to confirm knockdown. The expression of the gene of interest was calculated by normalising against the housekeeper gene, and two-tailed, independent t-tests were performed in Prism 6 (GraphPad) to test for level of knockdown of the gene, with significance determined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

5.3.5 Live/Dead staining protocol

To enable analysis of cell viability at the end of the assay I used a terminal stain with Propidium Iodide (PI; Invitrogen P3566) and Hoechst (ThermoFisher 62249). A concentrated stock of Live/Dead reagent was prepared by adding 5µg/ml PI solution and 10µg/ml Hoechst to NB-A+. Two hours prior to the final timepoint of the assay the plates were removed from the incubator, seal carefully removed and 10µl of Live/Dead reagent was added to each well. The plate was returned to the incubator for the remaining time period and assayed as normal. The Hoechst can be imaged using the standard DAPI filter set: 361/497, and PI can be imaged using the standard Texas Red filter set: 535nm/617nm.

5.3.6 Nucleofection

RMS cells were nucleofected as per Chapter 4. The following shRNA plasmids were selected from the Dharmacon GIPZ Lentiviral Mouse shRNA Library (RMM5829):

Table 9 shRNA constructs used for kinase gene knockdown

Reference	Gene Target	Vector	Sense Sequence
Abl1#1	Abl1	pGIPZ	CTCTTATAAATGACATGTA
Abl1#2	Abl1	pGIPZ	GAACCACCATTCTACATAA
Hunk#1	Hunk	pGIPZ	CAAAGAGATCTTTCTTTAT
Mertk#1	Mertk	pGIPZ	CTGTTATATTCCCGATTAA
Mertk#2	Mertk	pGIPZ	CGGAACATAAAAATGTCAA
MuSK#1	MuSK	pGIPZ	GGAGCTTAAGGATCCATAA
MuSK#2	MuSK	pGIPZ	CCACTCCTTCTGAATCCTA
Nek9#1	Nek9	pGIPZ	TCCAAGACACAAGATGTCT
Nek9#2	Nek9	pGIPZ	AGATGTTTACCCAACTACA
Pik3cb#1	Pik3cb	pGIPZ	CTCACATCGGTCAAAGATA
Pik3cb#2	Pik3cb	pGIPZ	CTGGAGAAGCTTGAAGATA

5.3.7 Imaging

Imaging was primarily completed using the GE InCell 6000 High Content Imager. This system comprises both brightfield and a line scanning laser confocal microscope making it ideal for quick 3D cell imaging, albeit at a slightly poorer resolution than standalone point scanning confocal microscopes. A protocol was established on the InCell software that imaged the centre of each well of the Greiner ULA plate. The starting plane was set by the microscope's in built 'autofind' function and 9 Z slices were imaged at 25µm per well, which amounted to a total section of 200µm. Prior to starting the imaging experiment a trial scan was completed to check the position of the spheroid in each image. Should any spheroid be dramatically off-centre or shifted in the z-plane the acquisition field was manually set for that well. All images were taken with the 10X 0.45NA objective. The InCell has four excitation filters (UV, Blue, Green & Red) and four capture filters (DAPI, GFP, RFP and brightfield). All scans included a brightfield image using filters: Red/brightfield and exposure 0.2s. When fluorescence was used the exposure was set based on the individual signal level of that experiment. To scan each plate it took around 15 minutes on brightfield only and around 25 minutes when using fluorescence.

5.3.8 Image analysis

To manually quantify the spheroid migration the individual brightfield image stacks were loaded into ImageJ and a maximum intensity projection created. Unlike explants and hanging drop aggregates the migration from each spheroid is relatively symmetrical. This property was exploited to provide a quick and simple quantification method. An ellipse was drawn around the spheroid such that it satisfied the following criteria: 1) it was centred on the spheroid, 2) it cut through at least 6 neuroblast deemed to have migrated from the spheroid, 3) it was round (Circularity > 0.8). When there are a few single cells that had migrated far from the spheroid individually these were ignored so not to skew the analysis. The area was then calculated and saved as a “Region of Interest”. In some instances, the “Find Edges” function was applied to assist in visualising individual cells.

To accurately determine the effect of each compound whilst accounting for plate-to-plate variability a ‘Z score’ was calculated. This method of creating a Z score is based on the finding that most drugs are inactive, and therefore can serve as a control. In the “Z score” method, the mean score of all of the compounds on a plate are subtracted from the raw value of the compound of interest, and this difference is divided by the standard deviation of all of the values (Malo *et al.*, 2006).

5.4 Results

5.4.1 Screen of the GSK Published Kinase Inhibitor Sets 1 and 2 identifies compounds that modulate neuroblast migration.

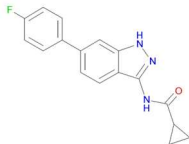
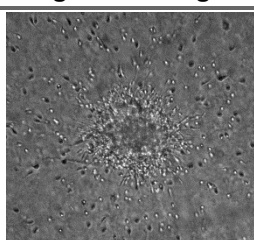
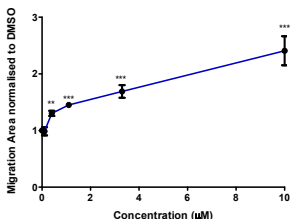
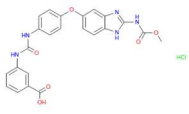
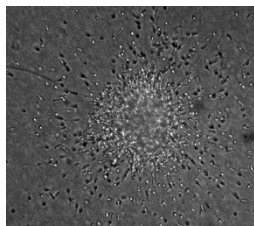
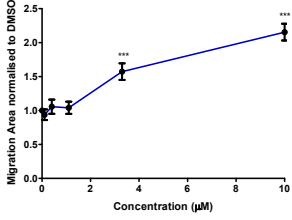
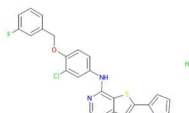
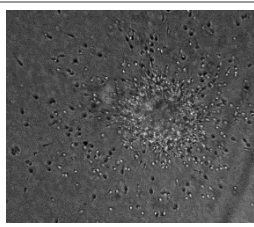
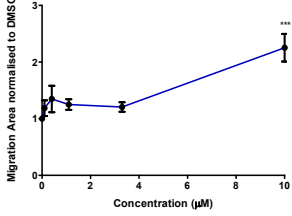
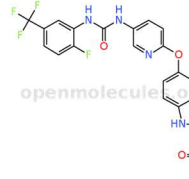
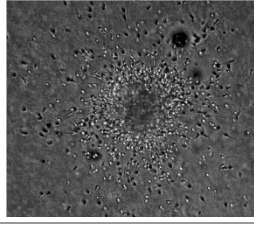
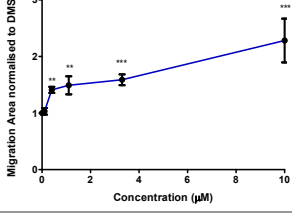
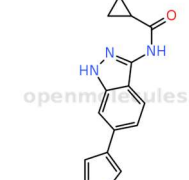
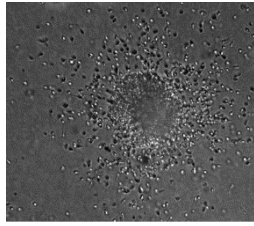
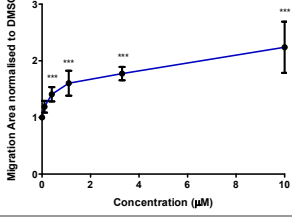
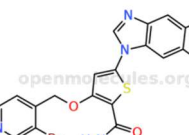
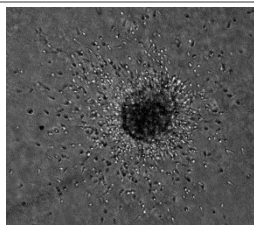
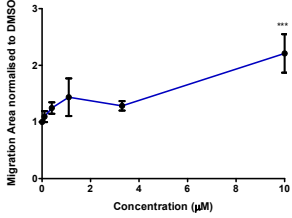
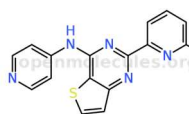
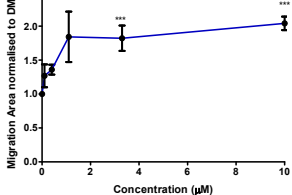
Driven by the need to identify new treatment options for brain regeneration the previously described spheroid assay was used to screen the small molecule kinase inhibitor libraries PKIS1 and PKIS2 for effects on the migration of SVZ neuroblasts. The screen was completed over four separate experiments with each compound being tested in duplicate at 10µM. Migration was quantified manually at the 24-hour time point for each sample. Despite plating the same starting number of cells each time, the size of the spheroid generated and thus migration area was not consistent between rounds of the screen, likely down to increased cell death. So each week was analysed separately, normalised against the DMSO controls and pooled at the end of the screen. Cut offs for high and low migration were introduced by comparing migration levels to those of

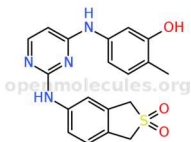
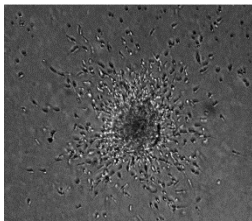
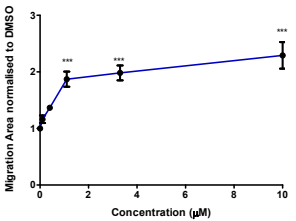
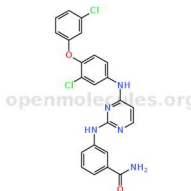
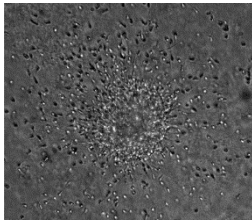
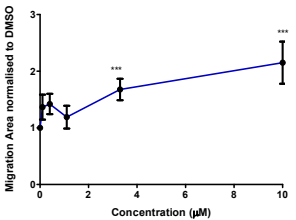
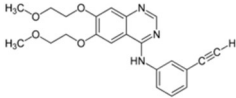
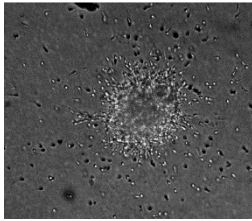
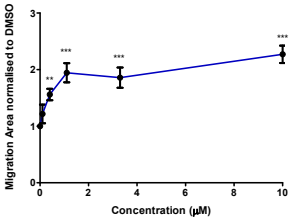
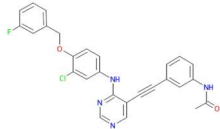
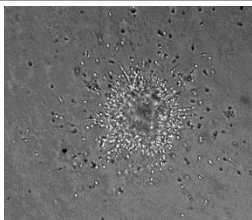
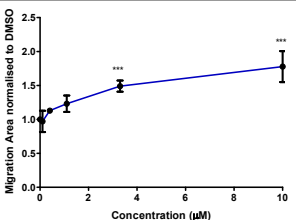
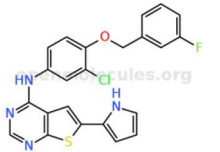
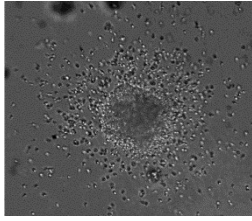
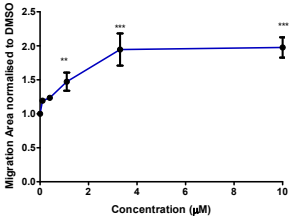
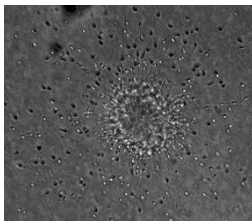
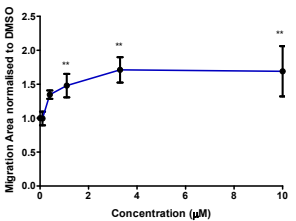
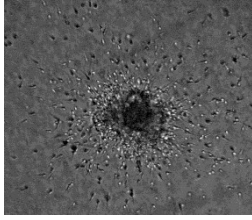
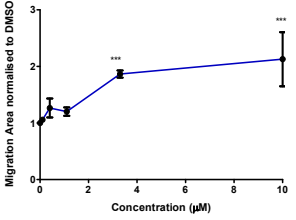
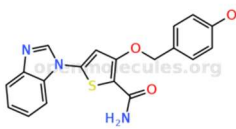
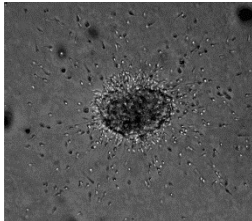
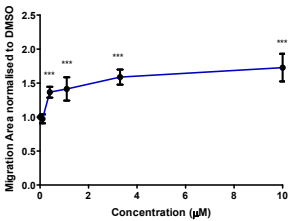
control DMSO treatment. To be categorised as a 'high migrating pool' compounds needed to produce a migration area at least 4 standard deviations away from the mean of the controls. This yielded 24 compounds with migration areas in the screen ranging from 1.7x to 3.0x that of controls. To be considered as a 'low migration compound' the migration area again had to be at least 4 standard deviations away from the mean of the controls and also not toxic. This yielded 36 compounds with migration areas ranging from 0.17x to 0.35x that of controls. In total 181 compounds were deemed toxic.

The compounds selected as putative modulators of migrations were then triaged into a dose response confirmation experiment. Each compound was tested in triplicate at concentrations ranging 0 μ M up to 10 μ M, and dose response curves were made to identify the concentration that produces the greatest migration phenotype. Table 10 shows the 21 compounds out of the 24 tested that significantly increased migration at the maximum 10 μ M concentration, representative images and, where possible, the compound structure. The profile of the dose response curves varies across the hit compounds with some showing significant increases in migration at the lower concentrations (GW607049C, GW459135A, GSK2336394A, GW579362A and GSK1024304A), while others showed little response apart from the highest concentration (GW693881A and GW854278X). The initial screen was performed blind: the primary kinase targets of the small molecule inhibitor hits were unknown at the time of treatments and active compounds were selected solely based on migration area. These compounds represent several different chemical classes and were originally published as inhibitors of a range of kinase targets. The intended targets for which the compounds were designed are also listed in the table 10. EGFR was the most common intended target of the pro-migratory compounds (5/21). This may be because a large number of compounds in the PKIS were designed to target EGFR, but equally this may still be an interesting target. The VEGFR2 was the next most common intended target (4/21) followed by Polo Like Kinase 1 (PLK1) from the compounds that increased migration.

Table 11 shows the compounds that significantly reduced the migration area in comparison to DMSO. All 36 compounds tested significantly reduced neuroblast migration at the highest concentration of 10 μ M. Of these, p38 α was the most common single target (10/36), followed by GSK3 (6) and Akt (4). Several compounds (7/36) have unspecified molecular targets.

Table 10. Kinase inhibitors that increased migration of neuroblasts from 3D spheroids. Compounds are listed together with their target (if known), chemical structure, representative image of migration at 24hrs, and the extent of migration above the DMSO control for 5 different concentrations of the compound (N=3). Un-paired two-way t-test completed for each data point against DMSO with *** p<0.001, ** p<0.01 and * p<0.05.

Compound	Chemical Structure	Migration Image	Results												
SB-735467 Intended Target: GSK3b			 <table><caption>Approximate data for SB-735467</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.2</td></tr><tr><td>2</td><td>1.4</td></tr><tr><td>4</td><td>1.8</td></tr><tr><td>10</td><td>2.4</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.2	2	1.4	4	1.8	10	2.4
Concentration (μM)	Migration Area normalised to DMSO														
0	1.0														
1	1.2														
2	1.4														
4	1.8														
10	2.4														
GW701427A Intended Target: TIE2/VEGFR2			 <table><caption>Approximate data for GW701427A</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.0</td></tr><tr><td>2</td><td>1.1</td></tr><tr><td>4</td><td>1.6</td></tr><tr><td>10</td><td>2.2</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.0	2	1.1	4	1.6	10	2.2
Concentration (μM)	Migration Area normalised to DMSO														
0	1.0														
1	1.0														
2	1.1														
4	1.6														
10	2.2														
GW693881A Intended Target: EGFR/ERBB2			 <table><caption>Approximate data for GW693881A</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.2</td></tr><tr><td>2</td><td>1.2</td></tr><tr><td>4</td><td>1.3</td></tr><tr><td>10</td><td>2.3</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.2	2	1.2	4	1.3	10	2.3
Concentration (μM)	Migration Area normalised to DMSO														
0	1.0														
1	1.2														
2	1.2														
4	1.3														
10	2.3														
GW681170A Intended Target: TIE2/VEGFR			 <table><caption>Approximate data for GW681170A</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.2</td></tr><tr><td>2</td><td>1.3</td></tr><tr><td>4</td><td>1.7</td></tr><tr><td>10</td><td>2.3</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.2	2	1.3	4	1.7	10	2.3
Concentration (μM)	Migration Area normalised to DMSO														
0	1.0														
1	1.2														
2	1.3														
4	1.7														
10	2.3														
SB-732932 Intended Target: GSK3a/GSK3b			 <table><caption>Approximate data for SB-732932</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.2</td></tr><tr><td>2</td><td>1.4</td></tr><tr><td>4</td><td>1.8</td></tr><tr><td>10</td><td>2.3</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.2	2	1.4	4	1.8	10	2.3
Concentration (μM)	Migration Area normalised to DMSO														
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1	1.2														
2	1.4														
4	1.8														
10	2.3														
GW854278X Intended Target: PLK1/PLK2			 <table><caption>Approximate data for GW854278X</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.3</td></tr><tr><td>2</td><td>1.3</td></tr><tr><td>4</td><td>1.4</td></tr><tr><td>10</td><td>2.2</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.3	2	1.3	4	1.4	10	2.2
Concentration (μM)	Migration Area normalised to DMSO														
0	1.0														
1	1.3														
2	1.3														
4	1.4														
10	2.2														
GW869641X Intended Target: ALK5			 <table><caption>Approximate data for GW869641X</caption><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.3</td></tr><tr><td>2</td><td>1.8</td></tr><tr><td>4</td><td>1.8</td></tr><tr><td>10</td><td>2.1</td></tr></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	1	1.3	2	1.8	4	1.8	10	2.1
Concentration (μM)	Migration Area normalised to DMSO														
0	1.0														
1	1.3														
2	1.8														
4	1.8														
10	2.1														

GW579362A Intended Target: Unknown			
GW659008A Intended Target: Unknown			
GW459135A Intended Target: EGFR/ERBB2			
GW703087X Intended Target: EGFR			
GW707818B Intended Target: EGFR/ERBB2			
GSK336313A Intended Target: Unknown			
GSK346294A Intended Target: PLK1/PLK2			
GSK1024304A Intended Target: PLK1/PLK2			

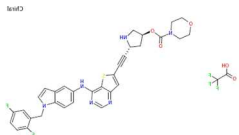
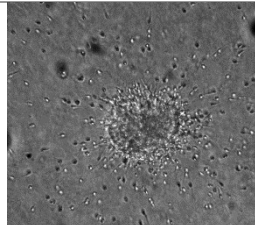
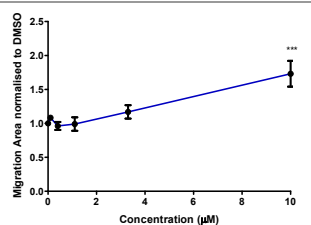
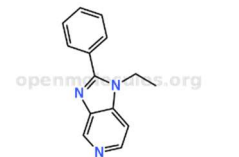
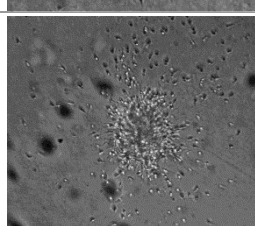
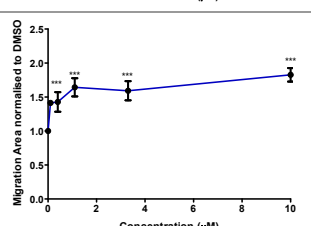
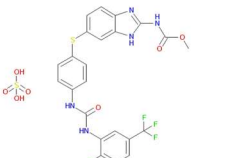
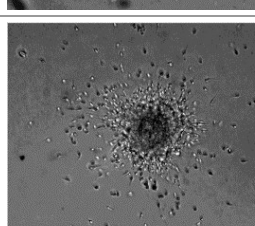
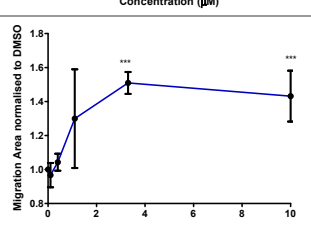
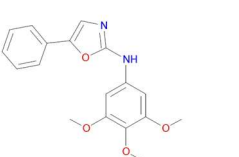
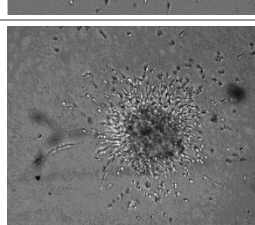
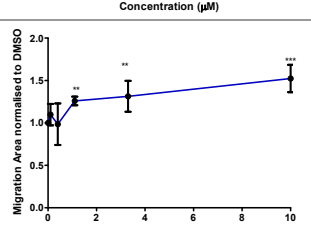
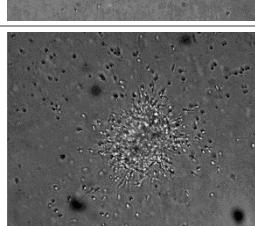
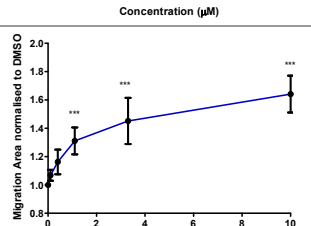
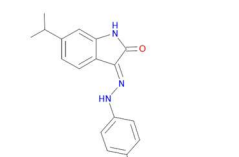
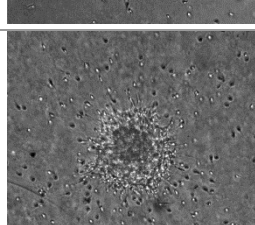
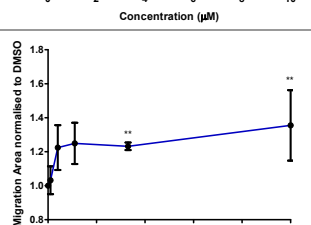
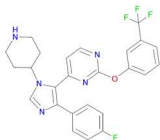
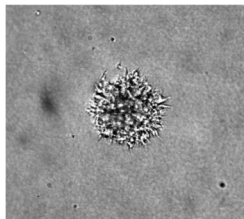
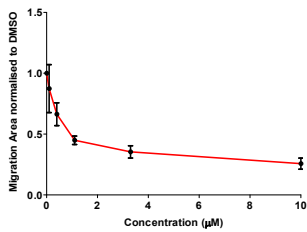
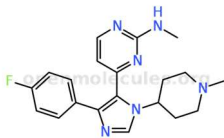
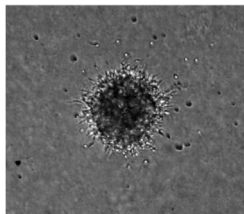
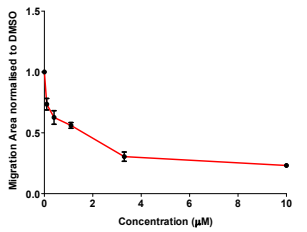
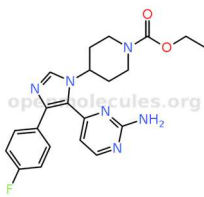
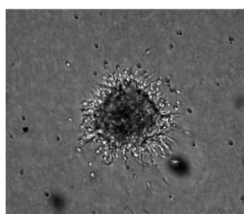
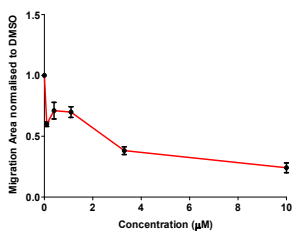
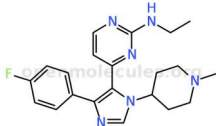
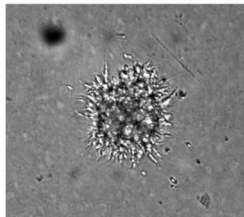
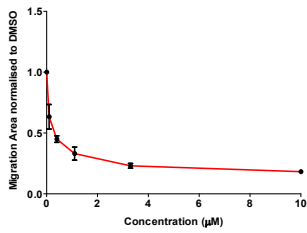
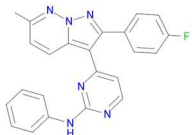
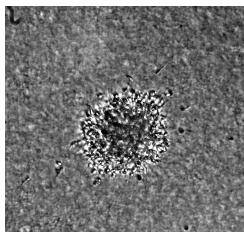
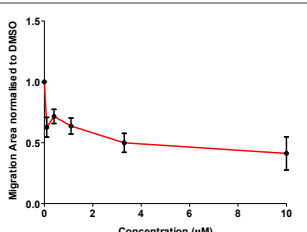
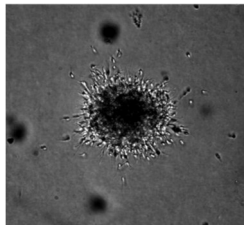
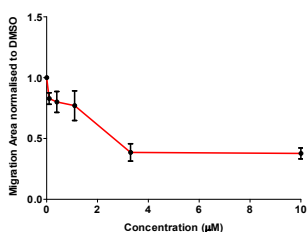
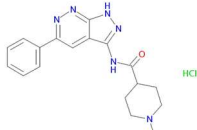
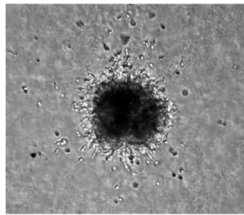
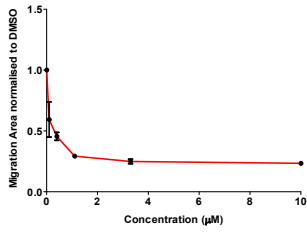
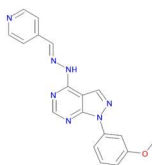
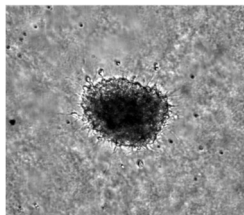
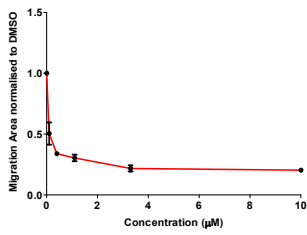
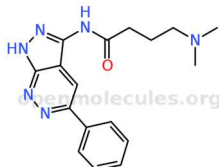
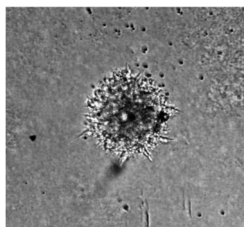
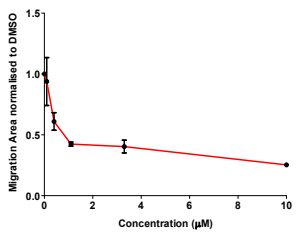
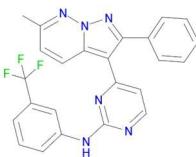
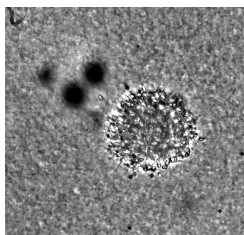
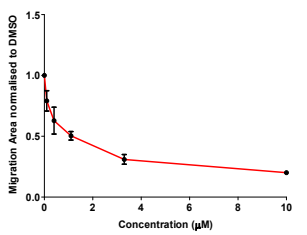
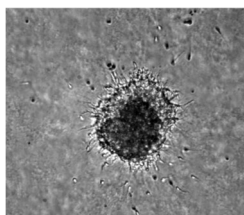
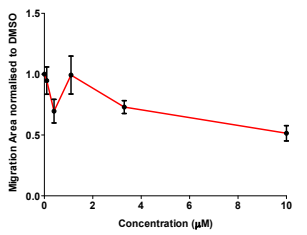
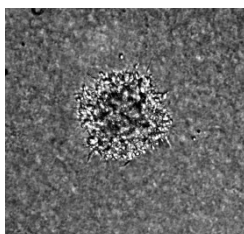
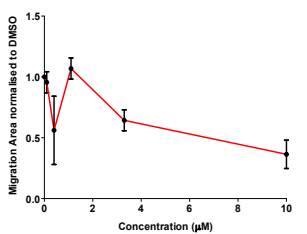
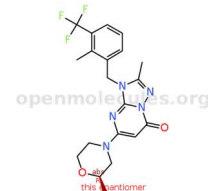
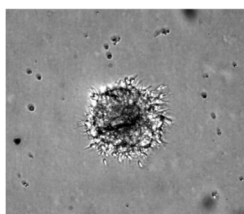
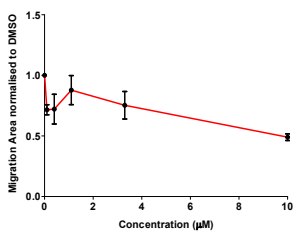
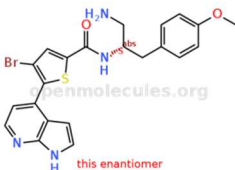
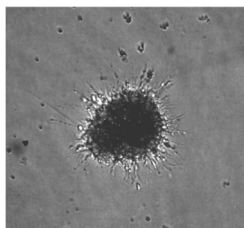
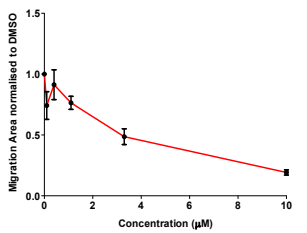
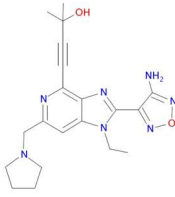
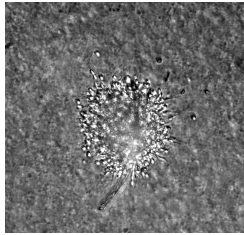
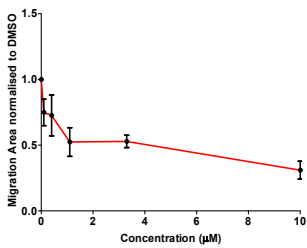
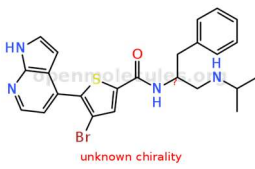
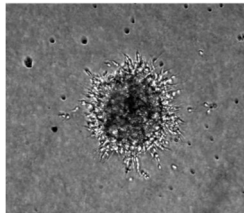
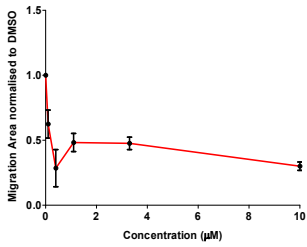
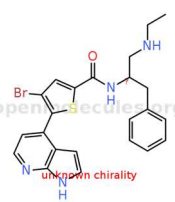
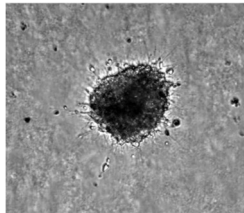
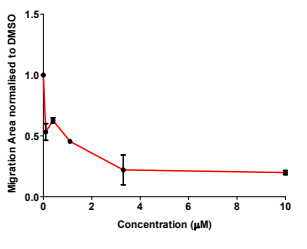
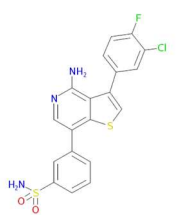
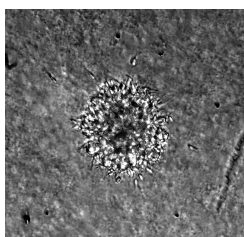
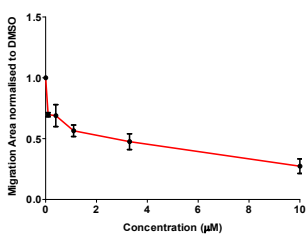
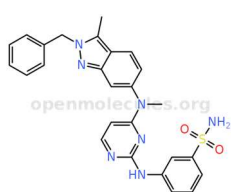
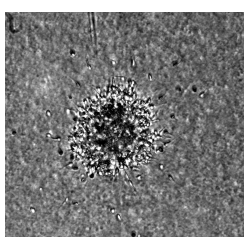
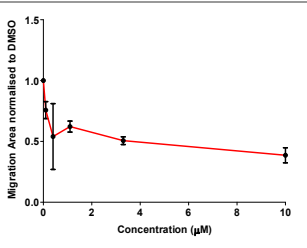
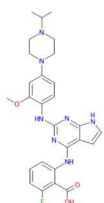
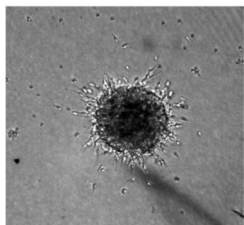
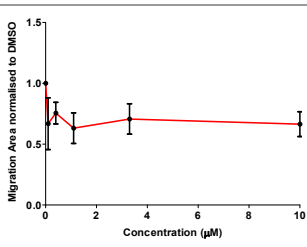
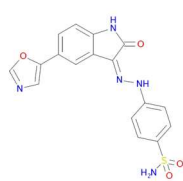
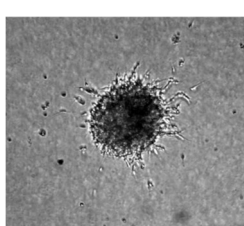
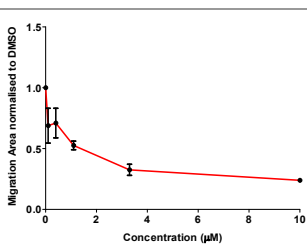
GSK259178A Intended Target: EGFR/ERBB2			 <table><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.0</td></tr><tr><td>2</td><td>1.0</td></tr><tr><td>4</td><td>1.1</td></tr><tr><td>10</td><td>1.7</td></tr></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	1	1.0	2	1.0	4	1.1	10	1.7
Concentration (µM)	Migration Area normalised to DMSO														
0	1.0														
1	1.0														
2	1.0														
4	1.1														
10	1.7														
SB-733416 Intended Target: MSK-1			 <table><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.4</td></tr><tr><td>2</td><td>1.5</td></tr><tr><td>4</td><td>1.5</td></tr><tr><td>10</td><td>1.8</td></tr></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	1	1.4	2	1.5	4	1.5	10	1.8
Concentration (µM)	Migration Area normalised to DMSO														
0	1.0														
1	1.4														
2	1.5														
4	1.5														
10	1.8														
GW607049C Intended Target: TIE2/VEGFR2			 <table><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.3</td></tr><tr><td>2</td><td>1.5</td></tr><tr><td>4</td><td>1.5</td></tr><tr><td>10</td><td>1.4</td></tr></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	1	1.3	2	1.5	4	1.5	10	1.4
Concentration (µM)	Migration Area normalised to DMSO														
0	1.0														
1	1.3														
2	1.5														
4	1.5														
10	1.4														
GW627512B Intended Target: VEGFR2			 <table><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.1</td></tr><tr><td>2</td><td>1.2</td></tr><tr><td>4</td><td>1.3</td></tr><tr><td>10</td><td>1.5</td></tr></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	1	1.1	2	1.2	4	1.3	10	1.5
Concentration (µM)	Migration Area normalised to DMSO														
0	1.0														
1	1.1														
2	1.2														
4	1.3														
10	1.5														
GSK2336394A Intended Target: PIK3CB			 <table><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.1</td></tr><tr><td>2</td><td>1.3</td></tr><tr><td>4</td><td>1.4</td></tr><tr><td>10</td><td>1.6</td></tr></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	1	1.1	2	1.3	4	1.4	10	1.6
Concentration (µM)	Migration Area normalised to DMSO														
0	1.0														
1	1.1														
2	1.3														
4	1.4														
10	1.6														
GW300653X Intended Target: CDK2			 <table><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr><tr><td>0</td><td>1.0</td></tr><tr><td>1</td><td>1.2</td></tr><tr><td>2</td><td>1.2</td></tr><tr><td>4</td><td>1.2</td></tr><tr><td>10</td><td>1.3</td></tr></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	1	1.2	2	1.2	4	1.2	10	1.3
Concentration (µM)	Migration Area normalised to DMSO														
0	1.0														
1	1.2														
2	1.2														
4	1.2														
10	1.3														

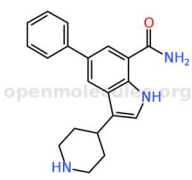
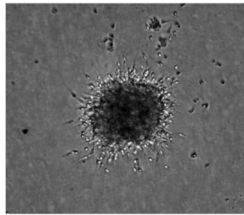
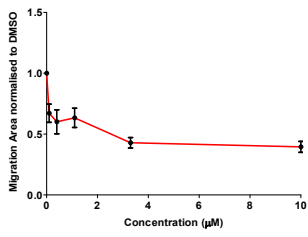
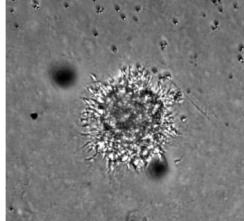
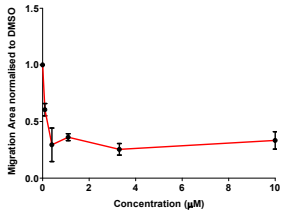
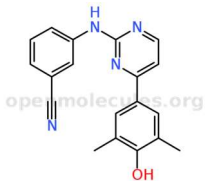
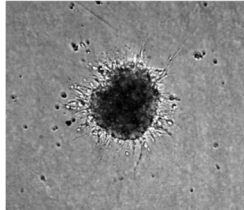
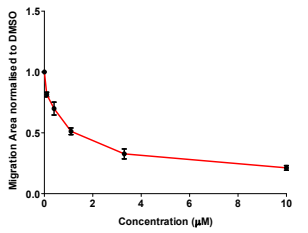
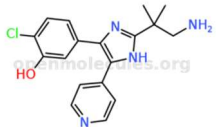
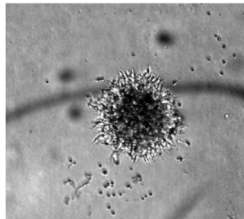
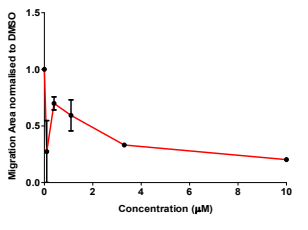
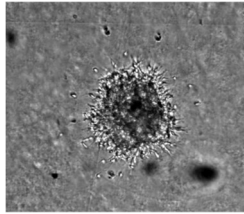
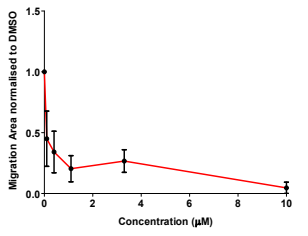
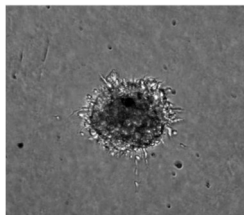
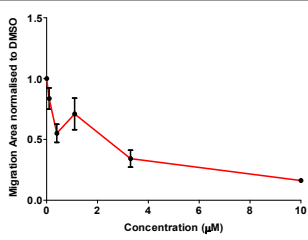
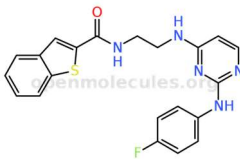
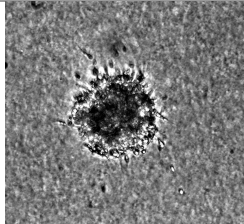
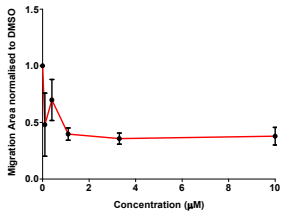
Table 11. Kinase inhibitors that decreased migration of neuroblasts from 3D spheroids. Compounds are listed together with their target (if known), chemical structure, representative image of migration at 24 hours, and distance of migration measured compared to the DMSO control for 5 different concentrations of the compound. N=3. Un-paired two-way t-test completed for each data point against DMSO with *** p<0.001, ** p<0.01 and * p<0.05.

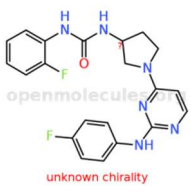
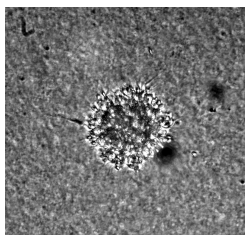
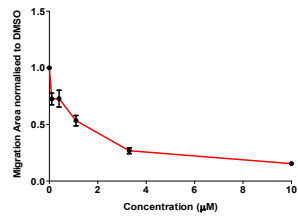
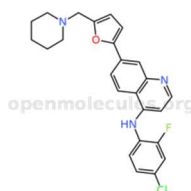
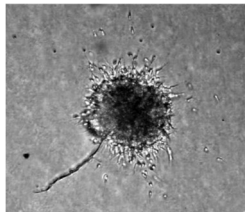
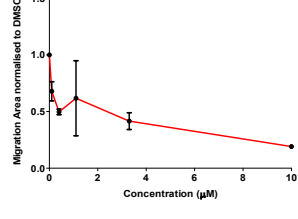
Compound	Chemical Structure	Migration Image	Results												
SB-251505 Intended Target: p38a			<table><caption>Approximate data for SB-251505</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.4</td></tr><tr><td>3</td><td>0.25</td></tr><tr><td>10</td><td>0.25</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.5	1	0.4	3	0.25	10	0.25
Concentration (μM)	Migration Area (normalized to DMSO)														
0	1.0														
0.5	0.5														
1	0.4														
3	0.25														
10	0.25														
SB-220455 Intended Target: p38a			<table><caption>Approximate data for SB-220455</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.4</td></tr><tr><td>3</td><td>0.25</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.5	1	0.4	3	0.25	10	0.2
Concentration (μM)	Migration Area (normalized to DMSO)														
0	1.0														
0.5	0.5														
1	0.4														
3	0.25														
10	0.2														
SB-253228 Intended Target: p38a			<table><caption>Approximate data for SB-253228</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.4</td></tr><tr><td>3</td><td>0.4</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.5	1	0.4	3	0.4	10	0.3
Concentration (μM)	Migration Area (normalized to DMSO)														
0	1.0														
0.5	0.5														
1	0.4														
3	0.4														
10	0.3														
SB-226879 Intended Target: p38a			<table><caption>Approximate data for SB-226879</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.3</td></tr><tr><td>3</td><td>0.25</td></tr><tr><td>10</td><td>0.25</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.5	1	0.3	3	0.25	10	0.25
Concentration (μM)	Migration Area (normalized to DMSO)														
0	1.0														
0.5	0.5														
1	0.3														
3	0.25														
10	0.25														
SB-239272 Intended Target: p38a			<table><caption>Approximate data for SB-239272</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.4</td></tr><tr><td>3</td><td>0.3</td></tr><tr><td>10</td><td>0.25</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.5	1	0.4	3	0.3	10	0.25
Concentration (μM)	Migration Area (normalized to DMSO)														
0	1.0														
0.5	0.5														
1	0.4														
3	0.3														
10	0.25														
SB-219980 Intended Target: P38a/p38b			<table><caption>Approximate data for SB-219980</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.4</td></tr><tr><td>3</td><td>0.4</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.5	1	0.4	3	0.4	10	0.3
Concentration (μM)	Migration Area (normalized to DMSO)														
0	1.0														
0.5	0.5														
1	0.4														
3	0.4														
10	0.3														

<div>SB-253226</div> <div>Intended Target: p38α</div>			 <table><caption>Approximate data for SB-253226</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.4</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.6	2	0.5	4	0.4	10	0.3
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.6																
2	0.5																
4	0.4																
10	0.3																
<div>SB-222903</div> <div>Intended Target: P38a/p38b</div>			 <table><caption>Approximate data for SB-222903</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.3</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.6	2	0.5	4	0.3	10	0.2
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.6																
2	0.5																
4	0.3																
10	0.2																
<div>SB-219952</div> <div>Intended Target: P38a/p38b</div>			 <table><caption>Approximate data for SB-219952</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.7</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.4</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.7	2	0.5	4	0.4	10	0.3
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.7																
2	0.5																
4	0.4																
10	0.3																
<div>SB-226605</div> <div>Intended Target: P38a/p38b</div>			 <table><caption>Approximate data for SB-226605</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.3</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.6	2	0.5	4	0.3	10	0.2
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.6																
2	0.5																
4	0.3																
10	0.2																
<div>GW833373X</div> <div>Intended Target: GSK3b</div>			 <table><caption>Approximate data for GW833373X</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.7</td></tr><tr><td>2</td><td>0.6</td></tr><tr><td>4</td><td>0.5</td></tr><tr><td>10</td><td>0.4</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.7	2	0.6	4	0.5	10	0.4
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.7																
2	0.6																
4	0.5																
10	0.4																
<div>SB-742034-AC</div> <div>Intended Target: GSK3a/GSK3b</div>			 <table><caption>Approximate data for SB-742034-AC</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.7</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.4</td></tr><tr><td>10</td><td>0.4</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.7	2	0.5	4	0.4	10	0.4
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.7																
2	0.5																
4	0.4																
10	0.4																
<div>SB-678557-A</div> <div>Intended Target: GSK3a/GSK3b</div>			 <table><caption>Approximate data for SB-678557-A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.4</td></tr><tr><td>10</td><td>0.4</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.6	2	0.5	4	0.4	10	0.4
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.6																
2	0.5																
4	0.4																
10	0.4																

GW784752X Intended Target: GSK3b			 <table><caption>Approximate data for GW784752X</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.4</td></tr><tr><td>1</td><td>0.35</td></tr><tr><td>2</td><td>0.3</td></tr><tr><td>4</td><td>0.25</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.4	1	0.35	2	0.3	4	0.25	10	0.2
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.4																
1	0.35																
2	0.3																
4	0.25																
10	0.2																
SB-627772-A Intended Target: GSK3a/GSK3b			 <table><caption>Approximate data for SB-627772-A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.5</td></tr><tr><td>1</td><td>0.45</td></tr><tr><td>2</td><td>0.4</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.5	1	0.45	2	0.4	4	0.35	10	0.3
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.5																
1	0.45																
2	0.4																
4	0.35																
10	0.3																
GW829906X Intended Target: GSK3b			 <table><caption>Approximate data for GW829906X</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.6</td></tr><tr><td>1</td><td>0.5</td></tr><tr><td>2</td><td>0.45</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.6	1	0.5	2	0.45	4	0.35	10	0.2
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.6																
1	0.5																
2	0.45																
4	0.35																
10	0.2																
GSK2358994A Intended Target: PI3K			 <table><caption>Approximate data for GSK2358994A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.7</td></tr><tr><td>1</td><td>1.0</td></tr><tr><td>2</td><td>0.8</td></tr><tr><td>4</td><td>0.7</td></tr><tr><td>10</td><td>0.5</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.7	1	1.0	2	0.8	4	0.7	10	0.5
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.7																
1	1.0																
2	0.8																
4	0.7																
10	0.5																
GSK2286295A Intended Target: PI3K			 <table><caption>Approximate data for GSK2286295A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.6</td></tr><tr><td>1</td><td>1.1</td></tr><tr><td>2</td><td>0.8</td></tr><tr><td>4</td><td>0.6</td></tr><tr><td>10</td><td>0.4</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.6	1	1.1	2	0.8	4	0.6	10	0.4
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.6																
1	1.1																
2	0.8																
4	0.6																
10	0.4																
GSK2373690A Intended Target: PI3K			 <table><caption>Approximate data for GSK2373690A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.9</td></tr><tr><td>2</td><td>0.8</td></tr><tr><td>4</td><td>0.7</td></tr><tr><td>10</td><td>0.5</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.9	2	0.8	4	0.7	10	0.5
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.9																
2	0.8																
4	0.7																
10	0.5																
GSK1389063A Intended Target: Akt			 <table><caption>Approximate data for GSK1389063A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.9</td></tr><tr><td>1</td><td>0.8</td></tr><tr><td>2</td><td>0.7</td></tr><tr><td>4</td><td>0.5</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.9	1	0.8	2	0.7	4	0.5	10	0.2
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.9																
1	0.8																
2	0.7																
4	0.5																
10	0.2																

<div>GSK1000163A</div> <div>Intended Target: Akt3</div>			 <table><caption>Approximate data for GSK1000163A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.55</td></tr><tr><td>4</td><td>0.5</td></tr><tr><td>10</td><td>0.35</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.6	2	0.55	4	0.5	10	0.35
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.6																
2	0.55																
4	0.5																
10	0.35																
<div>GSK1383281A</div> <div>Intended Target: Akt</div>			 <table><caption>Approximate data for GSK1383281A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.4</td></tr><tr><td>1</td><td>0.5</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.45</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.4	1	0.5	2	0.5	4	0.45	10	0.3
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.4																
1	0.5																
2	0.5																
4	0.45																
10	0.3																
<div>GSK1383280A</div> <div>Intended Target: Akt</div>			 <table><caption>Approximate data for GSK1383280A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.6</td></tr><tr><td>1</td><td>0.5</td></tr><tr><td>2</td><td>0.4</td></tr><tr><td>4</td><td>0.25</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.6	1	0.5	2	0.4	4	0.25	10	0.2
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.6																
1	0.5																
2	0.4																
4	0.25																
10	0.2																
<div>GW856804X</div> <div>Intended Target: TIE2/VEGFR2</div>			 <table><caption>Approximate data for GW856804X</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.7</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.55</td></tr><tr><td>4</td><td>0.5</td></tr><tr><td>10</td><td>0.3</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.7	1	0.6	2	0.55	4	0.5	10	0.3
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.7																
1	0.6																
2	0.55																
4	0.5																
10	0.3																
<div>GW787226A</div> <div>Intended Target: VEGFR</div>			 <table><caption>Approximate data for GW787226A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.6</td></tr><tr><td>1</td><td>0.55</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.45</td></tr><tr><td>10</td><td>0.35</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.6	1	0.55	2	0.5	4	0.45	10	0.35
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.6																
1	0.55																
2	0.5																
4	0.45																
10	0.35																
<div>GSK1819799A</div> <div>Intended Target: IGF1R</div>			 <table><caption>Approximate data for GSK1819799A</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.6</td></tr><tr><td>1</td><td>0.55</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.6</td></tr><tr><td>10</td><td>0.6</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.6	1	0.55	2	0.5	4	0.6	10	0.6
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.6																
1	0.55																
2	0.5																
4	0.6																
10	0.6																
<div>GW276655X</div> <div>Intended Target: CDK2</div>			 <table><caption>Approximate data for GW276655X</caption><thead><tr><th>Concentration (µM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.7</td></tr><tr><td>1</td><td>0.55</td></tr><tr><td>2</td><td>0.45</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.25</td></tr></tbody></table>	Concentration (µM)	Migration Area normalised to DMSO	0	1.0	0.5	0.7	1	0.55	2	0.45	4	0.35	10	0.25
Concentration (µM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.7																
1	0.55																
2	0.45																
4	0.35																
10	0.25																

<div>GSK301362A</div> <div>Intended Target: IKK</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for GSK301362A</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.65</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.6</td></tr><tr><td>4</td><td>0.45</td></tr><tr><td>10</td><td>0.4</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.65	1	0.6	2	0.6	4	0.45	10	0.4
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.65																
1	0.6																
2	0.6																
4	0.45																
10	0.4																
<div>GW813349X</div> <div>Intended Target: PLK1/PLK2</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for GW813349X</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.35</td></tr><tr><td>1</td><td>0.3</td></tr><tr><td>2</td><td>0.25</td></tr><tr><td>4</td><td>0.25</td></tr><tr><td>10</td><td>0.35</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.35	1	0.3	2	0.25	4	0.25	10	0.35
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.35																
1	0.3																
2	0.25																
4	0.25																
10	0.35																
<div>GSK1440913A</div> <div>Intended Target: Unknown</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for GSK1440913A</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.55</td></tr><tr><td>1</td><td>0.5</td></tr><tr><td>2</td><td>0.45</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.25</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.55	1	0.5	2	0.45	4	0.35	10	0.25
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.55																
1	0.5																
2	0.45																
4	0.35																
10	0.25																
<div>SB-477794-AAA</div> <div>Intended Target: Unknown</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for SB-477794-AAA</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.65</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.5</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.25</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.65	1	0.6	2	0.5	4	0.35	10	0.25
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.65																
1	0.6																
2	0.5																
4	0.35																
10	0.25																
<div>GW494601A</div> <div>Intended Target: Unknown</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for GW494601A</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.45</td></tr><tr><td>1</td><td>0.35</td></tr><tr><td>2</td><td>0.3</td></tr><tr><td>4</td><td>0.3</td></tr><tr><td>10</td><td>0.1</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.45	1	0.35	2	0.3	4	0.3	10	0.1
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.45																
1	0.35																
2	0.3																
4	0.3																
10	0.1																
<div>GSK2181306A</div> <div>Intended Target: Unknown</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for GSK2181306A</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.55</td></tr><tr><td>1</td><td>0.75</td></tr><tr><td>2</td><td>0.65</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.55	1	0.75	2	0.65	4	0.35	10	0.2
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.55																
1	0.75																
2	0.65																
4	0.35																
10	0.2																
<div>GSK1379874A</div> <div>Intended Target: Unknown</div>	<div></div>	<div></div>	<div></div> <table><caption>Approximate data for GSK1379874A</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area (normalized to DMSO)</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.65</td></tr><tr><td>1</td><td>0.4</td></tr><tr><td>2</td><td>0.35</td></tr><tr><td>4</td><td>0.35</td></tr><tr><td>10</td><td>0.35</td></tr></tbody></table>	Concentration (μM)	Migration Area (normalized to DMSO)	0	1.0	0.5	0.65	1	0.4	2	0.35	4	0.35	10	0.35
Concentration (μM)	Migration Area (normalized to DMSO)																
0	1.0																
0.5	0.65																
1	0.4																
2	0.35																
4	0.35																
10	0.35																

<p>GSK1379722A</p> <p>Intended Target: Unknown</p>	 <p>openmolecules</p>		 <table><caption>Approximate data for GSK1379722A graph</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.8</td></tr><tr><td>1</td><td>0.6</td></tr><tr><td>2</td><td>0.4</td></tr><tr><td>4</td><td>0.3</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	0.5	0.8	1	0.6	2	0.4	4	0.3	10	0.2
Concentration (μM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.8																
1	0.6																
2	0.4																
4	0.3																
10	0.2																
<p>GW560116X</p> <p>Intended Target: Unknown</p>	 <p>openmolecules</p>		 <table><caption>Approximate data for GW560116X graph</caption><thead><tr><th>Concentration (μM)</th><th>Migration Area normalised to DMSO</th></tr></thead><tbody><tr><td>0</td><td>1.0</td></tr><tr><td>0.5</td><td>0.6</td></tr><tr><td>1</td><td>0.5</td></tr><tr><td>2</td><td>0.4</td></tr><tr><td>4</td><td>0.3</td></tr><tr><td>10</td><td>0.2</td></tr></tbody></table>	Concentration (μM)	Migration Area normalised to DMSO	0	1.0	0.5	0.6	1	0.5	2	0.4	4	0.3	10	0.2
Concentration (μM)	Migration Area normalised to DMSO																
0	1.0																
0.5	0.6																
1	0.5																
2	0.4																
4	0.3																
10	0.2																

5.4.2 Analysis of the binding targets of the small molecules indicates potential genes that regulate neuroblast migration.

The PKIS is comprised of inhibitors that function via competitive binding with the cofactor ATP. Because the ATP binding pocket is conserved but not identical across the kinome, many of the PKIS compounds have some degree of promiscuity in their kinase activity. Consequently, the kinase for which a compound was originally prepared might not be the only kinase inhibited, and importantly the original kinase may not be a contributor to the phenotype resulting from compound treatment.

Both the PKIS1 and PKIS2 have been characterised to try to provide insights into their likely molecular targets. The PKIS1 was analysed using the Nanosyn enzyme assay panel to determine the binding affinity to 224 recombinant human kinases (Elkins *et al.*, 2016) and the PKIS was analysed using the KINOMEScan completion binding assay against 468 kinases. The results for both provide an affinity value equivalent to percentage of the kinase bound to the inhibitor compared to DMSO (Drewry *et al.*, 2017). It is important to note that neither panel is exhaustive so will not identify all potential interactions and that there are many kinases characterised for PKIS2 that are absent from the PKIS1 data set. It is also important to note that affinity is not necessarily correlated with potency.

Due to time and financial restraints we could not complete a full algorithmic systems analysis of the results. Instead we sought to select the most likely candidate kinase targets from the compounds screened for confirmation studies. The primary method used was to select candidate hit kinases from the affinity matrix for compounds that were validated to increase and decrease

migration. Tables 13-16 contain all hit compounds from the screen, the migration area relative to control, claimed affinity to a selection of protein kinases, and the total number of kinases for each compound where the affinity is greater than 80. Only kinase targets that are the top target for at least one compound are listed. To account for the promiscuous polypharmacology only hits in which there were 2 or fewer kinases with an affinity greater than 90 were analysed. The top two kinase hits were then selected for each compound as potential regulators of migration. The second approach we used was to analyse the distribution of binding affinities across the entire data set to identify kinase targets that are concentrated in the higher and lower migration groups. The rationale for this is that the results from the Nanosyn and KINOMEscan profiles do not necessarily perfectly correlate with inhibition efficiency, so simply screening for hits greater than 90 will indiscriminately mask targets that have low affinity. Conversely, there may be kinases that have a consistently high affinity that are picked up in the screen, but do not actually have any functional activity. The cut offs used were if the migration area was $>1.5\times$ control compounds were allocated to the high migrating pool and if it was $<0.5\times$ control the compounds were allocated to the low migrating pool. All toxic compounds were removed from the analysis. The average affinity of each kinase to all compounds in the two categories was then calculated, compared and ranked. The kinases with a Δ high:low above 5 were considered as potential hits, where inhibition may increase migration for both PKIS1 and PKIS2 (see Table 12). The kinases with a Δ high:low less than -5 were considered as potential hits where inhibition decreases migration for PKIS1 (see Table 12). For PKIS2 the number of putative hits was >200 using -5 as the threshold, and so it was adjusted to -13. It is important to note that kinases may still be selected even if they do not themselves participate in the biological process, provided they are pharmacologically linked to kinases that do.

The lists of putative hits selected from each analysis method were then compared (Figure 34). Several receptor tyrosine kinases were selected as robust pro-migration targets. These included EGFR, ErbB2, ErbB4, EphA2, TrkA, TrkC, PDGFR, Tie2, FLT3 and KIT. Other kinases selected included four members of the Polo-Like Kinase (PLK) family: PLK1, PLK2, PLK3, and LOK; NimA-related protein kinase 9 (NEK9); hormonally up-regulated Neu-associated kinase (HUNK); muscle specific kinase (MUSK); and Aurora C kinase (AURKC). Anti-targets that inhibited migration included GSK3 α , GSK3 β , MAPK14/P38 α , IGF1R, IR, CDK2, Protein Kinase D1, Casein Kinase 1 (CK1 α) and dual specificity Yak 1-related kinase 1B (Dyrk1B).

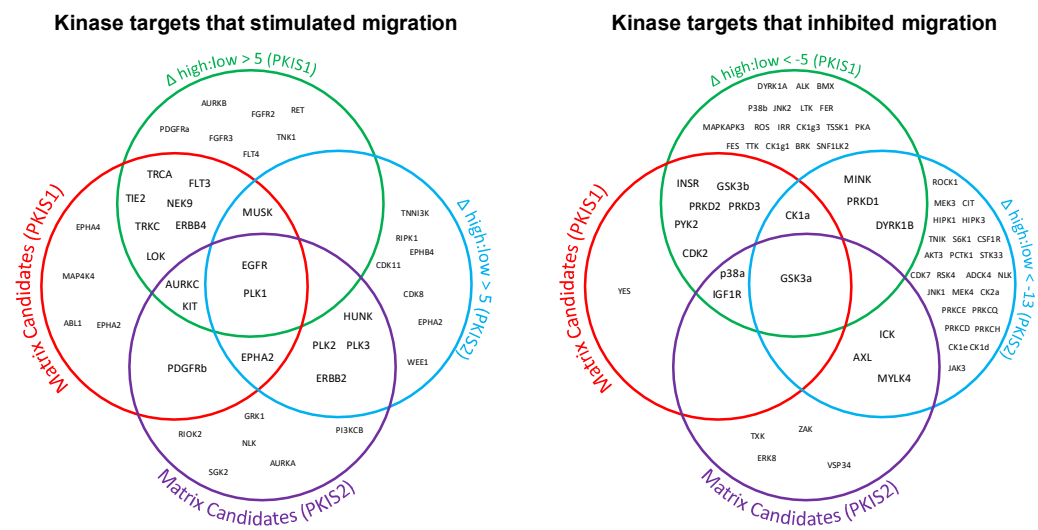


Figure 34. Comparison of kinase targets selected by analysis of the PKIS1 and PKIS2 data sets
Venn diagram of the putative kinase targets selected by the analysis of the kinase targets for the 'hit' compounds and the relative kinase affinity to high and low migrating compounds for both PKIS1 and PKIS2.

Table 12 Analysis of the average affinity ratio between high and low migrating compounds.
Ranking of kinases selected as putative biological targets through comparison of average affinity to compounds with either high (>1.5X control) or low (<0.5X control) migration areas.

Kinases with highest Δ high:low				Kinases with lowest Δ high:low			
PKIS1				PKIS2			
Gene	Mean Affinity		Δ	Gene	Mean Affinity		Δ
	High	Low			High	Low	
LOK	25.9	11.9	14.0	ERBB2	32.8	20.1	12.7
FLT3	21.9	8.1	13.8	RIPK1	18.4	8.3	10.0
TRKC	21.8	8.0	13.8	PLK2	17.2	7.8	9.4
PDGFR α	26.6	13.2	13.4	PLK1	15.2	6.3	8.9
AURORA-C	21.3	8.5	12.8	MUSK	16.3	7.4	8.9
TRKB	20.4	7.8	12.6	CDK11	18.4	10.1	8.3
KIT	18.2	7.4	10.8	PLK3	13.8	6.7	7.1
TRKA	16.1	6.1	10.0	EPHB4	16.6	10.0	6.6
AURORA-A	18.8	10.9	7.9	CDK8	26.0	19.4	6.6
EGFR	22.1	14.4	7.7	EGFR	36.8	30.5	6.3
FGFR2	13.8	6.4	7.4	EPHA2	18.1	12.3	5.8
ERBB4	27.6	20.4	7.2	TNNI3K	20.5	14.9	5.6
AURORA-B	15.7	8.5	7.1	WEE1	10.5	5.5	5.0
RET	21.2	14.6	6.6	HUNK	14.4	9.4	5.0
FLT4	16.5	10.0	6.5				
MUSK	15.5	9.1	6.4				
FGFR3	11.4	5.1	6.2				
TIE2	16.5	10.4	6.1				
NEK9	10.9	5.2	5.7				
TNK1	20.3	15.2	5.1				
PLK1	13.2	8.2	5.0				

PKIS1				PKIS2			
Gene	Mean Affinity		Δ	Gene	Mean Affinity		Δ
	High	Low			High	Low	
P38 α	11.6	46.3	-34.7	CSNK1A1	11.5	39.9	-28.4
CK1a	4.5	27.8	-23.3	CSNK1E	25.8	51.3	-25.5
P38 β	8.4	27.0	-18.6	CSNK1D	21.5	44.5	-23.0
PYK2	10.3	26.3	-16.1	MEK3	8.3	29.2	-20.9
JNK2	6.0	20.9	-14.9	CSNK1A1L	7.2	27.6	-20.4
INSR	4.6	19.1	-14.6	PRKCH	8.3	28.4	-20.1
LTK	4.2	18.1	-14.0	HIPK1	8.1	27.1	-19.0
IGF1R	2.7	15.5	-12.9	PRKCD	5.0	23.7	-18.7
GSK3B	12.0	24.4	-12.5	YSK4	34.1	52.7	-18.6
FER	4.9	17.2	-12.3	ROCK1	16.0	33.5	-17.5
PRKD2	4.6	16.9	-12.3	HIPK2	5.2	22.7	-17.5
MAPKAPK3	4.9	16.9	-12.0	DYRK1B	7.8	24.7	-16.9
GSK3A	12.3	23.4	-11.1	HIPK3	12.2	28.9	-16.8
ALK	5.2	16.1	-10.9	PRKCE	11.4	27.8	-16.4
PRKD3	4.0	14.8	-10.8	TNIK	20.4	36.3	-15.9
PRKD1	4.8	15.4	-10.6	S6K1	8.3	23.3	-15.0
ROS	11.6	21.8	-10.2	CSF1R	18.7	33.5	-14.8
DYRK1A	7.1	17.1	-10.0	AKT3	4.5	19.2	-14.7
IRR	1.5	11.4	-9.9	CSNK2A1	6.3	20.9	-14.5
CK1-g3	3.1	11.9	-8.9	MINK	25.8	40.2	-14.4
TSSK1	2.9	11.2	-8.3	CIT	31.9	46.3	-14.4
DYRK1B	4.0	12.3	-8.3	JAK3	13.0	27.3	-14.3
PKA	2.7	10.7	-8.1	GSK3A	14.2	28.4	-14.2
FES	2.6	10.2	-7.7	PCTK1	4.5	18.7	-14.2
TTK	10.4	17.1	-6.7	MYLK4	13.5	27.6	-14.1
CLK2	7.5	14.2	-6.7	AXL	17.8	31.6	-13.9
BMX	6.3	12.6	-6.4	STK33	10.3	24.1	-13.8
CK1-g1	3.2	8.9	-5.7	CDK7	9.9	23.7	-13.7
BRK	9.0	14.4	-5.4	PKAC-alpha	7.1	20.7	-13.6
SNF1LK2	9.1	14.5	-5.3	RSK4	19.1	32.6	-13.5
MINK	19.2	24.5	-5.2	ADCK4	21.0	34.5	-13.5
				PRKCC	12.7	26.2	-13.5
				PRKD1	14.3	27.6	-13.3
				MEK4	18.0	31.3	-13.3
				ICK	10.8	24.1	-13.3
				JNK1	21.7	34.9	-13.2
				NLK	22.6	35.6	-13.0

Table 13. Matrix of compounds that stimulated migration from PKIS1 and their indicated kinase targets

All compounds selected from PKIS1 that had migration areas at least 4 standard deviations larger than the DMSO controls. Listed next to the kinases is the relative migration compared to control and the affinity of each compound to kinases targets from the nanosyn affinity assay. Also listed is the total number of kinases that that compound binds to with affinity greater than 80 and 90, which can be used to judge promiscuity. Note: the Nanosyn value for GW607049C:ErbB4 was blank on the published data set.

Compounds that stimulated migration (PKIS1)																												
Compound	x Control	>90	>80	ABL1	BRK	SRC	YES	EPHA2	EPHA4	DDR2	MUSK	TRKA	TRKC	RET	KDR	KIT	FLT3	PDGFR α	PDGFR β	TIE2	EGFR	ERBB4	MAP4k4	LOK	PLK1	AURORA-C	NEK9	
GW607049C	3.03	0	4	72	1	13	6	90	82	83	79	83	70	74	47	44	33	58	17	71	3		7	73	1	6	0	
GW703087X	2.70	0	0	2	2	6	2	2	1	8	7	0	2	11	13	2	2	15	7	7	77	57	3	4	3	15	2	
SB-735467	2.43	0	0	69	3	6	7	-2	4	2	5	4	7	4	19	5	4	15	8	7	4	12	70	9	2	7	6	
GW300653X	2.39	0	0	7	1	14	9	-2	5	0	4	26	35	34	4	32	44	34	30	3	2	2	62	2	1	7	5	
GSK317315A	2.27	1	3	1	-1	0	1	-4	0	-2	22	3	6	4	14	15	8	35	37	5	3	3	4	81	99	0	84	
GW627512B	2.23	3	4	22	4	72	47	-1	1	9	14	23	25	16	48	99	91	70	74	5	14	-3	26	11	2	72	8	
GSK237701A	2.22	5	9	58	4	41	18	7	27	12	78	12	63	52	72	27	25	100	95	42	3	14	19	88	97	51	92	
GW627834A	2.04	0	0	10	3	20	18	1	8	3	3	13	25	4	11	80	62	17	26	2	2	9	24	4	2	6	5	
GW804482X	1.99	1	1	1	1	-3	1	2	3	0	-4	-8	-2	7	3	-4	-4	14	8	3	2	13	18	39	92	4	8	
GSK259178A	1.96	0	1	4	5	9	8	0	3	3	5	1	3	5	11	2	3	12	8	11	76	86	6	8	11	9	12	
GW824645A	1.95	0	1	9	4	10	12	1	10	3	14	19	28	18	15	85	67	36	38	5	3	11	70	16	3	0	9	
GW794607X	1.94	0	7	86	51	82	83	5	17	8	50	7	37	55	65	24	18	78	79	36	33	43	44	28	1	59	0	
GW701427A	1.94	0	0	15	0	8	2	-5	5	49	3	3	4	14	13	19	6	39	16	7	-2	0	6	7	1	38	2	
SB-437013	1.93	0	1	12	42	9	8	2	8	4	3	2	2	9	18	-1	2	10	4	83	3	23	8	78	4	15	0	
GW683134A	1.91	7	19	94	4	37	22	83	87	80	83	85	90	84	72	92	71	85	58	82	-1	-5	5	77	1	82	7	
GW693881A	1.90	0	1	5	6	7	9	-6	6	-1	6	3	2	12	12	2	2	16	9	9	77	69	14	12	4	-2	9	
SB-633825	1.88	1	2	43	85	11	21	1	18	-5	6	1	4	22	48	5	1	24	13	79	6	44	15	95	4	13	18	
GSK300014A	1.86	0	1	5	6	15	6	-1	10	6	9	7	4	7	9	8	7	14	11	11	84	77	10	13	8	9	9	
GW780159X	1.83	0	0	8	11	14	18	1	15	-6	2	0	5	6	67	8	2	28	15	6	7	28	60	14	4	-2	12	

Table 14 Matrix of compounds that inhibited migration from PKIS1 and their indicated kinase targets

All compounds selected from PKIS1 that had migration areas at least 4 standard deviations smaller than the DMSO controls. Listed next to the kinases is the relative migration compared to control and the affinity of each compound to kinases targets from the nanosyn affinity assay. Also listed is the total number of kinases that that compound binds to with affinity greater than 80 and 90, which can be used to judge promiscuity.

Compounds that inhibited migration (PKIS1)																										
Compound	x Control	>90	>80	YES	PYK2	LTK	ALK	INSR	IGF1R	DDR2	PDGFR β	ERBB4	CK1a	PRKD3	PRKD2	TSSK1	CDK2	CDK3	JNK2	P38 α	GSK3A	GSK3B	CLK2	DYRK1A	TTK	
GW784752X	0.34	6	19	73	16	6	8	7	5	9	81	21	11	9	9	5	77	49	11	16	90	89	35	72	19	
SB-686709-A	0.33	0	2	0	1	0	2	1	-1	1	5	1	-1	0	0	-1	20	17	2	-1	90	89	3	61	2	
GW811168X	0.32	5	10	57	6	2	-2	1	-5	8	78	8	6	-2	-3	-1	68	21	5	5	81	79	18	64	-19	
GW827099X	0.30	5	15	97	44	6	8	18	3	83	70	95	73	48	52	3	4	-3	85	93	60	76	1	3	19	
SB-253228	0.30	0	1	0	12	2	2	0	-5	2	11	5	15	6	4	3	-1	-1	4	84	3	3	0	0	14	
SB-678557-A	0.30	0	2	3	15	5	7	5	4	1	8	24	4	5	6	7	6	6	6	5	86	84	2	34	15	
SB-226879	0.30	0	1	9	18	5	5	3	5	3	21	12	73	11	15	7	-1	0	37	86	5	7	2	4	2	
SB-253226	0.28	0	0	3	7	4	5	4	6	2	10	14	21	5	5	5	3	-2	6	71	3	2	0	4	15	
GW833373X	0.28	2	10	92	48	3	6	8	5	83	68	89	71	81	87	0	1	3	81	89	45	67	2	5	6	
GSK1000163A	0.28	0	0	6	-7	0	0	0	-1	6	12	0	-1	6	7	10	-1	-1	-2	0	22	19	1	2	5	
GSK1392956A	0.27	3	7	12	97	89	70	117	93	1	5	16	13	21	17	85	4	-3	57	10	0	-1	83	13	82	
SB-220025-A	0.27	1	2	16	4	1	2	5	4	0	26	9	86	26	38	-1	6	-1	7	91	4	6	2	4	0	
SB-223133	0.27	2	2	40	2	1	3	0	-1	13	30	46	90	19	23	-2	-5	-1	53	93	6	14	1	6	0	
GW829906X	0.26	2	6	92	59	3	4	25	5	47	85	79	59	51	57	4	3	-1	82	74	68	76	3	3	11	
SB-220455	0.25	0	1	13	6	5	4	5	3	2	30	19	73	17	16	3	1	0	11	83	7	8	14	6	-8	
SB-239272	0.24	0	1	-1	7	2	0	3	-4	2	6	15	31	2	4	2	-2	-2	14	86	-2	-2	-1	-2	6	
GW276655X	0.24	1	3	12	20	3	0	7	-2	2	50	14	3	7	9	12	94	89	2	3	67	65	36	81	53	
SB-251505	0.24	0	1	7	14	3	4	6	4	4	12	11	32	6	5	4	2	0	13	80	4	4	3	6	8	
GSK1751853A	0.23	1	5	1	93	81	82	88	88	-1	5	60	11	8	9	32	2	-1	18	7	2	4	45	10	48	
GSK2186269A	0.22	3	7	-2	90	90	85	91	98	1	3	6	2	4	4	19	-2	2	2	4	1	1	44	4	25	

Table 15. Matrix of compounds that stimulated migration from PKIS2 and their indicated kinase targets

All compounds selected from PKIS2 that had migration areas at least 4 standard deviations greater than the DMSO controls. Listed next to the kinases is the relative migration compared to control and the affinity of each compound to kinases targets from the KINOMEScan affinity assay. Also listed is the total number of kinases that that compound binds to with affinity greater than 80 and 90, which can be used to judge promiscuity.

Compounds that stimulated migration (PKIS2)																											
Compound	x Control	>90	>80	AURKA	CDC2L5	CDKL2	EGFR	ERBB2	ERK8	FLT4	GRK1	HUNK	KIT	MEK5	MYLK2	NLK	PDGFRB	PIK3CB	PLK1	PLK2	PLK3	RET	RIOK2	SGK2	SLK	SRPK1	YSK4
GSK306886	2.43	5	5	30	0	19	99	99	0	98	4	57	34	26	3	44	46	0	0	0	0	98	3	0	8	100	0
GW579362	2.40	75	124	100	0	41	55	17	18	100	2	8	100	100	32	98	100	0	0	0	4	100	66	0	99	99	99
GSK175726	2.40	3	4	90	0	32	38	22	0	0	100	0	40	49	11	17	21	40	0	3	1	10	92	0	8	0	0
GSK346294	2.37	2	3	8	0	5	32	15	0	1	0	0	20	18	0	19	22	5	100	96	87	24	0	10	59	20	3
GW466413	2.18	1	4	0	0	5	28	35	8	9	14	93	17	35	0	0	5	0	0	0	0	24	2	0	8	21	11
GSK2336394	2.15	1	1	0	0	22	28	20	0	3	0	6	14	1	0	0	15	98	0	0	0	14	6	1	22	37	26
SB-601273	2.11	138	164	62	41	100	88	93	0	96	6	55	100	100	98	99	100	30	26	27	52	100	29	2	99	50	99
GSK1024304	2.08	3	5	2	0	18	4	28	0	1	0	14	23	32	0	0	31	100	97	90	86	26	80	10	9	29	15
GW681170	2.05	32	38	78	69	98	12	22	95	79	0	7	100	51	0	30	100	5	0	0	0	100	0	1	36	25	87
SB-735464	2.03	0	2	18	23	57	0	21	32	0	14	0	33	31	77	86	67	4	0	37	13	0	40	0	11	36	82
SB-732932	2.00	4	9	13	47	95	0	0	92	0	0	0	56	50	97	46	80	8	2	24	0	0	16	4	0	45	95
GW854278	1.97	5	8	0	5	0	27	11	0	68	0	0	24	99	0	0	58	0	100	100	99	20	14	3	96	44	61
SB-733416	1.97	1	1	0	98	9	37	0	23	27	6	0	8	37	0	11	16	0	0	0	0	23	0	9	28	11	0
GW824645	1.95	3	4	4	0	0	0	4	3	0	23	58	90	70	9	0	96	0	0	20	23	0	0	100	0	0	0
GW683134	1.91	41	54	0	0	85	44	9	82	100	0	0	100	85	75	6	100	0	0	0	0	100	7	14	83	51	93
GW659009	1.81	14	22	0	0	15	100	93	10	0	0	0	17	98	0	0	36	0	0	0	30	0	53	4	0	37	3

Table 16 Matrix of compounds that inhibited migration from PKIS2 and their indicated kinase targets

All compounds selected from PKIS2 that had migration areas at least 4 standard deviations smaller than the DMSO controls. Listed next to the kinases is the relative migration compared to control and the affinity of each compound to kinases targets from the KINOMEScan affinity assay. Also listed is the total number of kinases that that compound binds to with affinity greater than 80 and 90, which can be used to judge promiscuity.

Compounds that inhibited migration (PKIS2)																															
Compound	x Control	>90	>80	AMPK-alpha1	AXL	CDK2	DAPK3	ERK8	FLT3	GSK3A	ICK	IGF1R	LYN	MYLK2	MYLK4	NIM1	PDGFRB	PHKG2	PIK3C2B	PIK3C2G	PIK3CB	PIK3CD	PIK3CG	PLK1	PLK2	PLK3	TKK	VPS34	ZAK	p38-alpha	
GSK1440913	0.34	3	4	17	51	89	5	99	48	94	79	0	20	76	100	12	60	15	0	24	0	47	67	0	0	0	35	70	16	0	
SB-738481	0.34	5	8	0	37	87	0	84	92	93	95	0	11	89	19	100	90	0	7	0	13	5	12	3	3	12	20	0	15	0	
GSK2373693	0.33	5	6	0	4	11	95	26	0	0	15	10	0	6	2	11	65	0	99	89	0	100	94	0	0	0	0	97	15	66	
GSK1660437	0.33	3	3	48	68	99	6	66	33	12	0	0	74	0	0	0	99	0	0	0	13	0	0	0	5	0	20	0	97	32	
SB-711880	0.32	3	6	90	18	99	21	82	28	87	26	0	0	85	0	0	0	100	14	0	0	0	17	0	0	0	0	0	0	0	
SB-229482	0.31	0	1	0	48	22	0	0	27	0	45	0	15	21	11	54	29	16	0	0	37	0	34	11	0	0	0	50	4	89	
GW813349	0.30	3	4	0	2	13	0	9	8	17	0	9	33	0	24	5	0	0	24	17	0	0	24	97	92	93	9	84	0	0	
GSK1287544	0.29	2	3	17	74	40	0	43	83	0	23	2	0	30	100	15	28	0	0	0	100	0	0	0	14	0	18	28	59	0	
GSK1379874	0.28	3	3	0	10	5	0	0	0	0	0	0	0	0	0	33	26	0	0	0	0	0	0	0	0	0	100	16	100	20	
GSK1383281	0.28	20	35	57	74	11	37	2	68	7	11	4	0	1	79	0	74	16	0	0	0	0	0	0	13	38	79	0	73	0	
SB-219980	0.28	9	11	0	9	0	0	0	0	16	1	0	0	0	0	10	20	0	17	52	4	35	7	12	28	10	0	0	0	100	
SB-742034-AC	0.27	1	6	9	13	69	17	2	7	98	22	0	2	89	25	0	30	4	16	13	2	0	4	3	24	5	0	0	0	13	
GSK2286295	0.27	4	5	0	100	7	15	2	0	8	3	12	98	2	0	0	37	19	22	0	99	89	50	10	0	0	19	97	6	0	
GW494601	0.26	36	53	0	21	2	15	0	28	23	0	13	86	24	0	0	100	0	13	2	0	22	0	30	0	0	98	34	84	50	
GSK301362	0.26	7	10	6	47	30	40	0	47	0	14	0	4	40	99	15	67	0	0	0	0	6	2	10	34	0	0	0	0	0	
GSK2358994	0.24	2	3	5	3	0	6	13	10	4	4	0	0	0	0	0	2	0	70	64	100	82	45	0	0	16	7	100	12	0	
GW560116	0.24	7	9	0	0	2	25	0	0	0	0	24	0	27	5	0	95	0	0	0	0	0	0	0	0	23	22	0	36		
GSK1581427	0.24	33	54	55	86	18	19	30	68	0	24	0	0	19	100	22	75	19	0	0	0	0	0	0	0	0	1	0	0	0	
GSK2373690	0.23	2	2	0	0	24	8	0	0	13	1	41	0	2	100	0	14	0	0	0	97	65	21	0	0	0	3	59	9	0	
GW787226	0.23	22	30	17	73	39	0	3	99	0	0	34	84	0	0	0	100	0	5	0	0	0	0	0	15	17	0	17	0	0	
SB-219952	0.22	10	11	100	19	0	0	0	24	0	70	0	9	20	21	31	23	0	56	69	0	16	64	23	2	0	16	35	9	95	
GSK986310C	0.22	12	29	39	59	28	59	0	23	24	16	0	34	7	94	18	80	44	0	0	0	0	3	1	0	0	0	56	0	0	
SB-222903	0.21	6	7	0	0	0	25	6	0	3	0	0	0	0	0	0	17	0	0	0	0	0	0	0	4	0	0	0	0	99	
SB-249175	0.21	14	18	1	54	0	0	2	39	0	65	0	12	0	19	44	8	0	23	1	0	18	45	9	24	23	10	24	15	99	
GSK2276055	0.20	11	14	0	0	0	23	0	0	98	31	0	0	3	48	30	63	6	9	0	11	3	21	7	18	0	76	0	69	8	
GSK1723980	0.20	84	99	74	74	99	0	98	66	74	32	0	98	0	0	0	100	0	7	0	0	0	0	0	0	0	93	0	99	27	
SB-710363	0.19	18	27	0	37	97	32	90	82	99	99	0	23	79	31	0	32	0	0	0	0	0	2	0	10	0	0	0	23	0	
GSK2008607	0.18	115	145	97	98	99	34	94	70	53	77	8	99	0	0	1	100	0	49	0	0	0	0	0	4	95	0	99	81		
GSK1389063	0.18	67	100	91	95	40	18	51	98	0	64	0	0	52	99	13	96	24	0	0	52	2	0	0	27	27	30	35	0	0	
GSK1383280	0.17	25	41	49	63	0	28	3	69	0	18	23	0	22	86	20	75	20	0	0	0	0	0	30	47	0	0	15	11		
GSK2181306	0.16	127	170	89	100	44	100	5	61	98	68	19	7	78	60	67	100	94	12	23	100	34	86	31	5	0	26	88	0	0	
GSK1379722	0.16	6	6	2	0	30	5	31	1	0	100	100	0	12	0	15	8	0	0	0	0	0	0	0	0	16	0	0	0	6	

STRING is an accumulated database of known and predicted protein-protein interactions, including both direct (physical) and indirect (functional) associations (Szklarczyk *et al.*, 2017). It is an interesting tool to consult and utilise for this screen as it can help to interrogate the kinases that are likely central to the molecular mechanism behind the migration phenotype. Proteins that were hits in the screen and have known associations with other proteins that were also hits could be important in future experiments that investigate the migration process. Figure 35 shows the STRING connections between the kinases that were identified as promoters of migration in the RMS spheroid assay. The stem cell growth factor receptor Kit may be an interesting kinase to investigate further as it links two different protein pathway hubs. PLK1, PLK2 and PLK3 were all hits for increasing migration and have known interactions with other proteins that produced the same phenotype and could have important roles in regulating migration. EGFR, the most common target of the compounds that increased migration (as shown in Table 10), has many interactions with other proteins that significantly altered migration, including Kit.

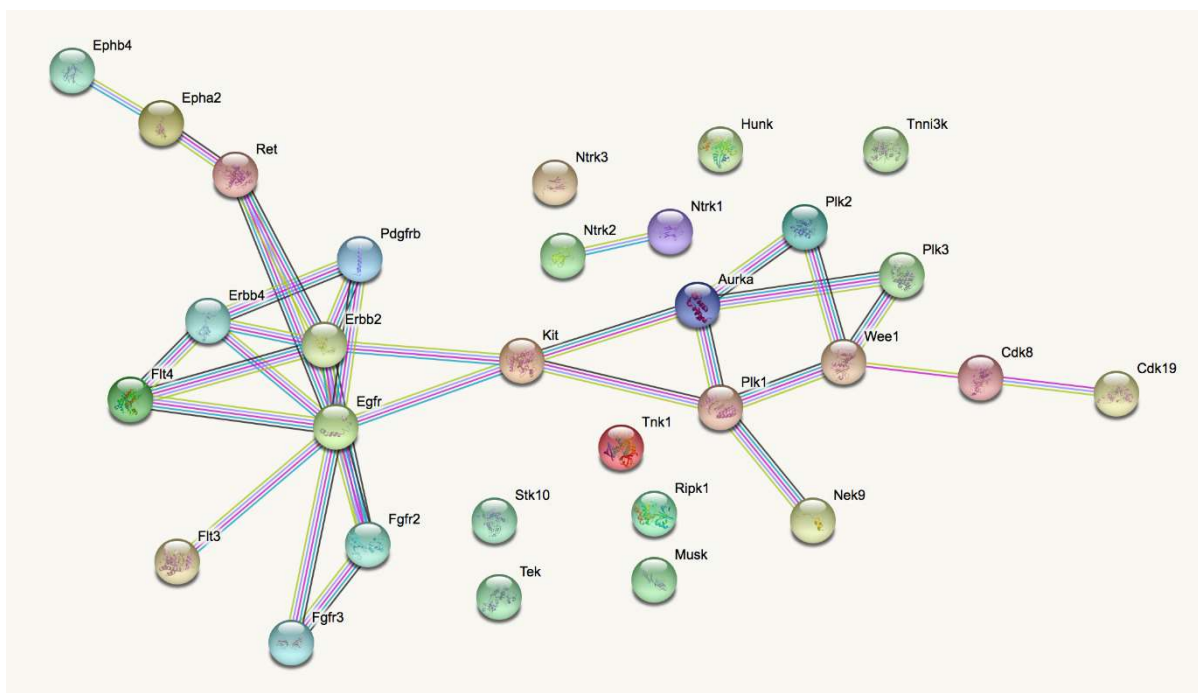


Figure 35 STRING representation of kinase targets that increased migration in the PKIS screen.

Figure 36 shows kinases that decreased migration and the known interactions between these kinases from the accumulated STRING databases (Snel, 2000). Mapk14, an alias for p38a, which was the most common target of compounds that decreased migration is central to the STRING protein interaction map. It is closely associated with Mapk11, an alias for p38b, which was also a significant target for decreasing migration, and both proteins have many interactions in the network. The number of kinases that decreased migration that are closely associated with one another suggests that there may be a common mechanism in this process.

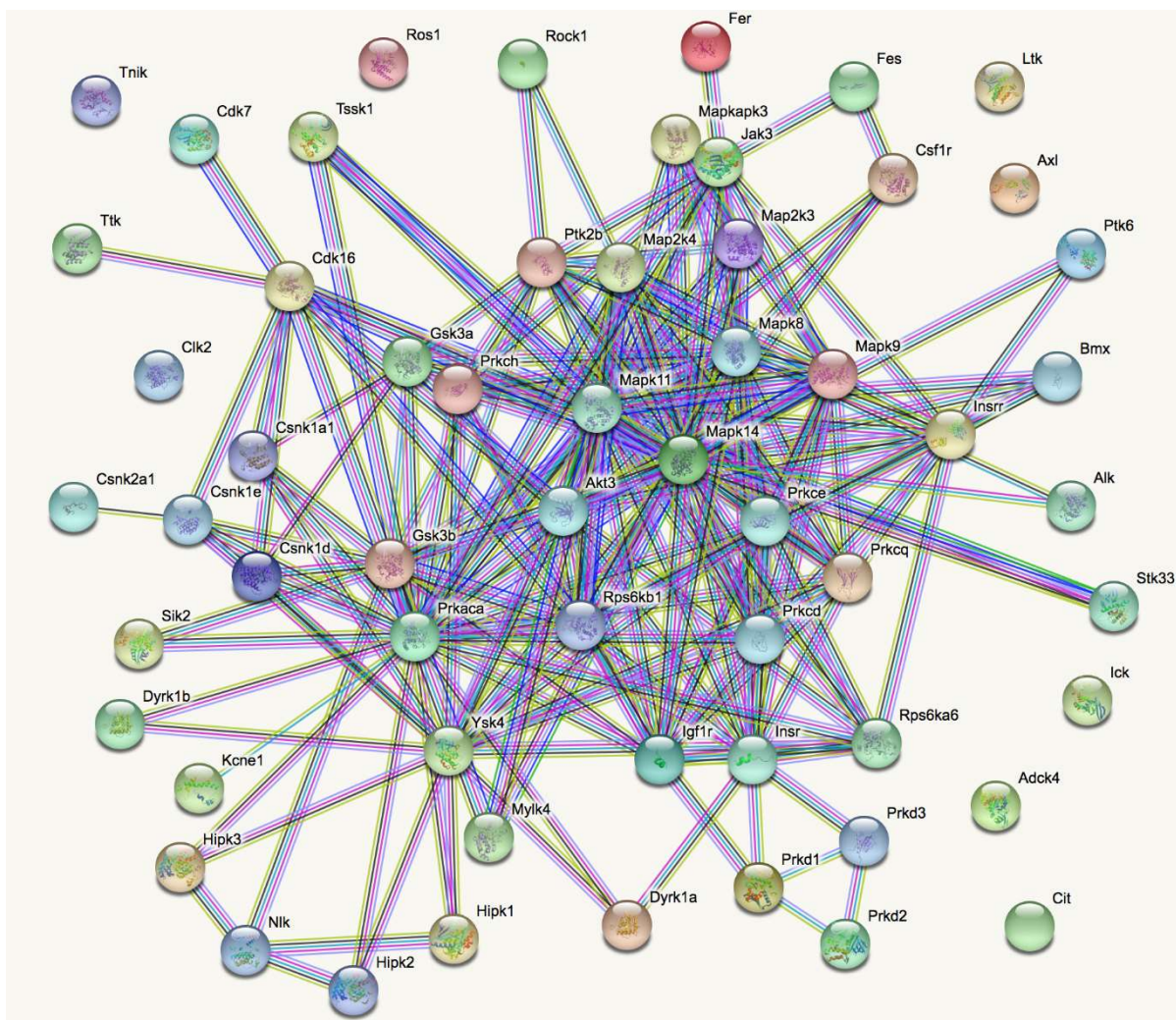


Figure 36 . STRING representation of kinase targets that decreased migration in the PKIS screen.

5.4.3 Inhibition of EGFR has a promigratory effect in the presence of insulin signalling

Cross talk between IGF and EGF signalling is well documented in multiple cell lines (Jin & Esteva, 2008). I have previously shown that there may also be a potential interaction involving EGF and IGF in relation to neuroblast migration from SVZ explants (see Chapter 2). The results from the kinase inhibitor screen add impetus to this line of investigation. To confirm the result from the screen that EGFR inhibition stimulates migration and IGFR inhibition blocks neuroblast migration the spheroid migration assay was repeated with known specific inhibitors of EGFR and IGFR. The inhibitors used were Afatinib, a selective EGFR inhibitor with IC50 0.5nM, and BMS754807 a selective IR/IGF1R inhibitor with IC50 of 1.8nM.

For the confirmation of the migration screen in neuroblast spheroids the cell culture medium was substituted for a medium with no insulin to enable the study of the effects of IGF1 supplementation on the effects of the two inhibitors. Spheroids were cultured overnight, as described in the methods, and then washed and re-plated in Neurobasal media without insulin the following morning. The spheroids were then treated with the inhibitors for 30 minutes prior to embedding and cultured in the media containing EGF, IGF or a combination for 24 hours. The results are shown in Figure 37A/B. As expected, stimulation with IGF1 significantly increased the migration and this effect was entirely blocked by inhibition of IGF1R by BMS754807. EGF stimulation alone significantly decreased migration compared to DMSO control, the effect of which was blocked by Afatinib addition. Inhibition of EGFR by Afatinib did not result in a significant increase in migration when used alone, however when co-stimulated with IGF1 it did result in a significant increase in migration.

EGF has previously been shown to stimulate the migration of SVZ neural stem cells and many cancer cell lines in vitro (Aguirre *et al.*, 2005; Gonzalez-Perez and Quinones-Hinojosa, 2010; Sasaki *et al.*, 2013). To explore the effect of EGFR inhibition on the migration of neural stem cells, an experiment using spheroids comprised primarily of SVZ stem cells was completed. To do this secondary neurospheres were cultured from P4 wild type mice, dissociated and plated in spheroid plates to form 10 uniform spheroids per condition, each containing 10,000 cells. These were then embedded in Matrigel and grown in complete medium supplemented 100nM of either the IGF1R inhibitor BMS754807 or EGFR inhibitor Afatinib. This confirmed that contrary to the neuroblast spheroids inhibiting both IGF1R and EGFR blocked migration entirely (Figure 37C/D).

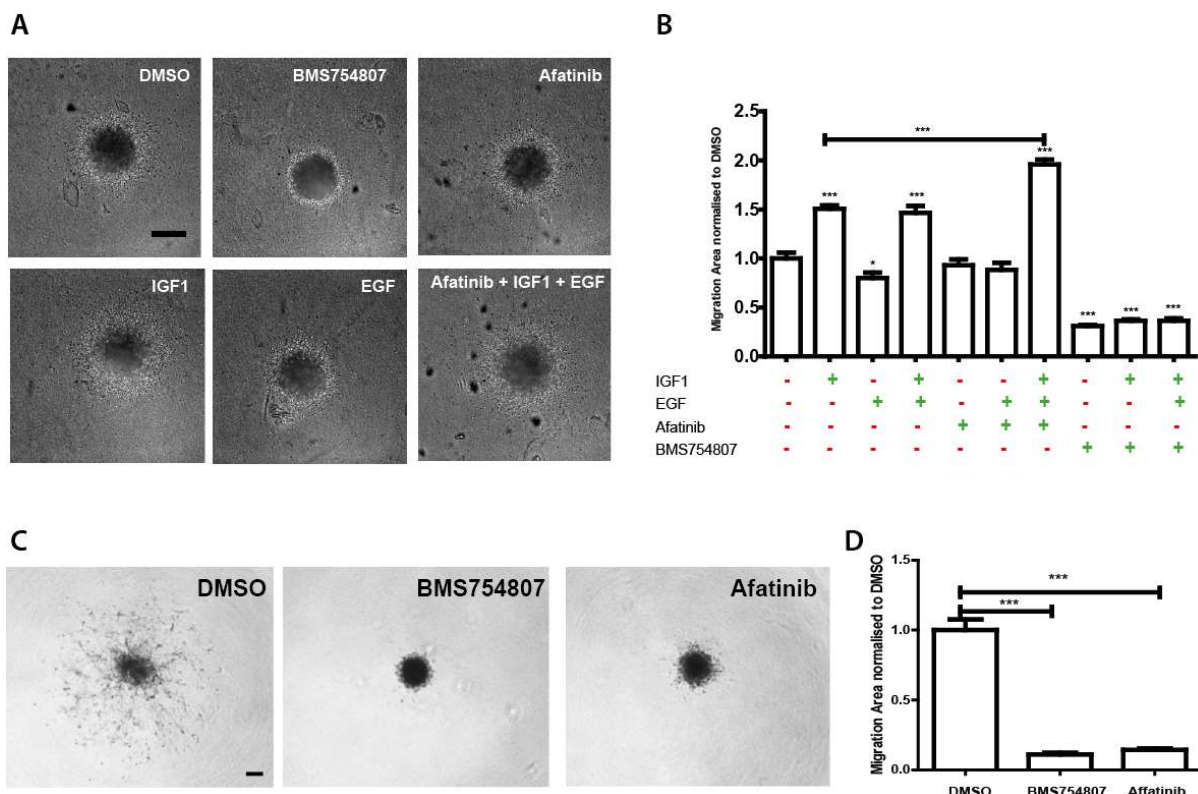


Figure 37 Migration of neuroblasts when cultured with specific inhibitors of EGFR and IGF1R.

(A) Example images of neuroblast migration in the presence of IGF1 and EGF, with and without IGF1R and EGFR inhibitors, BMS754807 and Afatinib. (B) Quantification of migration areas from neuroblast spheroids normalised to the DMSO control (N=6). Afatinib increased the migration of neuroblasts when cultured with IGF1. Error bars equal to SEM. (C) Example images of stem cell migration from neurospheres when cultured with Afatinib and BMS754807. Scale bars = 100µm. (D) Quantification of migration area showed that inhibition of both IGF1R and EGFR blocked migration of stem cells from SVZ neurospheres. Data from one out of two biological replicates each with 8 technical replicates. Error bars equal SEM. Multi-comparison ANOVA were performed followed by Tukey Test for each variable pair; *** p<0.001, ** p<0.01 and * p<0.05.

5.4.4 Inhibition of PLK1 with an orthogonal compound does not promote neuroblast migration

PLK1 was indicated to be a modulator of migration in both PKIS1 and PKIS2 libraries. To investigate whether PLK1 is the bioactive target of the compounds selected in the screen we completed a confirmation experiment with an orthogonal compound. GSK461364 is a potent PLK1 inhibitor developed by GSK and shares the same chemotype as the PKIS libraries (IC₅₀=2.2nM). Volasertib is also a potent PLK1 inhibitor (IC₅₀=0.87nM), but with a different chemotype from the compound from the PKIS screen.

Figure 38 shows migration of cells from spheroids in the presence of these two inhibitors. At the highest concentration of 1µM there was a significant increase in migration for spheroids treated with GSK461364 (p<0.01). There was also a significant decrease in migration distance for spheroids treated with the orthogonal compound Volasertib (p<0.01). The differing results indicate

that PLK1 inhibition may not be solely responsible for promoting neuroblast migration, and that the compounds in the screen likely acted through a PLK1-independent pathway, and may have other actions, or perhaps polypharmacology.

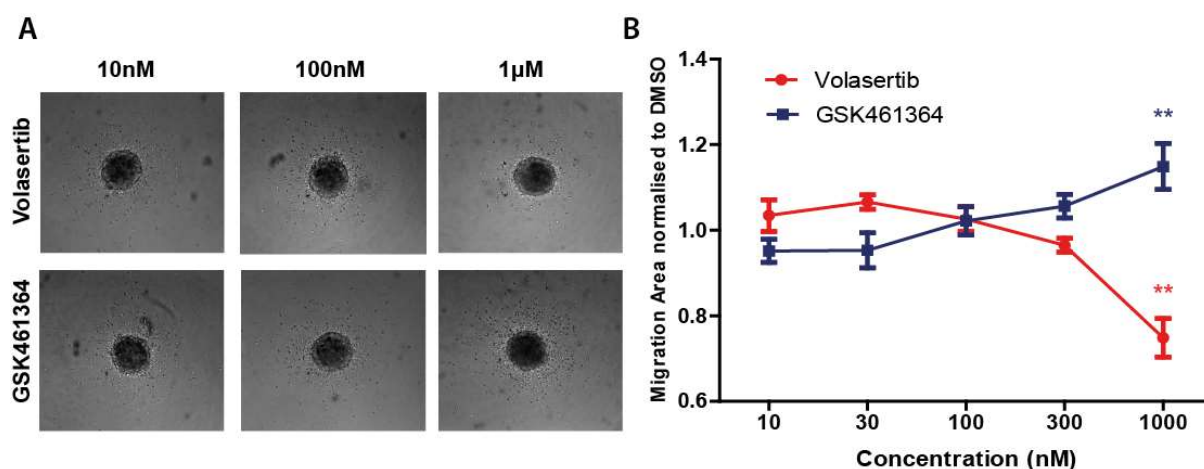


Figure 38 Migration of neuroblasts in spheroid assay treated with PLK1 inhibitors

The effect of ortholog PKIS inhibitor Volasertib and GSK461364 on neuroblast migration were tested at concentrations 10nM-1µM. Volasertib did not increase migration and was toxic at the higher concentrations. Data from one out of two biological replicates each with 3 technical replicates. Un-paired two-way t-test completed for each data point against DMSO control with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

5.4.5 Inhibition of MuSK and PIK3CB promotes the migration of neuroblasts

A common criticism of phenotypic assays is that they tend to yield the same 'low-hanging fruit' in terms of mechanisms of action and thus provide relatively little contribution to the identification of novel therapeutic strategies. The data from this screen was not immune to this bias so the next priority was to investigate some of the less well studied kinases that were implicated in the migration of neuroblasts by the spheroid screen.

Six different kinases were selected for validation using genetic knockdown. The kinases MUSK, NEK9, ABL1 and HUNK were selected based on analysis of the affinity matrices discussed earlier. PIK3CB was selected because it was the only indicated binding partner of GSK2336394A, a compound validated to increase migration. The RTK MERTK was also selected based on analysis of an early annotation of the PKIS1 library. Knock downs for each kinase were generated with shRNAs and verified by qPCR. Neurosphere migration assays were also performed for all six knock downs. 10,000 nucleofected neurosphere cells were formed into uniform spheres overnight, embedded in Matrigel and then left to migrate for 24 hours. Figure 39 shows the

knockdown efficiencies and the migration areas for the neurospheres. Based on the results from the shRNA confirmation qPCR showing the poor efficiency of the knockdown, it was not surprising that there was no difference in migration area for MERTK, ABL1, NEK9, or HUNK. There was, however, a 1.65-fold increase in migration area for MUSK shRNA knockdown when compared to controls ($p < 0.01$), with these neurospheres showing the best knockdown efficiency. There was also a non-significant increase in migration detected for PIK3CB, with the expression level of PIK3CB was 0.4 of the control.

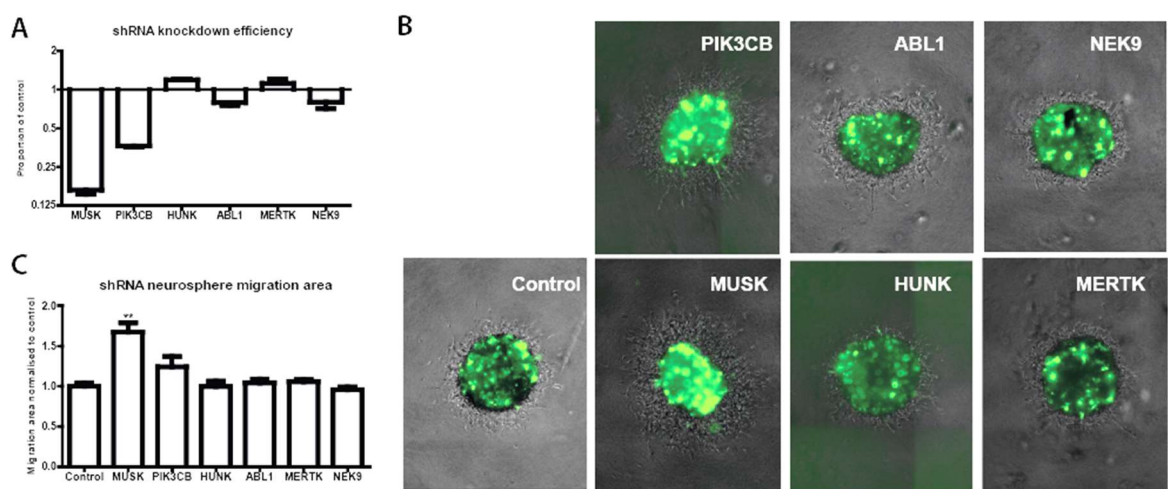


Figure 39 Genetic knockdown of kinase targets.

(A) Efficiency of the shRNA knockdown of the genes for kinase targets: MUSK, PIK3CB, MERTK, ABL1, NEK9 and HUNK relative to a control plasmid. From the genes tested only plasmids targeting MuSK and PIK3CB were effective. **(B)** Images of migration from neurospheres that had been nucleofected with shRNA plasmids targeting kinase genes. **(C)** Quantification of migration area around neurospheres nucleofected with shRNA plasmids targeting kinase genes. Knockdown of MuSK significantly increased migration ($N=6$). Multi-comparison ANOVA were performed followed by Tukey Test for each variable pair; *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

The genes MUSK and PIK3CB, which showed the most success in regulating migration in the neurosphere migration in Figure 39, were then investigated by knock down in the RMS spheroid assay. Migration of neuroblasts expressing plasmids for pGIPZ (GFP controls), anti-MUSK and anti-PIK3CB were measured (shown in Figure 40). Knockdown of both MUSK and PIK3CB significantly increased the average distance migrated compared to control cells. Interrogation of the Allen Brain Atlas also shows that PIK3CB, MUSK and its ligand LRP4 are specifically highly expressed in the ventricular region, further supporting their role in neurogenesis.

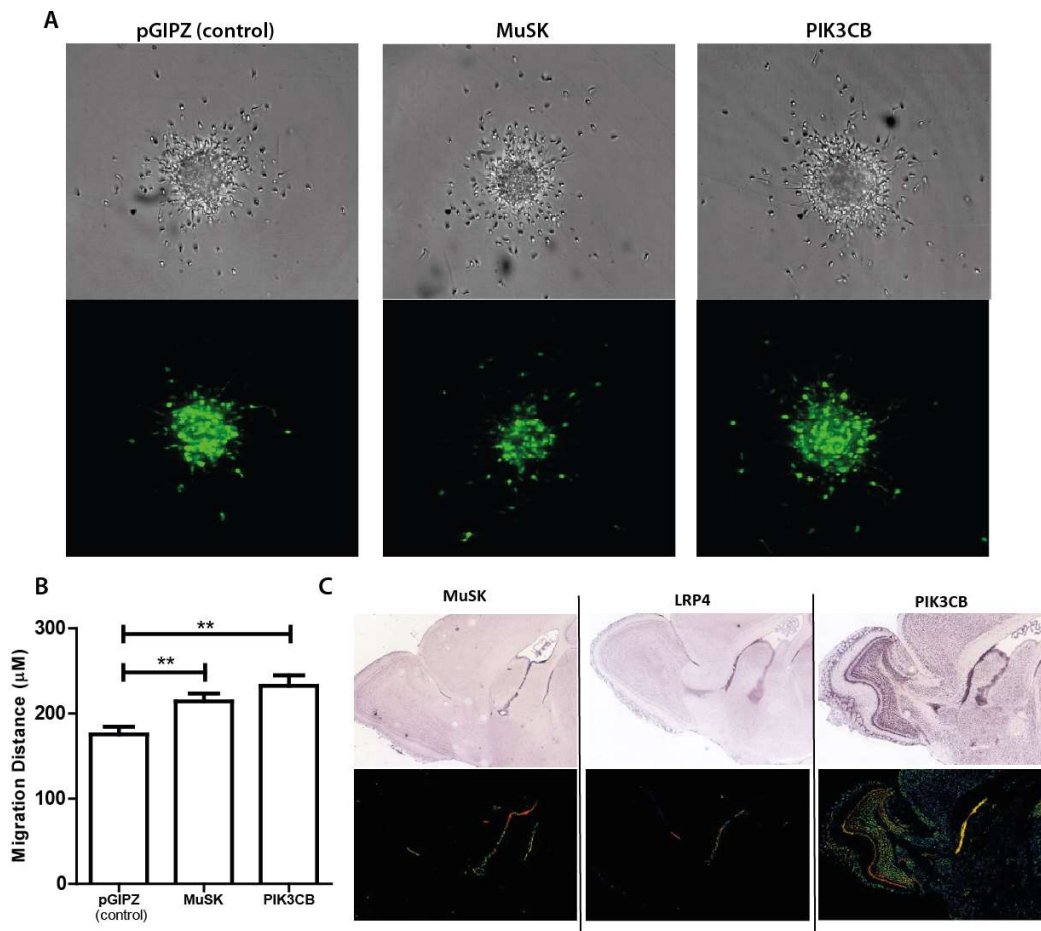


Figure 40 Knockdown of MuSK and PIK3CB increased migration of neuroblasts from 3D spheroids. **(A)** Representative images of RMS spheroids nucleofected with shRNA plasmids targeting MuSK and PIK3CB. **(B)** Quantification of the distance migrated by nucleofected neuroblasts shows that knockdown of both MuSK and PIK3CB increased the average distance travelled. Data shown from one out of three biological replicates, each with 8 technical replicates. Un-paired two-way t-test completed with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. **(C)** Images showing the expression of MuSK, LRP4 and PIK3CB in sagittal mouse brain sections (taken from Allen Brain Atlas).

5.5 Discussion

5.5.1 Small molecule kinase inhibitors modulate migration of neuroblasts from RMS spheroids.

The results presented in this chapter show the potential to use the 3D spheroid migration assay to assess the modulation of SVZ neuroblast migration by small molecules. 21 compounds were found to successfully increase migration of neuroblasts. Neuroblasts were most responsive to these compounds at different concentrations, for example GW607049C, GW459135A, and GSK2336394A increased migration at lower concentrations, whereas GW693881A and GW854278X only gave a significant effect at the highest concentration. When choosing compounds to take forward for potential therapeutic purposing the effective dose may be important to consider. 36 compounds significantly reduced migration of neuroblasts, with all compounds effective at decreasing migration distance at 10 μ M.

EGFR and PLK1 were the most common intended targets of the pro-migratory compounds. Further investigation of the PKIS is needed to determine whether this is due to a disproportionately large number of compounds in PKIS being designed to target EGFR and PKIS, or because these were the most effective targets. For compounds that decreased migration p38 α was the most common single target. Over a third of the compounds that significantly inhibited migration had promiscuous targets, suggesting that multiple pathways were disrupted, which could prevent compensatory molecular mechanisms from becoming activated.

None of the compounds that were found to be toxic have been thoroughly analysed, and this may be useful to do in the future to identify if there are common targets amongst these toxic inhibitors. This may be important both in determining mechanistically what genes are essential in neuroblast migration, and also what targets may not be good to focus on for therapeutic purpose. A more thorough analysis of the structure of the compounds selected by a medicinal chemist is still required. It is possible that some compounds may be able to be modified to be more effective or better suited for medicinal use.

5.5.2 Inhibition of MuSK and PIK3CB increases the migration of neuroblasts.

A primary objective of this screen was to identify key kinase pathways and previously uncharacterized kinases that may have key regulatory roles in neuroblast migration. Interestingly, the inhibition of several receptor tyrosine kinases was indicated to increase neuroblast migration. These included: EGFR, ErbB4, FGFR, VEGFR3, KIT, PDGFR β , TrkA and Ephs. It is likely that this cluster is in part due to the promiscuity of the molecular probes used, nevertheless it indicates that cellular functions relating to proliferation and differentiation may be actively shut down during neuroblast migration. This corresponds with literature evidence that many of these receptors are significantly downregulated in neuroblasts and that migration is perturbed by genetic disruption of either the ligand or receptor (Kim *et al.*, 2009; Ramos *et al.*, 2013; Sato *et al.*, 2016; Todd *et al.*, 2017). This result raises the concern that cancer therapies targeted against RTKs may result in a more invasive phenotype. A more rigorous analysis using specific genetic knockdown of RTKs is needed to investigate this further.

Using a specific inhibitor of the EGFR we were able to show that inhibition of EGFR on its own does not increase neuroblast migration, but it is able to reverse the inhibition of migration caused by exogenous EGF. This indicates that like IGF2, EGF acts as a paracrine signalling molecule on neuroblasts. Curiously it was also shown that inhibition of EGFR further sensitizes the spheroids to IGF1, thus indicating some cross talk between the two signalling pathways. This is not entirely surprising, albeit the underlying mechanism is yet to be elucidated. In Chapter 2 of this thesis it was shown that EGF can inhibit the IGF1 driven increases in neuroblast migration *ex vivo* SVZ explants. It is known that IGF1R stimulation is necessary for EGF and FGF2 expansion of SVZ neurospheres (Arsenijevic *et al.*, 2001). Activation of the IGF1R by high levels of insulin in standard culture media in the presence of EGFR activation results in increased levels of phosphohistone 3, phospho-retinoblastoma protein and cyclin D. The increase in the levels of these proteins was not observed in cell culture media that contained either high levels of insulin alone or low levels of insulin plus EGF, which indicates that activation of both the IGF1R and EGFR are necessary for cell-cycle progression (Alagappan *et al.*, 2014). ErbB:IGF1R synergies and cross talk have been studied in greater detail in various other cell types. Both the type I receptor tyrosine kinase family members (ErbB/1–4) and the type II receptor tyrosine kinase family members (IGF1R and IR) have been found to form homodimers or heterodimers with each other (Nahta *et al.*, 2005; Morgillo *et al.*, 2006; Jin and Esteva, 2008). For instance, IGF1

stimulates a novel EGFR-dependent signalling transduction through the physical interaction between IGF1R and EGFR, activating the ERK pathway and inducing proliferation in human mammary epithelial cells (Ahmad *et al.*, 2004). The response, measured by pAkt, of breast cancer cell lines to IGF is not correlated with IGF1R expression, but strongly inversely correlated with ErbB2 and EGFR expression (Niepel *et al.*, 2014). Inhibition of EGFR by gefitinib or erlotinib, sensitizes cells to the IGF1R-IRS1-Akt axis by dissociating IRS1 from EGFR (Buck *et al.*, 2008; Knowlden *et al.*, 2008). IGF1R signalling can also 'transactivate' EGFR signalling by regulating the matrix metalloprotease-dependent release of the ligands for EGFR activation, such as amphiregulin, heparin-binding EGF or TGF α (Roudabush *et al.*, 2000; Desbois-Mouthon *et al.*, 2006). Finally, there is evidence that EGFR and IGF1R interact via a complex involving integrins and Focal Adhesion Kinase (FAK) (Ross, 2004; Fujita *et al.*, 2013).

Inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta isoform (PIK3CB) was found to significantly increase neuroblast migration. PIK3CB is one of the four catalytic subunits of PI3K. In Chapter 2 of this thesis we showed that pan inhibition of all four subunits by LY294,002 reduced migration from explants. Interestingly although in non-neuronal cells these catalytic subunits may have partially redundant functions, there is increasing evidence that in neurons their roles are more specialised, and confined to distinct receptor-dependent pathways. For instance, while the key mediator of IGF1R signalling is PIK3CA (Knight *et al.*, 2006), PIK3CB is the major catalytic isoform downstream of the ErbB4 (Law *et al.*, 2012). Activation of PIK3CB by ErbB4 is orchestrated via Yes-associated protein (YAP), the nuclear effector of Hippo signalling (Yan *et al.*, 2017). It is therefore tempting to consider the opposing function of IGF1R and the ErbBs in neuroblasts migration may involve differential activation of PIK3CA and PIK3CB. PIK3CB is also the predominant subunit associated with GPCRs (Guillermet-Guibert *et al.*, 2008). The phosphatase and tensin homologue (PTEN), a negative regulator of PI3K activity, was shown to preferentially bind to PIK3CB compared to other PI3K catalytic subunits. PTEN deletion has been shown to disrupt neuroblast migration and, like PIK3CB, has been linked to autism (Zhu *et al.*, 2012). However, the specific regulation of PIK3CB by PTEN in the SVZ remains unstudied.

Genetic validation with shRNA knockdown further supported a function of Muscle-specific tyrosine kinase receptor (MuSK) in neuroblast migration. MuSK is a receptor tyrosine kinase that is mainly expressed in the muscle and plays an essential role in neuromuscular junction (NMJ) formation (Garcia-Osta et al. 2006). MuSK is critically involved in redistributing the acetylcholine receptors (AChRs) in the postsynaptic apparatus. This function requires the interaction of MuSK with the heparan-sulfate proteoglycan Agrin and Low-density lipoprotein receptor-related protein 4 (Lrp4). In muscle, the activation of MuSK via the ErbB–neuregulin pathway results in an increase of AChR expression and triggers the activation of signalling pathways that lead to postsynaptic differentiation (Burden *et al.*, 2013).

The role of MuSK in the central nervous system is poorly understood, however it has been reported to have a role in memory consolidation (Garcia-Osta *et al.*, 2006; Sun *et al.*, 2016). MuSK is concentrated at excitatory synapses, marked by coexpression of PSD-95 in the CNS and according to the Allen Brain Atlas MuSK, Lrp4 and Agrin expression is evident in the SVZ and hippocampus (Ksiazek *et al.*, 2007). Mice and zebrafish deficient in MuSK have defects in neural crest migration (Banerjee *et al.*, 2011). In the absence of MuSK, streaming neural crest cells, which are normally confined to the central region of each somite, are dispersed and present throughout the somite. Specific knockdown of Agrin in the SVZ by postnatal electroporation disrupted integration, morphological differentiation and survival of new olfactory bulb interneurons (Burk *et al.*, 2012).

Inhibition of MuSK is not an especially attractive prospect for therapeutic development due to the high chance of negative side effects. In humans, mutations in MuSK are associated with congenital myasthenic syndromes (CMS), which are characterized by varying degrees of muscle weakness due to impaired neuromuscular transmission. Patients with myasthenia gravis, an autoimmune disease associated with deficient acetylcholine receptor transmission and impaired olfaction, often also present anti-MuSK antibodies (Leon-Sarmiento *et al.*, 2012). Nevertheless, further characterisation of the role of MUSK in adult neurogenesis is warranted.

The PLK family was also suggested by analysis of the hits of the screen. However, upon validation via an orthologous PLK1 inhibitor the increased migratory phenotype was not confirmed. It is therefore likely that increase in migration is directed by inhibitions of a yet

unknown kinase target. It is not unknown for PLK to come up in a phenotypic screen, only to be found later not to be the biological mechanism. The leukaemia drug Rigosertib was identified by a phenotypic screen and first entered the clinic annotated as a PLK1 inhibitor. However, it has since also been described as a multi-targeted PI3K inhibitor and its preclinical efficacy was attributed to downregulation of cyclin D1 and to the induction of oxidative stress through unknown mechanisms. It is also possible that the increased migration detected in the screen was due to other members of the PLK family, which contains at least five PLKs (PLK1-PLK5) and several related kinases. Hence a more thorough analysis using combinations of genetic knockdowns are required to fully preclude a role of the PLKs in neuroblast migration. Close scrutiny of the screen data reveals one potential option for this, the closely related kinase: LOK/Stk10. STK10 is a negative regulator of MAP3K1 and JNK that has been shown to be a potent negative regulator of lymphocyte migration (Belkina *et al.*, 2009). According to the Allen Brain Atlas it is also specifically expressed in the anterior SVZ and is therefore an attractive candidate to explore in the next round of target validations.

5.5.3 Kinase targets indicated to inhibit the migration of neuroblasts.

The phenotypic assay also provided data on the potential kinase targets of the compounds that blocked migration. This data can be used in the future to provide potential therapeutic strategies for cancer or in research into the mechanisms of adult neurogenesis in the RMS. The kinase targets of the compounds that inhibited migration are yet to be further validated experimentally. Nevertheless, initial analysis of the affinity matrices both provides confidence in the assay by identifying pathways known to be critical to neuroblast migration and also suggests a role of some lesser studied kinases. It is beyond the scope of this thesis to discuss each potential kinase hit in detail but a few warrant further investigation.

The mitogen-activated protein (MAP) kinase family are well-documented regulators of migration in various cell types (Huang, 2004). Several are indicated by the screen as potential mediators of neuroblast migration, including MAPK8/Jnk1, MAPK9/Jnk2, MAPK14/p38a and MAPK11/p38b. Of these p38 α can be found within the top hits selected by all methods used to interrogate the screen data set. The role of p38 α in the SVZ remains incompletely understood but evidence supports a

role in switching between proliferation and migration. It is highly expressed in the brain from E10 and is retained in the SVZ and Choroid Plexus of the adult brain (Sato *et al.*, 2008; Kim and Wong, 2012; Yoshioka *et al.*, 2015). Pharmacological inhibition has been shown to increase proliferation and decrease migration of SVZ stem cells *in vitro* (Sato *et al.*, 2008; Yoshioka, *et al.*, 2015). A cell permeable p38 enhances migration *in vitro* without affecting cell survival or differentiation (Hamanoue *et al.*, 2016). p38 also correlates with poor prognosis in several types of cancers, including glioblastoma (Goldsmith *et al.*, 2018). p38 inhibitors have, therefore, attracted significant attention for use in chemotherapy, with at least 22 different compounds being investigated in Phase I/II. Yet despite their potential, high toxicity and off-target effects have severely limited their therapeutic value and none have reached Phase III. It is therefore of significant interest to identify the downstream effectors of p38. One possibility proposed by the screen data set in MAPK-activated protein kinase 3 (MAPKAPK3). This is a known substrate of p38 that is essential for migration of smooth muscle and endothelial cells (Rousseau *et al.*, 1997; Hedges *et al.*, 1999). This mechanism has also been shown to regulate the surface expression of EGFR (Frey *et al.*, 2006).

Protein Kinase B (Akt) is a key effector in the IGF1R/PI3K/Akt pathway that exists as three isoforms: Akt1, Akt2 and Akt3. The screen data only indicates Akt3 as a modulator of neuroblast migration. This is interesting because Akt3 is found in high concentrations in the brain and evidence from knockout animals indicate a significant role in brain development. Akt1^{-/-} mice display impaired overall growth, whereas Akt2^{-/-} mice are insulin intolerant, demonstrating a diabetes-like syndrome (Fayard, 2005). Akt3^{-/-} mice, however, have a normal glucose metabolism, have approximately normal body weight, but have a 25% reduction in brain mass. In humans, mutations in Akt3 result in focal malformations of cortical development and epilepsy (Baek *et al.*, 2015). It would therefore be interesting to investigate the specific role of Akt3 in IGF signalling in the SVZ.

Dual specificity Yak 1-related kinase 1A (DYRK1A) and the closely related DYRK1B, HIPK1 and HIPK2 all came up as potential targets for compounds that inhibited migration. *DYRK1A* is the human orthologue of the *Drosophila minibrain (mnb)* gene, named because flies with a mutation

in this gene have smaller brains and distinct learning and behavioural defects (Tejedor *et al.*, 1995). In humans, truncating mutations in the DYRK1A gene cause primary microcephaly (Courcet *et al.*, 2012) and autism (Roak *et al.*, 2013; van Bon *et al.*, 2016). Several lines of evidence suggest that the Dyrk1A may also underlie the mental retardation phenotype of Down's syndrome (Yin *et al.*, 2017). A promising recent study utilised a potent inhibitor DYRK1A to rescue aberrant radial migration and dendritic spine development in a mouse model the disease (Nakano-Kobayashi *et al.*, 2017).

Misshapen-like kinase 1 (MINK1) is a member of the germinal centre kinase (GCK) family that has not been studied at all in relation to SVZ neurogenesis despite previously been implicated in cerebral development. It is abundantly expressed in the mouse brain, up-regulated during postnatal mouse cerebral development and it has been shown to activate various pathways involved in migration including JNK, p38 and Akt (Dan *et al.*, 2000). The *C. elegans* orthologue, MIG-15, is required for migration of central nerve cells (Q cells) (Su *et al.*, 1998). While the *Drosophila* ortholog Misshapen (Msn) has been shown to regulate embryonic dorsal closure, photoreceptor axon pathfinding, and planar cell polarity (Ruan *et al.*, 2002). Recently it has been suggested that MINK1 may serve as a novel activation pathway of JNK1 essential for correct growth cone, synapse formation, and neural stress response (Crawley *et al.*, 2017; Larhammar *et al.*, 2017)

Protein Kinase C (PKC) is another family of kinases strongly associated with neural migration that is also identified in the screen. Interestingly, however it is the related Protein Kinase D (PKD) family that is indicated more strongly by the screen data. The PKDs are downstream of PKC and there is ample evidence that they regulate cell migration and invasion in other cell types, including glioblastoma cell lines, by modulating membrane and integrin transport (Azoitei *et al.*, 2011; Bernhart *et al.*, 2013). The function of this family of kinases in the SVZ is still to be determined.

Casein Kinase 1 (CK1/CSNK1a1), a key component of the Wnt/ β -catenin signalling pathway, and the related Casein Kinase 2 (CK2/CSNK2a1), are also indicated as potential regulators of neuroblast migration (Cruciat, 2014). Both CK1 and CK2 are abundantly expressed in the brain, yet very little is known about their function (Faust and Montenarh, 2000). Recently, however, the inhibition of CK2 has gained a lot of interest as a potential strategy to block the invasion of glioblastoma (Pencheva *et al.*, 2017).

5.5.4 Challenges and Limitations

Kinase inhibitor promiscuity is generally considered beneficial in phenotypic drug screening. Due to the large array cellular processes mediated by kinases and the complex adaptive system that is intracellular signalling, the promiscuity permits the selection of favourable combinations of interactions that otherwise would be challenging to predict. However, promiscuity inevitably makes it difficult to disentangle the precise mechanisms through which these kinase inhibitors exert their effects. The PKIS libraries are well annotated, however the affinity data available is not exhaustive and each compound may potentially interact with multiple unknown pathways. Selecting compounds with multiple modes of action via phenotypic screens is currently considered the preferred drug discovery strategy (Peters, 2013). However, compound promiscuity means that it is difficult to make conclusions about targets that regulate neuroblast migration. Further experiments to interrogate the targets identified is now needed to identify the pathways involved in neuroblast migration in the SVZ.

The major translational impact of this project lies in the potential for discovery of novel targeted therapies designed to drive regeneration after injury or prevent invasion of brain cancer. This discovery would drastically change patient survival outlook and lead to a previously unknown molecular pathway that has wide therapeutic application. However, it poses the question of whether or not identifying the specific mechanism of action is actually necessary. Especially given that a substantial number of FDA-approved drugs (estimates range from 7% to 18%) lack a defined molecular target. It also highlights the critical limitation of this study at the moment: Will increasing the migration of neuroblasts from 3D RMS spheroids result in an improved clinical outcome? Gaining clarity to that question in humans will require considerable time, but *in vivo* confirmation of the efficacy of the small molecules identified in mouse models of stroke and injury is achievable. It is important to consider that the majority of the compounds in the PKIS are molecular probes and will not have the pharmacodynamics and oral bioavailability required for *in vivo* treatment.

With regards to the running of this screen it was limited by the usual confines of a DPhil project. The GSK PKIS libraries were only supplied in sufficient quantity to run the screen and for one round of confirmation. This meant that we were unable to repeat non-ideal experiments or complete extensive follow up on the individual compounds selected. The genetic validation stages of this project also proved very troublesome. The initial aim was to triage the kinase genes

identified by the small molecule screen into a secondary focused screen using lentiviral based shRNA knockdown. However, this was not possible and the use of nucleofection substantially limited the number of genes that could be validated. It is recommended that prior to any future screens using the spheroid assay that arrayed genetic manipulation is optimised.

5.5.5 Future Work

The large diversity in chemical structures of hit compounds suggests that neuroblast migration may be promoted through modulating a variety of target kinases and corresponding host networks. Additive effects may therefore be achieved by co-treating spheroids with hits that act via distinct biological targets. Applying machine learning algorithms may also help to illuminate the most relevant biological targets and systems. One potential option would be to use Support Vector Machines (SVM). This is a computer-based learning algorithm that recognizes patterns given examples belonging to each of two categories and builds a model that can classify new examples into either category. Thus, it can be used to predict kinase inhibition profiles that will have maximal biological effect. This method is still limited by the Nanosyn and KinomeSCAN annotations and the pharmacological linkage of kinases. Nevertheless, it is a necessary next step in the exploration of this data set. The predictions will become candidates for follow up genetic knockdown experiments, or chemical probe development. Once specific compounds or kinases are selected it will be important to investigate the stage of migration that is being targeted. Other complimentary migration and chemotaxis assays should be utilised at this stage. These should include live cell time lapse imaging to study individual cell dynamics. It is also absolutely essential that any effect is validated *in vivo*.

Neuroblast migration is coordinated and regulated by multiple signalling pathways both intrinsic and external. An interesting direction for future research will be to identify the downstream convergence points of the kinases. For this purpose, the development of a technique to monitor kinase activity in live cells will be valuable. Several Förster resonance energy transfer (FRET) and kinase translocation reporter (KTR) based biosensors have been developed that could be utilised (Maryu, Matsuda and Aoki, 2016; Sizaire and Tramier, 2017). Comparative phosphoproteomics of cells following genetic knockdown or overexpression of specific kinases will also be useful.

Chapter 6 Discussion

6.1 Thesis Summary

6.1.1 IGF1 and IGF2 stimulate the migration of SVZ neuroblasts

Several growth factor and neurotrophic factors have previously been suggested as modulators of neuroblast migration. To investigate the mechanism of action and explore the potential of these proteins as neuroregenerative therapeutics, a panel of growth factors were tested for their effect on the migration of neuroblasts from SVZ explants. Only IGF1 and IGF2 significantly increased the migration of neuroblasts in an ex-vivo explant model of migration. While this does not necessarily negate an *in vivo* function for other growth factors, it suggests that they may modulate neuroblast migration indirectly.

To determine how IGFs increase neuroblast migration from explants we manipulated the IGF1R and the two major downstream signalling cascades it activates: MAPK/ERK and PI3K/AKT. Inhibiting the IGF1R with the small molecule PPP blocked the migratory response to IGF1, indicating that the phenotype is instructed via the IGF1R not the IR. The small molecule LY294,002, which inhibits the catalytic subunit of PI3K, blocked the IGF1 induced stimulation of SVZ neuroblast migration, while the ERK inhibitor U0126 did not. Together these results indicate that neuroblast migration is instructed via the IGF1R/PI3K/Akt pathway. Albeit this remains to be conclusively demonstrated with more precise pharmacological or genetic manipulations.

The IGF family includes ligands, receptors and IGF binding proteins (IGFBPs). Classically, there are three ligands: Insulin, IGF1 and IGF2, three cell surface receptors: insulin receptor (IR), IGF1R and IGF2R and at least seven IGFBPs. We found several genes in this family to be differentially expressed in the RMS compared to the SVZ. Stem cells in the SVZ are proliferative until they begin their migratory route via the RMS. Therefore, we predict the differentially regulated genes in the RMS compared to the SVZ tissue to have a role in the migration process. The IGF1R, IR, IGFBP1, and IGFBP2 were upregulated in the RMS, whereas IGFBP3 was suppressed. These genes could be further investigated to determine how they influence the migration switch following neurogenesis.

The function of IGF1 and IGF1R in neuroblast migration in the SVZ has been described previously in the literature (Hurtado-Chong *et al.*, 2009; Maucksch *et al.*, 2013; Chaker *et al.*, 2015). A recent paper by Nieto Guil *et al.* (2017) described the live cell dynamics of IGF1R

mediated neuroblast migration in the embryonic cerebral cortex. Using Golgi and actin specific stains on IGF1R-null neuroblasts they show that IGF1R signalling is necessary for correct polarity, orientation and axon formation; and that this is dependent on PI3K. It is likely, but not certain that a similar mechanism regulates postnatal RMS neuroblast migration and an analogous live cell imaging study is recommended.

Within the SVZ neurogenic niche there is a complex assortment of different signalling molecules and recently more significance has been given to the cross talk between the different signalling pathways. For example, IGF1R signalling has been shown to be necessary for EGF and FGF2 mediated proliferation of SVZ derived neurospheres (Adepoju *et al.*, 2014; Alagappan *et al.*, 2014). Co-stimulation with IGF1 has also been shown to modulate the function of CNTF and BDNF *in vitro* (Konagaya *et al.*, 2011). Interestingly when explants were cultured in the presence of EGF and IGF1, neuroblast migration was not increased. Therefore, indicating contradictory cross talk between EGF and IGF signalling that directs the initiation of migration. It also implies removal of basal insulin signalling may have masked the response to the other growth factors tested. Using the more accurate 3D RMS spheroid assay we were able to show that stimulation with EGF does inhibit neuroblast migration and that this is reversed by inhibition of the EGFR. Curiously it was also shown that inhibition of EGFR further sensitizes the spheroids to IGF1, thus supporting cross talk between the two signalling pathways. ErbB:IGF1R synergies and cross talk have been studied in great detail in various other cell types; albeit the underlying mechanism is debated. The response of breast cancer cell lines to IGF1 is strongly inversely correlated with ErbB2 and EGFR expression (Niepel *et al.*, 2014). Inhibition of EGFR by gefitinib or erlotinib, sensitizes cells to the IGF1R-IRS1-Akt axis by dissociating IRS1 from EGFR (Knowlden *et al.*, 2008; Buck *et al.*, 2008). The ErbB and IGF1R/IR have been found to form heterodimers with each other, which can modify intracellular signalling (Morgillio *et al.*, 2006; Nahta *et al.*, 2005; Jin & Esteva, 2008). IGF1R signalling can also 'transactivate' EGFR signalling by regulating the matrix metalloprotease-dependent release of the ligands for EGFR activation, such as amphiregulin or heparin-binding EGF or TGF α (Roudabush *et al.*, 2000; Desbois-Mouthon *et al.*, 2006). There is, also evidence that EGFR and IGF1R interact via a complex involving integrins and Focal Adhesion Kinase (FAK) (Ross, 2004). Investigating these mechanisms further in the SVZ may instruct one of the most evasive aspects in adult neurogenesis, the proliferation:migration transition .

6.1.2 Maternal transmission of the loss of function *Igf2r*^{1656A} allele increases proliferation in the VZ/SVZ of the neocortex

While the function of IGF1R and IGF in SVZ neurogenesis has been studied extensively, less attention has been paid to IGF2. We investigated the neural phenotype of a novel mouse model, in which a single knockin point mutation results in a loss of Igf2 binding to the IGF2R without disruption to other binding and catalytic functions. Homozygotes (*Igf2r*^{1656A/1656A}) developed perinatal mortality (>90%) confirming a critical function of IGF2R in development. The paternal IGF2R allele is considered to be silenced in all tissue during early embryology. However, maternal allele transmission of *Igf2r*^{1656A/+p} resulted in only partial neonatal lethality (~60%), potentially indicating a partial loss of imprinting and rescue by wild type paternal allele. Maternal allele transmission of *Igf2r*^{1656A/+p} resulted in embryo brain and body overgrowth, and increased proliferation in the neocortical VZ and SVZ, consistent with stimulation of cell proliferation of radial glial stem cells and intermediate progenitors, respectively. No significant difference was detected in the proliferation of progenitors in the LGE of *Igf2r*^{1656A/+p} embryos indicating that the regulation of IGF2R and IGF2 signalling may differ between these two structures. No gross disruption of the cortical layer V/VI marker, CTip2, was observed, suggesting neither neuronal migration or cortical layering were significantly disrupted. It has subsequently been brought to our attention that IGF2R imprinting may be lost in neurons. So additional work is needed to draw firm conclusions from this study.

6.1.3 Phenotypic screen of neuroblast migration

The molecular mechanisms that regulate postnatal neuroblast migration is an understudied field in developmental biology. This is in part due to the absence of high fidelity *in vitro* migration assays. Conventional platforms, such as the transwell migration assay, are not suitable because culturing neuroblasts in a monolayer removes interactions between cells and the extracellular matrix preventing the formation of the characteristic neuroblast chains. Moreover, the stiffness of the plasticware flattens the cells further altering their motility (Kawaguchi, Kageyama and Sano, 2017). These issues prompted the development of culture systems that recapitulate more complex cell diversity and extracellular matrix interactions, such as ex-vivo explants, organotypic slices, and hanging drop assays. However, the high technical requirements of these methods have restricted their application to the study of a few targets of interest.

This thesis developed a novel phenotypic assay that utilises the cost-effective ULA round bottomed plates to enable a robust evolution of the hanging drop assay (Falenta *et al.*, 2013). Each preparation of the 3D RMS spheroid migration assay can be conducted by a single experimenter within a 3-day period. Spheroids can be successfully formed with as few as 300 and as many as 40,000 cells, but the optimum for screening compounds was found to be 5,000 cells per well. Up to six 96 well plates, equating to 576 individual samples, can be tested per dissection of 10 mouse pups using only standard laboratory equipment and reagents. The spheroid assay is compatible with the vital dyes: CellTracker, Propidium Iodide, and Hoechst, and can therefore be used to study other cellular behaviours and mechanisms other than migration.

The spheroid migration assay was evaluated in a screen of the GSK PKIS, a library of around 900 small molecule kinase inhibitors. 21 compounds were successfully validated that stimulated the migration of neuroblasts. Neuroblasts were most responsive to these compounds at different concentrations, for example GW607049C, GW459135A, and GSK2336394A increased migration at lower concentrations, whereas GW693881A and GW854278X only gave a significant effect at the highest concentration. When choosing compounds to take forward for potential therapeutic purposing the effective dose may be important to consider. 36 compounds significantly reduced migration of neuroblasts, with all compounds effective at decreasing migration distance at 10 μ M. None of the compounds that were found to be toxic have been thoroughly analysed, and this may be useful to do in the future to identify if there are common targets amongst these toxic inhibitors. This may be important both in determining mechanistically what genes are essential in neuroblast migration, and also what targets may not be good to focus on for therapeutic purpose. A more thorough analysis of the structure of the compounds selected by a medicinal chemist is still required. It is possible that some compounds may be able to be modified to be more effective or better suited for medicinal use.

The 3D spheroid migration described in this thesis has the potential to dramatically increase our understanding of the molecular mechanisms that instruct neuroblast migration. The Src family kinase inhibitor PP2, the PKC inhibitors BIM1 and Ro318220, and 11 novel nonannotated compounds have previously been identified as disruptors of migration in a phenotypic assay of cortical radial migration (Jossin *et al.*, 2003; Zhou *et al.*, 2007). However, to my knowledge, no other phenotypic screen has successfully identified positive modulators of postnatal neuroblast migration.

6.1.4 Protein kinase regulation of neuroblast migration

A primary objective of the small molecule inhibitor screen was to identify key kinase pathways and previously uncharacterized kinases that may have key regulatory roles in neuroblast migration. Analysis of the high affinity binding partners of the compound library suggested many kinase targets. The pro-migration targets selected included the RTKs: EGFR, ErbB2, ErbB4, EphA2, TrkA, TrkC, PDGFR, Tie2, FLT3, MUSK and KIT; and intracellular kinases PLK1, PLK2, PLK3, LOK, NEK9, HUNK and AURKC. Anti-targets of compounds that inhibited migration included GSK3 α , GSK3 β , P38 α , IGF1R, INSR, CDK2, PRKD1, MINK1, CK1 α and Dyrk1B. Because the ATP binding pocket is conserved across the kinome, there is a high degree of pharmacological linkage between targets. Thus, related proteins that are not part of the mechanism of action may still be selected. It is also true that closely related proteins can compensate for the loss of function of individual kinases. Thus, confirming the mechanism of action for the putative target kinases is not trivial and will require dedicated independent investigation.

Genetic knockdown of MuSK via shRNA confirmed that disruption of this gene results in an increase in the average distance migrated by neuroblasts from a spheroid. To my knowledge this is the first time that a functional role for MuSK has been found in adult neurogenesis. MuSK is expressed in the CNS and according to the Allen Brain Atlas is localised to the ventricular regions. It is more commonly associated with synapse formation in skeletal muscle (Burden *et al.*, 2013). Interestingly it has been shown to have a crucial function in restricting neural crest migration in both zebra fish and mice (Banerjee *et al.*, 2011). In the absence of MuSK, streaming neural crest cells, which are normally confined to the central region of each somite, are dispersed and present throughout the somite. The researchers suggest that the MuSK dependent guidance cue is mediated via the non-canonical Wnt/Planar Cell Polarity (PCP) pathway involving the ligand Wnt11. Several core members of the Wnt/PCP pathway are known to regulate the migration, proliferation and differentiation of neuroblasts at different stages of the RMS (please see Hirota *et al.*, 2016 for review). Interestingly Wnt11, and another MuSK ligand Wnt4a, have high physiological expression in the SVZ that is significantly reduced following brain injury in mice (Chavali *et al.*, 2018). So, it is possible that MuSK acts to confine neuroblasts to the SVZ/RMS via the Wnt/PCP pathway.

Another interesting consideration raised by MuSK is that cholinergic signalling may play a role in directing neuroblasts migration. MuSK regulation of acetylcholine has been shown to be critical for learning and memory in the hippocampus but there is less evidence of direct cholinergic input into the SVZ (Höglinger *et al.*, 2004; Garcia-Osta *et al.*, 2006; Winner *et al.*, 2009). Neural stem cells have been shown to express both nicotinic and muscarinic acetylcholine receptors, which have been indicated to modulate proliferation and differentiation *in vitro* (Zhou *et al.*, 2004). Activity dependent release of acetylcholine locally in the SVZ has also been shown to regulate proliferation and neuroblast production in an FGF dependent fashion (Paez-Gonzalez *et al.*, 2014). Thus, more detailed studies are required to determine whether acetylcholine also regulates neuroblast migration.

Genetic knockdown of PIK3CB via shRNA confirmed that disruption of this gene also results in an increase in the neuroblast migration. This result provides further support that the function of the four PI3K catalytic subunits is specialized in the CNS. RTKs may signal through any of the four subunit isoforms. PIK3CB has been found to be preferentially activated by ErbB4 and GPCRs, such as the metabotropic glutamate receptors (mGluRs) (Law *et al.*, 2012; Gross and Bassell, 2014). Interestingly PIK3CA is preferentially activated by IGF1R (Zhao *et al.*, 2006). It is therefore tempting to consider the opposing function of IGF1R and the ErbBs in neuroblasts migration may involve differential activation of PIK3CA and PIK3CB. Distinct functions for PIK3CA and PIK3CB have also been described in glioblastoma, with increased PIK3CB activity correlated with tumour proliferation and PIK3CA tumour invasion (Castellano *et al.*, 2016; Pridham *et al.*, 2017). A duplication in the PIK3CB gene locus has been associated with autism (Cuscó *et al.*, 2009). The phosphatase and tensin homologue (PTEN), a negative regulator of PI3K activity, was shown to preferentially bind to PIK3CB compared to other PI3K catalytic subunits. PTEN deletion has been shown to disrupt neuroblast migration and, like PIK3CB, has been linked to autism (Zhu *et al.*, 2012). However, the specific regulation of PIK3CB by PTEN in the SVZ remains unstudied. Elevated PI3KCB signalling is also associated with impaired higher order cognition in Fragile X Syndrome due to increased signalling through the mGluR5 (Gross *et al.*, 2015). Many components of the GABAergic and glutamatergic signalling pathways are known to regulate the migration, proliferation and differentiation of SVZ neuroblasts (Platel, Dave and Bordey, 2008). It is therefore, possible that the increase of migration following inhibition of PIK3CB signalling could

be mediated by modulating neurotransmission. Multiple selective inhibitors PI3K related proteins are currently in clinical trials for treatment of cancer (Engelman, 2009; Jansen *et al.*, 2015; Janku, 2017a). It is tempting to consider that some of these may have applications in neurological disorders. A more thorough investigation of this pathway in the SVZ is needed.

6.2 Limitations

6.2.1 IGF2R imprinting status in the SVZ

In writing this thesis I have discovered research that challenges the results from the study of a neural phenotype in the $Igf2r^{l1565Am/+p}$ mouse. The study design was reliant on the premise that the IGF2R gene is imprinted in all tissues from E6.5 (Lerchner and Barlow, 1997). Thus, the maternal transmission of the $Igf2r^{l1565A}$ gene was considered a loss of function model. However, it has now come to light that the $Igf2r$ may escape imprinting in the CNS (Hu *et al.*, 1998, 1999; Yamasaki *et al.*, 2005). This should have been caught earlier in the project and demonstrates the sensitivity of interdisciplinary collaborations. It also poses the dilemma of how to interpret the data from this thesis. If we accept that a functional IGF2R allele is expressed in the SVZ of the E19 $Igf2r^{l1565Am/+p}$ embryos studied then the increase in brain weight and proliferation is likely down to a general increase in circulating IGF2 in these mice, not a specific loss of local ligand titration. The absence of gross abnormalities in cortical layering fits this interpretation. Another more intriguing scenario is that the imprinting status of IGF2R is differently regulated in neural stem cells. The finding that biallelic expression is restricted to neurons and correlates with the onset of cortical neurogenesis support this hypothesis. Neural stem cells are themselves radial glia and by definition can give rise to astrocytes, neurons and oligodendrocytes. It is interesting to consider that the homozygotes $Igf2r^{l1565Am/l1565Ap}$ are perinatally lethal, thus supporting a yet unexplored functional role of the paternal allele. Characterising the imprinting status at the different stages of the neural lineage from radial glia through intermediate projector, projection neuron, astrocyte and oligodendrocyte is necessary before any conclusions can be affirmatively made from this study.

6.2.2 Pharmacology of the GSK PKIS Libraries

The results in this thesis successfully identified several small molecules that modulate the migration of neuroblasts. This is a valuable advance towards identifying novel strategies to treat brain disease, but the compounds identified are only starting points. The compounds in the PKIS libraries are specific molecular probes but are not drugs. They do not have the required pharmacological properties to be used *in vivo*.

The homology of kinase catalytic domains gives rise to kinase inhibitor promiscuity. Consequently, the kinase for which a compound was originally prepared might not be the only potentially inhibited kinase, and importantly the original kinase may not be a contributor to the phenotype resulting from compound treatment. The PKIS libraries are well annotated in comparison to other libraries, however the affinity data available is not exhaustive and each compound may potentially intersect with multiple unknown pathways. Affinity does not necessarily correlate with inhibition either. Selecting compounds with multiple modes of action via phenotypic screens is currently considered the preferred strategy for drug discovery (see Peters, 2013 for review). The therapeutic effects of multiple modes of action are often synergistic, whereas the adverse effects are not, thus reducing side effects. Blocking multiple pathways is also believed to prevent drug resistance. However, compound promiscuity makes drawing solid conclusions at the end of a DPhil thesis difficult. It will take several months of dedicated work to individually validate the targets identified. The major translational impact of this project lies in the potential to discover novel regenerative therapies. I personally do not have the expertise to fully interrogate the hit compounds. A trained medicinal chemist is needed to assist with evaluating the data set and deciding future strategy.

The prominent role of RTKs in neuroblast migration was underscored by this thesis. Yet, the systematic function of the individual receptors and their ligands remains an open question due to the aforementioned pharmacological linkage. Individual genetic knockdown of each RTK gene via electroporation of an shRNA DNA plasmid may assist with this. However, this method has proved challenging during the course of this thesis and should be avoided. The library of shRNA plasmids we utilised did not yield sufficient gene knockdown efficiency to permit the desired target evaluation in four out of the six genes tested. In addition, the technique requires very large

numbers of cells creating a restrictive bottleneck in workflow. If precise metrics for individual genes are desired in future it would be better to refine the assay for use with a genetic knockdown array, such as the Edit-R CrispR mouse kinase library (Dharmacon GP/GC-013500), combined with a gentle transfection method optimised for 96 well plates of neural cells, such as the Neuro Mag (Oz Bioscience KC30800). It will be important to be conscious of the differences between genetic screening and small molecules. Firstly, with small molecules you can be reasonably confident that the inhibition will occur homogenously in the cell population within a reasonably short incubation period. This is not the case for genetic approaches where transection efficiency may vary and the time course to functional reduction in protein concentration can take several days. In addition, the precision of genetic approaches, removes the benefits of drug polypharmacology. Such that combinatorial responses are missed and compensation between related proteins can mask functional mechanisms.

6.2.3 Interpretation of the spheroid migration data.

Cellular migration is a complex multistep process and it is important to acknowledge that defining a compound or kinase target as specifically pro- or anti- migratory from a single assay is ambiguous. The parameter measured in the inhibitor screen was the area of migration. This measurement integrates several cellular processes including: motility initiation, speed, persistence, directionality, polarity, chemo-attraction/repulsion and survival, among others. At this stage we do not know which individual process is the most relevant to the neurogenic response following brain injury. We cannot be certain that increasing the migration of neuroblasts from RMS spheroids will even provide a clinical benefit. Future studies are necessary to further characterise both the hit compounds and target kinases and better qualify the migratory mechanisms that provide the greatest therapeutic benefit. Complimentary migration, survival and proliferation assays should be considered along with *in vivo* validation in mouse models of brain injury.

Growth factors signalling is often associated with cell division. It is therefore not unexpected that inhibiting some RTKs increases the distance that neuroblasts travel as they will be less mitotically active. The spheroid assay was completed over 24 hour period to limit the linkage between proliferation and migration. However, it may be sensible in future experiments to measure or control for proliferation more accurately. Inhibitors of proliferation, such as Mitomycin-C, could be

used but these introduce ill-defined biological effects. A better solution may be to culture the spheroids in the presence of ethynyl-labeled (deoxy)uridine (EDU). Cells that have undergone divisions could then be detected at the end of the assay via cell permeable azide containing fluorescent dyes. Alternatively, the sequential dilution of lineage tracing vital dyes, such as CellTrace™, has been shown to successfully segment cells that have gone through cell division in migration assays (Glenn, Messner and Meldrum, 2016).

6.3 Future Directions

6.3.1 Further development and optimisation of the spheroid assay

The spheroid assay is a promising tool to study neuroblast migration, ask disease related questions and potentially screen for therapeutic compounds. Nevertheless, some issues remain that need to be addressed. The cellular composition of the spheroids more accurately approximates the *in vivo* environment than neurospheres, however much of the architecture of neural tissue is lost. For instance, regions of white matter and vasculature that neuroblasts associate with *in vivo* are not present. In the absence of circulation, the spheroids will not be exposed to the same composition of extracellular signals. Similarly, spheroids lack physiological sensory input. Some groups have begun to fuse two organoids of different composition to study the long-distance migration of neurons in the brain (Bagley *et al.*, 2017; Xiang *et al.*, 2017). Co-culturing RMS spheroids with spheroids composed of dissociated striatal cells could be considered for a better model of injury induced ectopic migration. Other groups have combined organotypic slices with tumour spheroids to study glioma invasion (Pencheva *et al.*, 2017). Both of these methods may more closely replicate *in vivo* physiology, but neither is without limitations and scalability is a concern.

A sensible initial step is to further investigate the initial composition of the RMS spheroids and to identify key parameters of the dissection and culture conditions that can drive large effects. In particular the internal arrangement of the spheroids needs to be studied and methods developed to make it predictable and reproducible. The assay is also based on two key steps involving

Matrigel and fetal bovine serum, which have unpredictable biochemical effects and vary between lots. Co-culturing the spheroids with endothelial or choroid plexus cells could be considered to more closely replicate the *in vivo* conditions. Many different research groups are now producing culture media and extracellular matrix gels that more closely replicate the neural physiology (Crapo *et al.*, 2013; Bardy *et al.*, 2015; Lozano *et al.*, 2015). Prior to any large scale discovery projects exploring the sensitivity of the assay to these systems should be considered. It will also be sensible to assay the initial and end point media concentration of components known to modulate migration. Commercially available cytokine antibody arrays, eg: Mouse Quantibody Cytokine Array Q4000 (RayBiotech QAM-CAA-4000), would serve as an instructive initial resource.

The spheroid migration assay described in this thesis been developed to study SVZ neuroblasts. However, there is no reason why it cannot be applied to study different primary cell types. The SVZ also gives rise to oligodendrocyte precursor cells (OPCs), which are another attractive therapeutic target in multiple sclerosis and are considered by many the most likely candidate as the tumour initiating cell in GBM. O'Meara *et al.* (2016) recently published a protocol to rapidly isolate OPCs from the mouse brain, which may serve as a starting point. Another consideration that needs to be made is whether or not we continue to use primary mouse cells. Concern is mounting about the clinical translatability of results from mouse experiments. Especially with the recent evidence suggesting a substantial difference between murine and human regulation of neurogenesis. It is therefore sensible to consider transitioning to human derived cells. At the beginning of my thesis, this was not an option. However, during the course of my project there has been substantial growth in the field of induced pluripotent stem cell (iPSC) derived cerebral organoids making these an increasingly attractive alternative.

Recently two groups have introduced fusion organoids to enable the study of interneuron migration (Bagley *et al.*, 2017; Xiang *et al.*, 2017). They describe the generation of two populations of organoids from human iPSCs specific to either the embryonic neocortex or MGE. The fusion of these two spheroids produces a dorsal-ventral axis and results in interneuron migration from the MGE to the neocortex. The interneurons replicate *in vitro* migration dynamics and functionally integrate with glutamatergic neurons. A subsequent study fused organoids from patients with Timothy syndrome, a monogenic disease associated with autism and epilepsy, and demonstrated that the interneurons display abnormal migratory saltations (Birey *et al.*, 2017).

Several other developmental disorders have now been studied in organoid systems. The 17p13.3 deletion leading to Miller–Dieker syndrome, a severe form of lissencephaly, has been challenging to study in animal models because mice are naturally lissencephalic. Two groups have developed forebrain organoid models of Miller–Dieker syndrome and found abnormalities in radial glia that can be rescued genetically and pharmacologically (Bershteyn *et al.*, 2017; Lefremova *et al.*, 2017). Organoids in which the tumour suppressor gene PTEN is deleted show increased proliferation and delayed neural differentiation, and this phenotype was attenuated by inhibition of PI3K/Akt signalling (Y. Li *et al.*, 2017). Cerebral organoids derived from microcephaly patients with mutations in the cell cycle-related gene CDK5RAP2 display an abnormal plane of division in cells located in the pseudo-VZ/SVZ (Lancaster *et al.*, 2013).

To my knowledge no research groups have studied adult neurogenesis in iPSC derived organoids yet so optimising the current protocols to provide sufficient postnatal neuroblasts will require some work. Nevertheless, if the ultimate aim is to better understand the biology of human adult neurogenesis, it is arguably a more sensible strategy than continuing with primary mouse cells.

6.3.2 Neuroregenerative Therapeutics

The early motivation of this thesis was to instruct the development of Smart Growth Factors for neuroregeneration. This initial goal may not have been achieved but we have identified several compounds that could form the basis of a new therapeutic class of small molecules. Confirming that increasing migration in the spheroid assays results in a therapeutic benefit *in vivo* is an essential next step. The 21 promigratory compounds should be analysed by someone with medicinal chemistry expertise to determine whether any could potentially be utilised *in vivo*. Clinical stage drugs developed against kinases targets indicated by the current screen could be tested as an alternative. TGX-221, AZD8186, SAR260301 and GSK2636771 are all selective inhibitors of PIK3CB and could be explored as a primary option (Janku, 2017b). Inhibitors of MuSK are not a sensible therapeutic target due to their high potential for toxic side effects on the peripheral nervous system. It is important to note that interrogation of the screen dataset has not been exhausted and further investigation of the targets identified is necessary. This includes all the putative hits yet to be validated as well as combinations suggested by future dual inhibitor studies.

An alternative to small molecule inhibition of intracellular kinases is to introduce cell permeable versions of active kinases to augment signalling. This has previously been demonstrated with a cell permeable version of p38 in a mouse model of ischemia (Hamanoue *et al.*, 2016).

6.3.3 Is adult neuroregeneration the best therapeutic target?

Five years ago, at the beginning of my DPhil, there was great optimism about the potential to harness the regenerative properties of adult neurogenesis for therapies in several human conditions. ¹⁴C radioisotope dating of neurons in the adult brain had demonstrated that the human basal ganglia contains a population of adult born cell (Ernst *et al.*, 2014). Although unconfirmed, it was suggested that these cells could originate from the neighbouring SVZ. It was also shown that these cells are depleted in patients with Huntington's Disease, thus providing a strong link between adult neurogenesis and a neurodegeneration. In the same year Scafidi *et al.*, demonstrated that intranasal delivery of heparin-binding EGF immediately after hypoxic injury decreases oligodendroglia cell death, increases the production of new oligodendroglial cells and promotes brain recovery (Scafidi *et al.*, 2014). Furthermore, this intervention diminished ultrastructural abnormalities and alleviated behavioural deficits in white-matter-specific paradigms. Thus, these results provided direct evidence that molecularly targeting neural progenitors is clinically feasible and potentially applicable to treating brain injury.

Recently, however, the debate has been reignited. Last year a thorough immunohistochemistry study in human brains found a marked decline of proliferating neural progenitors in early infancy (Dennis *et al.*, 2017). After the age of 3 years old, microglia were the only proliferating cells identified. Around the same time a beautiful paper by Paredes *et al.*, (2016) was able to capture the migration of postnatally born neuroblasts migrating in post mortem human brain slices. They describe extensive migration of DCX+ neuroblasts in both the RMS and the MMS. However, this migration occurred primarily in the first 3 months after birth, with few migrating cells persisting at 7 months and none detected after 2 years old. A much-discussed follow up paper that was released recently has also drawn into contention the presence of hippocampal neurogenesis in adult humans (Sorrells *et al.*, 2018). Interestingly, however, in this paper they used the SVZ as a staining control and were able to identify rare DCX+ cells with a migratory morphology in the two adults (aged 24 and 26) examined. The major barrier to studying adult neurogenesis is the

inaccessibility of living tissue. Studies such as these rely on imperfect post-mortem tissue and few immunohistological markers, such as DCX, nestin, Ki67 and NeuN, that are liable to post mortem degradation. Spontaneous somatic nuclear and mitochondrial mutations have been used as clonal marks to track cell lineage in human brains (Lodato *et al.*, 2015). After further development, these approaches may enable clonal analysis of endogenous adult NSCs in humans via single-cell sequencing. Novel non-invasive cell tracing techniques also have the potential to revolutionize the study of human adult neurogenesis.

While the adult neurogenesis debate persists, the potential to treat paediatric brain injury is far less contentious. Paediatric brain damage is sadly very common and can lead to significant morbidity and severe long-term neurologic and cognitive deficits, such as cerebral palsy, epilepsy, behavioural disorders, impaired vision, and language function (see Niimi and Levison (2017) for review). The SVZ of the human infant contains large number of PSA-NCAM and/or DCX expressing cells (Sanai *et al.*, 2011; Paredes *et al.*, 2017). It is clear from animal studies of paediatric injuries that the SVZ response is rapid and robust and the magnitude of this response is greater than in the mature brain. It is also evident that pharmacological intervention in neonatal brain injury can provide therapeutic benefit (Scafidi *et al.*, 2014). Therefore, while more studies are needed, there is a strong likelihood that this could lead to new regenerative therapeutics in human infants.

6.3.4 Targeting brain cancer metastasis.

Another potential application of the RMS spheroid migration is to study the invasion of brain cancers, such as GBM. An insidious feature of GBM is that single cells can detach from the primary tumour, invade normal brain tissue, blurring tumour margins, and establishing numerous microtumours at a distance from the primary tumour. This infiltrative nature of GBM makes complete surgical resection impossible despite great advancement in neurosurgical techniques, whereas resistance to conventional chemotherapy and radiation spares them from eradication. Therapies directed at blunting the migratory capacity of GBM is therefore a top priority in drug discovery. However, there is debate about the physiological relevance of current tumour invasion models (Dunn *et al.*, 2012). Conventional malignant glioma cell lines do not tend to exhibit invasive growth in orthotopic xenograft models. Transplanted tumour stem cells do invade and

can phenocopy the type of invasion observed radiographically in patients (Wakimoto *et al.*, 2012). However, few *in vitro* systems have replicated *in vitro* glioma migration with high fidelity (Bhat *et al.*, 2011).

The cell of origin of GBM is still debated, however, SVZ progenitors are a prominent suspect. Over half of all GBM tumour are in contact with the lateral ventricle and genetic profiling of tumour samples has revealed prevalent expression of SVZ specific markers, such as nestin and CD133. Whether or not SVZ stem cells are the cell of origin, the dynamics of the GBM individual cell migration is very similar to migrating neuroblasts: extending a prominent leading cytoplasmic process followed by a burst of forward movement in the cell body (Onishi *et al.*, 2011). Thus, it is highly likely that many of the same signalling mechanisms are involved.

In parallel to the development of this RMS spheroid assay, other research groups are now publishing GBM tumour invasions assays (Vinci *et al.*, 2015). These all have the same limitation as using SVZ neurospheres. They are not studying the invasive cells. Most either use immortalised cell lines or FACS purified CD133+ cancer stem cells, which are known to have very different amoeboid migration dynamics *in vitro*. Using RMS cells, therefore may offer the opportunity to screen for anti-invasive therapies in a more physiological model. Moreover, with the addition of known genetic manipulations, such as the IDH1(R132H) mutation that is expressed in over 80% of SVZ associated GBM, may enable us to study specific subsets of tumours (Bardella *et al.*, 2016).

This premise will need to be experimentally validated via tumour xenograft studies, however, the data from the kinase inhibitor screen does provide some promising insights. As previously mentioned inhibitors of RTKs, such as EGFR, VEGFR PDGFR, were among the classes of compounds that increased migration from RMS spheroids. Drugs targeting all of these have also been found to stimulate the invasion of GBM when used in the clinic. The most famous of these is the monoclonal antibody against VEGF, *Avastin*, which is still used to treat GBM despite providing limited clinical benefit. The PDGFR inhibitor, Imatinib, was the first targeted tyrosine kinase inhibitor to be approved for clinical use and remains first-line therapy for Philadelphia chromosome (Ph+)-positive chronic myelogenous leukaemia but failed in the clinic to treat GBM due to increased tumour invasion (Frolov *et al.*, 2016). Despite EGFR previously being associated with more invasive tumour behaviour and *in vitro* assays indicating that inhibition reduces cell

invasion, in the clinic EGFR inhibitors, such as Gefitinib and Afatinib, failed in the clinic for GBM (Franceschi *et al.*, 2007).

There are close to 70 anticancer drugs with specific molecular targets on the market today. More than half of these drugs target kinases (56%), and novel kinase inhibitors might be valuable for the treatment GBM invasion. I have not yet comprehensively explored the kinases targets of the compounds that blocked migration in the current screen and genetic knockdown validation experiments are necessary. Nevertheless, if we look at the top putative hits from the matrix analyses, they are all established neuro-oncology targets. Myriad studies have credentialed GSK-3 β as a therapeutic target in GBM (Kotliarova *et al.*, 2008; Korur *et al.*, 2009; Miyashita *et al.*, 2009). Likewise IGF1R is overexpressed in GBM and is correlated with poorer survival (Maris *et al.*, 2015). PPP, PQ401, GSK1838705A, and NVP-AEW541 are IGF-1R inhibitors that have all shown promising results pre-clinically (Pearson and Regad, 2017). However, cross reactivity or compensations by the IR have so far limited their therapeutic potential (Simpson *et al.*, 2017). Increased expression of p38 correlates with poor prognosis in several types of cancers, including glioblastoma (Yang *et al.*, 2013; Goldsmith *et al.*, 2018). However, despite it's potential as a target for chemotherapy none of the 22 different p38 inhibitors that have entered the clinic have reached Phase III due to high toxicity and off-target effects (Hammaker *et al.*, 2010). Strong expression of PRKD2 is reported in the tumour endothelium of GBM microvascular proliferations and it has been found to a potent regulator of GBM cell growth and migration *in vitro* and *in vivo* (Azoitei *et al.*, 2011; Bernhart *et al.*, 2013). Interestingly in myeloma cell migration PRKD2 is the downstream target of IGF signalling (Qiang *et al.*, 2004). CK2 is also an ongoing target in the treatment of GBM. Genome-wide analysis of GBMs revealed an increase in the CK2 expression in tumours (Zheng *et al.*, 2013) and it has been subsequently found to be a key regulator of tumour growth and invasion (Maya, Nitta and Li, 2014; Pencheva *et al.*, 2017). CX-4945 is the first clinical stage CK2 inhibitor being trialled for GBM that is reported to decrease GBM growth and stemness through β -catenin (Nitta *et al.*, 2015; Ferrer-Font *et al.*, 2017).

It would therefore be a rationale strategy to explore the potential to use the 3D RMS spheroid assay to discover novel anti-invasive molecular targets and drugs. An initial, essential step will be to validate that the compounds it selects are actually applicable to *in vivo* GBM invasion. As noted previously the compounds in the PKIS libraries are molecular probes and do not have the correct molecular properties to be administered *in vivo*. It is therefore sensible to complete a small

screen of clinical stage anticancer drugs and select candidates from that screen. Comparison of the invasion data from that screen with clinical outcome of those compounds will also provide further confidence as to whether this assay is a better predictor of clinical success than conventional *in vitro* preclinical assays.

6.3.5 IGF signalling in the SVZ

The results in this thesis confirm that IGF signalling increases the motility of SVZ neuroblasts, however there remain several unresolved questions regarding the molecular mechanism. Several mechanisms have been described in other cell types including a $\beta 1$ integrin/RACK1/FAK signalling complex (Cox *et al.*, 2015), activation of the NF- κ B-Snail axis (Kim *et al.*, 2007), upregulating SDF1/CXCR4 signalling (Li *et al.*, 2007; Zhu *et al.*, 2015) and regulating DDR1 expression, among others (Malaguarnera *et al.*, 2015; Matà *et al.*, 2016). The results from the inhibitor screen in this thesis can also be used as potential downstream targets in the pathway. These include: p38 α , GSK3 α , CK1 α , PRKD(1-3), Akt3, Jnk1, PIK3CB, Mink and Dyrk1B. Phosphoproteomic analysis of SVZ IGF1R^{-/-} stem cells or neurospheres cultured with an IGF1R function blocking antibody may provide initial insights into how these pathways integrate to regulate neuroblast migration.

Aside from the role of IGF1 and IGF2 in migration, the imprinting status of the IGF2R in the SVZ and its role in neurogenesis has emerged as a vital question from this thesis. This should be achievable via sequencing the cDNA of IGF2R mRNA amplified across the region containing the point mutation. This should be completed in FACS sorted cells if possible to determine the exact stages in the neural lineage that IGF2R imprinting is inactive.

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