The Regulatory Function of Raf-1 Kinase Inhibitory Protein in Adult Hippocampal Neurogenesis

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Adult hippocampal neurogenesis is the generation of new neuronal cells within the dentate gyrus of mammals. The process avails the hippocampus in its innate function of memory and notwithstanding any aberration, shows consistency throughout early adulthood until depreciating in old age. It begins with neural stem cells which undergo activation and division, mature over several weeks, and finally (of those that survive), integrate into existing neural circuitry. Several possible cellular pathways exist to mediate this neurogenic process, including the MAPK pathway. RKIP, a novel and elusive protein, is known to inhibit this pathway and regulate others, such as NF-kB, GSK3β, and GRK2. Owing to this expansive function is the finding of its role in promoting a neuronal fate in neural progenitor cells (NPCs). Here, I looked to delineate the role of RKIP throughout the entirety of the neurogenic process in mice. With voluntary exercise employed for neurogenesis induction, I discovered RKIP as functioning to reduce NPC proliferation while maintaining proper cell cycle kinetics. Furthermore, RKIP did not affect the overall formation of mature neuronal cells despite promoting a neuronal fate early in development.
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Author Contributions

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# Table of Contents

## Contents

Acknowledgements ........................................................................................................ iii
Author Contributions .................................................................................................... iv
Table of Contents ......................................................................................................... v
List of Tables ................................................................................................................ viii
List of Figures ............................................................................................................. ix
List of Appendices ....................................................................................................... x

### Chapter 1: Literature Review ................................................................................. 1-26

1 Adult Neurogenesis .................................................................................................. 1-20
   1.1 Hippocampal Anatomy and Function ................................................................. 1
   1.2 History and Discovery ......................................................................................... 2
   1.3 Non-neurogenic Regions ..................................................................................... 3
   1.4 Neurogenic Region: Lateral Ventricles ............................................................... 4
   1.5 Neurogenic Region: Hippocampus .................................................................... 5-17
      1.5.1 Facts and Function ....................................................................................... 5
      1.5.2 Cell Types and Progression ......................................................................... 7
      1.5.3 Electrophysiological Properties and Structural Development ..................... 11
      1.5.4 Molecular Mechanisms .............................................................................. 14
   1.6 Factors Affecting Neurogenesis - Exercise ....................................................... 19

2 The Multifunctional RKIP ....................................................................................... 22-26
   2.1 Discovery and Localization ................................................................................. 22
   2.2 Role in MAPK Pathway ...................................................................................... 22
   2.3 Role in NF-κB Pathway ..................................................................................... 23
   2.4 Phosphorylated RKIP ....................................................................................... 24
2.5 Role in GSK3β Pathway ................................................................. 25
2.6 Adult Hippocampal Neurogenesis ................................................... 28

Chapter 2: Research Objectives ............................................................... 31

Chapter 3: Methods ............................................................................. 32-37
1 Animals and Exercise Paradigms ....................................................... 32
2 Thymidine Analog Solution Preparation and Injections ....................... 32
3 Tissue Harvesting ............................................................................. 32
4 Immunofluorescence Staining ............................................................ 34
5 Immunohistochemistry Protocol .......................................................... 35
6 SDS-PAGE and Western Blot ............................................................... 35
7 Imaging and Quantification ................................................................ 37
8 Statistical Analyses ............................................................................ 37

Chapter 4: Results .................................................................................. 38-60
1 Neural Progenitor Cell Proliferation ................................................... 38
2 Type 2a and 2b Neural Progenitor Cell Proliferation ............................ 40
3 Type 3 Neural Progenitor Cell Proliferation ......................................... 42
4 Cell Cycle Kinetics ............................................................................ 44
5 Mechanism of Action: MAPK Pathway .............................................. 48
6 Mature Neuron and Glia Formation .................................................... 55

Chapter 5: Discussion ............................................................................ 58-68
1 Neural Progenitor Cell Proliferation ................................................... 58
2 Cell Cycle Kinetics ............................................................................ 61
3 Neural Progenitor Cell Differentiation ................................................ 62
4 Mechanism – MAPK Pathway ............................................................. 63
5 Maturation of Neuronal and Glial Cells .............................................. 65

Chapter 6: Future Directions and Conclusion ......................................... 68
Bibliography ................................................................. 71
Appendices ..................................................................... 80
List of Tables

Table 1: List of Regulators of Adult Hippocampal Neurogenesis

Table 2: List of Antibodies
List of Figures

Figure 1: Adult Hippocampal Neurogenesis .......................................................... 10
Figure 2: Regulators of Type 1 NSC Proliferation............................................. 16
Figure 3: RKIP Regulation................................................................................. 26
Figure 4: RKIP Cellular Pathway....................................................................... 27
Figure 5: Neural Progenitor Cell Proliferation ............................................... 39
Figure 6: Neural Progenitor Cell Subtype Proliferation................................. 41
Figure 7: Neural Progenitor Cell Differentiation ............................................ 43
Figure 8: Cell Cycle Kinetics............................................................................ 46
Figure 9: RKIP Mechanism of Action: the MAPK Pathway (IHC)................. 49
Figure 10: RKIP Mechanism of Action: the MAPK Pathway (IF)..................... 51
Figure 11: RKIP Mechanism of Action: MAPK Pathway (IF) and NPC Subtypes 53
Figure 12: SL-327 Efficacy: pERK Immunoblot............................................... 55
Figure 13: NPC Neuronal Cell Maturation....................................................... 57
Figure 14: NPC Astrocyte Cell Maturation....................................................... 81
List of Appendices

Table 2: List of Antibodies........................................................................................................................................... 80

Figure 14: NPC Astrocyte Cell Maturation............................................................................................................. 81
Chapter 1
Literature Review

1 Adult Neurogenesis
1.1 Hippocampal Anatomy and Function

The hippocampus is a bilateral structure inferior to the cerebral cortex located in the mid-region of the murine brain. It functions in the formation of declarative memories, spatio-temporal contextualization of memory, and spatial learning (Triviño-Paredes et al. 2016). The structure is comprised of four major morphologically and physiologically distinct areas: the Cornu Ammonis 1 (CA1), CA2, CA3, and the dentate gyrus (DG). Each region is intricately connected to one another through synaptic integration of the innumerable granule and pyramidal cells that generate the complex neural circuitry of the structure; briefly, the CA3 receives inputs from the DG, the CA1 from the CA3, and the CA3 from within itself. Of particular importance are the synaptic inputs from the entorhinal cortex (EC) to the DG and CA3 via the layer II perforant pathway and CA1 via the layer III perforant pathway, effectively joining the hippocampus to the greater function of the brain. The DG encompasses 3 distinguishable layers identifiable by the neural cell and microenvironment composition: the polymorphic cell layer (hilus), the granule cell layer, and the molecular layer (Amaral, Scharfman and Lavenex, 2007). Within the granule cell layer are the predominant granule cells responsible for pattern separation and glutamatergic inputs from entorhinal cortical neurons that synapse at the molecular layer. Upon closer examination, a sublayer within the granule cell layer becomes apparent in its distinct cellular composition: the subgranular zone (SGZ). This region outlines the boundary between the dentate gyrus and the polymorphic layer and more importantly, is the site of adult hippocampal neurogenesis where neural stem cells, neural progenitor cells, and GABAergic basket cells reside (Amaral, Scharfman and Lavenex, 2007). Careful examination of the granule cells within the dentate gyrus reveals the direction of their process extensions and sites of synaptic integration. Specifically, the apical dendrites project through the granule cell layer towards the molecular layer to synapse with GABAergic interneurons of the perforant path-associated cells and
commissural-associated pathway-related cells of the hilus (HIPP and HICAP, respectively) as well as glutamatergic afferent fibres of the layer II medial and lateral perforant pathways (MPP and LPP) originating from the entorhinal cortex. Concurrent with these integrations are additional GABAergic inputs that DG granule cells receive from the SGZ inhibitory interneuron basket cells which release γ-aminobutyric acid (GABA) and express parvalbumin, a calcium-binding protein. Inputs from beyond the hippocampus are also noted to synapse with granule cells of the DG. For example, glutamatergic fibres also integrate with granule cells at the molecular layer that originate from the close proximity of the presubiculum and the parasubiculum and from the following distal regions: the ventral tegmental area, raphe nuclei, septum, and local interneurons which transmit dopaminergic, serotonergic, acetylcholinergic, and GABAergic inputs, respectively (Toni and Schinder, 2015).

1.2 History and Discovery

The concept of neurogenesis in adulthood was a wild and contentious viewpoint for the greater part of neuroscience research. Its origins stemmed from the long-held belief that neuronal cells lose their potential to regenerate in adulthood. The beginning of a shift in this viewpoint came in the 1960s with the work of Altman and the arrival of autoradiograph technology use in neuroscience research. This technique made it possible to label proliferating cells with tritiated thymidine (thymidine-H³) and Altman (1962) was the first to implement its use for intracranial injections and autoradiograph labelling of the lesioned rat brains in the lateral geniculate nucleus of the thalamus. There they discovered the radionuclide-emitting thymidine labelled glial and neuronal cells, suggesting a state of proliferation in such cells. However, their findings were largely regarded as fallacious and did not sway the overarching consensus on the possibility of adult neurogenesis. The field did not witness resurgence until in the 1980s when Fernando Nottebohm discovered neuron formation in the seasonal song system of adult birds (Goldman and Nottebohm, 1983). Still, the concept was not easily accepted until once more in the 1990s when conclusive evidence was procured by Cameron and Gould (1994) in rats. The duo examined the effects of corticosterone on neurogenesis in the dentate gyrus of rats by performing adrenalectomy and administering thymidine-H³ to measure cellular proliferation by Nissl
staining. They observed and reported a marked increase in newborn neurons in the DG. Shortly thereafter, and of particular importance, was the work of Eriksson et al. in 1998 on adult hippocampal neurogenesis in humans. With the consent of their elderly cancer patients, the team administered bromodeoxyuridine (BrdU), a thymidine analog, to patients and collected post-mortem tissues to identify evidence of neurogenesis in the SGZ, granule cell layer, and hilus of the hippocampus using immunofluorescence microscopy. They, too, discovered formation of new neurons indicated by the antibody-based detection of BrdU in what appeared to be granule cells in the SGZ and granule cell layer. Since then, the discovery and validation of adult neurogenesis compelled numerous novel research projects into the prospects of adult neurogenesis in mammals and have provided further advances to the field. Many have focused on pinpointing the cranial regions of neurogenic potential, identifying the underlying molecular mechanisms, uncovering the behavioural consequences, and inferring possible clinical solutions for various diseases. These will be covered in the sections to follow.

1.3 Non-neurogenic Regions

As will be made clear in subsequent sections, adult neurogenesis has been primarily identified as specific to the lateral ventricles and hippocampus in mammals. However, numerous other regions have been identified as possessing neurogenic capabilities due in part to the presence of neural stem cells. It is important to note, however, that in discussing neurogenesis the conversation is exclusive to the process of proliferation, differentiation, migration, and survival of neural progenitor cells. Insofar as the following neural regions have a so-called neurogenic capability, these are in fact only constrained to forming glial cells: neocortex, striatum, amygdala, hypothalamus, prefrontal cortex, eyes, corpus callosum, optic nerve, spinal cord, and piriform cortex (Ihunwo, Temob and Dzamalala, 2016). Consequently, it may befall as a misnomer to identify such sites as neurogenic. Yet interestingly, neural stem cells from these regions do possess the ability to develop into neurons in vitro, speaking to the importance of the microenvironment of the neurogenic niche. Indeed, several transplantation studies further substantiate this observation in vivo; neural stem cells from such regions implanted to a more well-established neurogenic region (i.e. the hippocampus or lateral ventricles) have shown an
inherent ability to differentiate into either neuronal or glial cells. When performed vice-versa, however, the neural stem cells are only capable of glial cell differentiation (Alvarez-Buylla and Lim, 2004). Currently, the established conclusion drawn proposes that neural stem cells in these non-specific neurogenic sites contribute to the repair and replacement of cells following post-traumatic, excitotoxic, seizure-related, or ischemic brain injuries (Fidaleo, Cavallucci and Pani, 2017).

1.4 Neurogenic Region: Lateral Ventricles

The first of the two known fundamental regions of adult neurogenesis are the lateral ventricles. Specifically, the subependymal zone, also known as the subventricular zone (SVZ), harbours the neurogenic neural stem cells and lies adjacent to the single-cell layer of the ciliated ependymal cells lining the fluid-filled lateral ventricles of the forebrain. Between the lateral ventricles and the other primary neurogenic site, the hippocampus, the subventricular zone is known to foster a greater neurogenic potential according to the sheer number of newly formed neural cells it generates. This region functions to primarily produce the interneurons of the olfactory bulb, the region tasked with olfactory interpretation (Xu, Lakshman and Morshead, 2017). These interneurons are formed as a result of incremental migration of neuroblasts (immature neurons) from the subventricular zone to the olfactory bulb along the rostral migratory stream (RMS) in a process known as chain migration. Upon arrival at the olfactory bulb, the neuroblasts undergo differentiation and maturation to become the quintessential inhibitory interneurons of the lateral ventricles. This unique migratory process occurs by a saltatory mechanism involving process extension, swelling near the axon hillock and centrosomal migration, and finally, soma translocation of the neuroblasts (Schaar and McConnell, 2005). Migration is directed with the provision of directional cues along the migratory path in the form of chemoattractants released by the alluring olfactory bulb. This migration along the RMS takes place in a synchronized process whereby each neuroblast utilizes the other as scaffold to propel along blood vessels and astrocytic tunnels (Kaneko, Sawada and Sawamoto, 2017). Upon arrival at the olfactory bulb, the neuroblasts detach from their chained association and differentiate into one of three GABAergic interneurons: calretinin, calbindin, or tyrosine hydroxylase interneurons (Batista-Brito et al.,
2008), or into glutamatergic neurons (Brill et al., 2010). A more scrupulous investigation by Batista-Brito (2008) identified subtypes that comprise these interneurons using genetic fate mapping of Dlx1/2 precursors, genes required for olfactory bulb interneuron generation. He discovered 7 olfactory bulb interneuron subtypes dispersed within the glomerular, external plexiform, mitral cell, and granule cell layers. These subtypes were characterized by differences in soma and process morphology and synaptic connectivity with other neuronal olfactory bulb cells. Evidently, the vital aspects of adult neurogenesis involving the differentiation and maturation of neural stem cells in the lateral ventricles in fact occurs external to the site of its origin and requires a migratory process. Furthermore, as Batista-Brito has shown, there is a great degree of variability in the type of cell produced by such migrating immature neural cells. Although neurogenesis within the lateral ventricles shares a kinship of new neural cell formation and cell migration with the hippocampus, therein also lies the major differences between neurogenesis in both sites; the types of viable neural cells produced (two in the hippocampus versus eight in the olfactory bulb) and the migratory distance of such cells in their respective neural region (a few cell bodies in length traversed by hippocampal cells versus millimetres by lateral ventricle neuroblasts) (Xu, Lakshman and Morshead, 2017).

1.5 Neurogenic Region: Hippocampus

1.5.1 Facts and Function

The hippocampus is the second pivotal site of adult neurogenesis in mammals. Neural stem cells have been shown to develop into astrocytes or glutamatergic neuronal cells peppered throughout the granule cell layer of the dentate gyrus and evidence points to the microenvironment for assisting in the initiation and fate-determination of these stem cells. This microenvironment consists of a variety of cells, including: endothelial cells, astrocytes, microglia, mature neurons, and vascular components (Gage and Temple, 2013). Although much of the roles of such cells in this dynamic are unknown, what is currently understood of the function of the microenvironment in influencing the fate determination of the neural stem cells comes from transplantation studies. For example, stem cells isolated from the spinal cord, a non-neurogenic site, and transplanted
into the granule cell layer of the hippocampus gain the ability to develop into neurons. This is in addition to their otherwise limited astrocytic and oligodendrocytic fate in the spinal cord (Shihabuddin et al., 2000).

The magnitude of hippocampal neurogenesis in murine species has been highlighted to a conservative number of several thousand new cells per day. As an example, studies in rats have demonstrated an approximate net formation of 650,000 new neuronal cells produced over three months. Though the process is not without the prospect of apoptosis; in fact, much of these newly formed and matured neuronal cells are ill-fated. It is possible then that cell death is a regulatory mechanism for balancing the proliferation rate of newly formed cells and functions to remove those infrequently used (Snyder and Cameron, 2012). Nonetheless, both apoptosis and neurogenesis in the adult hippocampus are mediated by numerous intrinsic and extrinsic factors. Some, such as depression, reduce the formation rate of neuronal cells in the hippocampus. Others, such as the intrinsic effects of Sonic hedgehog, Notch1, growth factors, and bone morphogenic protein, and the extrinsic effects of exercise, anti-depressant medications, learning, and cerebral injuries, enhance neurogenesis by way of upregulating the rate of neural progenitor cell proliferation and survival (Alvarez-Buylla and Lim, 2004; Triviño-Paredes et al., 2016).

Cumulatively, this alludes to a regulatory function of the hippocampus to necessitate for both the abstinence of neural stem cells from entering a proliferative phase and promoting apoptosis of matured neural cells or an induction of neurogenesis to increase the neural cell population. Nevertheless, neural stem cells remain in a state of quiescence in the absence of such intrinsic or extrinsic signals to prevent exhaustion of the stem cell pool (Kempermann et al., 2004).

Intuitively, much of the original postulations behind the purpose of adult hippocampal neurogenesis suggested a role for the newly formed cells not unlike the innate function of the hippocampus. A series of studies testing this hypothesis arrived at the conclusion that adult hippocampal neurogenesis is indeed a recurrent process crucial for learning, memory formation, and pattern separation (Ma et al., 2017). A compelling study by Drapeau et al. (2003) provided some of the earliest evidence for this memory-related function in rats. They examined a possible correlation between adult hippocampal neurogenesis and cognitive function in aged (10-11 months old) rats using the Morris Water Maze (MWM) test. By recording the performance of the rats in the MWM test and measuring the levels of cell proliferation and neuronal maturation, they discovered rats categorized as impaired in their MWM performance (i.e. poorly preserved spatial
memory) had a strongly correlated diminished level of proliferating neural cells and newly matured neuronal cells compared to unimpaired rats. This particular study, and others similar in their use of aged mammal models, focused on age-related cognitive decline associated with reduced adult neurogenesis. Such studies therefore demonstrate a crucial attribute of adult neurogenesis: it is not consistent in its ability to produce new neural cells and is contingent upon the age of the mammal. In fact, numerous studies using murine models have clearly shown that the rate of adult neurogenesis declines with age; it is a process strongly upregulated in adolescence and young age, but depreciates significantly in late adulthood. An extremely detailed study examining the rate of adult hippocampal neurogenesis at various ages conducted by Abdallah et al. (2010) on mice demonstrated 1 month to be the age in which the highest rate of hippocampal neurogenesis is evident.

1.5.2 Cell Types and Progression

The neural stem cells mark the beginning of the multi-step process of adult hippocampal neurogenesis and are located in the subgranular zone of the dentate gyrus. Two distinct types of neural stem cells exist: radial glial-like stem cells and non-radial stem cells. The former possesses a triangular soma and thick radial apical processes penetrating through the granule cell layer and into the molecular layer; whereas the latter have no radial projections and instead extend their projections in a horizontal manner through the subgranular zone with thin branches projecting towards the hilus and the granule cell layer (Figure 1) (Gebara et al., 2016). Histological markers can be used to identify these neural stem cells. Specifically, the co-localization of glial fibrillary acidic protein (GFAP), an intermediate filament protein, Sox2, a transcription factor protein expressed by stem cells, and Nestin, a type VI intermediate filament protein, are indicative of the radial glial-like stem cells, hereafter referred to as Type 1 neural stem cells. On the other hand, and for the present time, the non-radial stem cells are only identifiable by the expression of Sox2 (Zhao, Deng, and Gage, 2008). Evidently, the type 1 neural stem cells appear to resemble a typical glial cell found elsewhere in the brain. Yet, these cells functionally differ from glial cells and are unique given the extension of their processes from the subgranular zone to the molecular layer and the presence of glutamate and GABA.
receptors (Toni and Schinder, 2016). Rather than supporting neurons as glia cells typically do, these type 1 cells are distinguished by their restricted capacity to replicate and undergo the intricate process of adult hippocampal neurogenesis. Moreover, these cells are capable of both symmetric and asymmetric division to produce new neural cells and maintain the pool of type 1 NSCs, however the precedent in which one mode of division is preferred over the other is not yet clear. It is also important to bear in mind that while the microenvironment of the hippocampus constitutes various cell types, these type 1 neural stem cells are limited to producing only astrocytes and neuronal cells that are typical of the dentate gyrus; the granule cells produced add to excitatory granule neurons previously established which receive their input from the entorhinal cortex and project their output via the mossy fibre tract to the CA3 (Kempermann, Song and Gage, 2015).

The approximate timeframe for the completion of the neurogenic process is 3-4 weeks and is identifiable by the three developmental cell stages within the dentate gyrus: an intermediate neural progenitor cell (type 2a and 2b cells) stage, an immature neuronal (type 3 cell) stage, and finally, a mature dentate granule cell (Jin, 2016). As mentioned previously, type 1 neural stem cells remain quiescent until the introduction of an intrinsic or extrinsic stimuli. This halt of their progression through neurogenesis is due in entirety to the parvalbumin-expressing (PV+) interneurons (basket cells) in the dentate gyrus that release GABA to activate the γ2 subunit-containing GABAA receptors (GABAAARs). Upon stimulation, type 1 cells exit quiescence and enter a proliferative phase. Through asymmetric division, the activated type-1 cell can produce a daughter type-1 cell and a type 2a intermediate neural progenitor cell (NPC) (Song et al., 2012). The latter cells are transiently amplifying and histologically identifiable by their expression of Nestin and Sox2 but, unlike type 1 cells, do not express GFAP, indicating a loss of the initial glia-like morphology (Kempermann, Song and Gage, 2015). The type 2a cells can undergo further rounds of cell division as they progress towards a differentiated state. Cells of the next phase are labelled as intermediate type 2b NPCs and begin to express markers of a differentiating granule cell, namely, NeuroD1, Prox1 (Kempermann, Song and Gage, 2015), and doublecortin (DCX), a microtubule-associated protein (Kempermann et al., 2004). Within the first 3-5 days of development, both type 2a and 2b intermediate neural progenitor cells express polysialylated neuronal cell adhesion molecule (PSA-NCAM), a marker of developing and migrating cells, and continue proliferating. Upon completion of their proliferative phase the NPCs exit their mitotic
state and begin differentiating, acquiring properties resembling an underdeveloped neuron. Hereafter, such cells are referred to as immature neuronal cells (type 3 cells) (Kempermann et al., 2004). Type 3 cells are post-mitotic given their low or absent proliferative activity and identifiable with markers such as NeuN, calretinin, and DCX (Kempermann, Song and Gage, 2015). Another frequently used marker for distinguishing type 1, 2, and 3 neural cells is the proliferation marker, Ki67. This nuclear protein is detected in all phases of the cell cycle and exclusively labels proliferating cells (Bullwinkel et al., 2005). Given the proliferative nature of these neural cells, it was Kronenberg et al.’s (2003) investigation into the adult hippocampal neurogenic process in mice that successfully identified the appropriate proliferative markers to be used as tools in highlighting and differentiating the subtypes of neural progenitor cells. Like studies before them, they distinguished proliferating neural progenitor cells using transgenic green fluorescence protein (GFP)-expressing mice with a Nestin promoter. They found that a class of cells expressed GFP but were morphologically distinct from type 1 cells and yet another class of cells expressed both GFP and DCX. They labelled these cells as type 2a and type 2b intermediate neural progenitor cells, respectively. Finally, they found some cells to express DCX but not GFP and identified these as type 3 cells. Importantly, they found Ki67 to be useful in identifying both type 2a and type 2b cells since Ki67 labelled both cell types in conjunction with GFP but not type 1 or 3 cells.

Finally, the maturation of immature neuronal cells is identifiable by a switch from the expression of their calcium-binding protein, calretinin, to that of calbindin and continued expression of NeuN after approximately 3-4 weeks of development (Kempermann, Song and Gage, 2015). Despite what may appear as a relatively short period of time for neural cell maturation, such mature granule cells are not functionally integrated until the establishment of synaptic connections with neuronal cells of the CA3 and molecular layer of the hippocampus (Toni and Schinder, 2016).
Figure 1. Adult Hippocampal Neurogenesis Schematic diagram of the multi-step process involving type 1 neural stem cells (NSC), type 2a neural progenitor cells (NPC), type 2b neural progenitor cells, type 3 immature neuronal cells, and the mature neuron. Time course for full development to mature neuron is approximately 28 days. Both NSC types are shown here: non-radial and radial glia-like. Only the latter proceed through the neurogenic process, beginning in the subgranular zone (SGZ) and slowly moving towards the granule cell layer (GCL). Meanwhile, dendritic processes extend through the GCL into the molecular layer and axonal projections penetrate the polymorphic layer (hilus). Listed below are the various proteins and markers expressed at different stages of the process. Note: the width of the dentate gyrus layers represented here are not proportional to the actual dentate gyrus (i.e. the GCL is significantly larger than the SGZ).
1.5.3 Electrophysiological Properties and Structural Development

Morphologically-speaking, type 3 immature neuronal cells are identifiable by the extension of their dendritic processes towards the middle molecular layer of the dentate gyrus for excitatory GABAergic and glutamatergic inputs from the entorhinal cortex perforant pathway neurons; the projection of their axons towards the CA3 region to synapse with existing CA3 pyramidal cells; and the development of dendritic spines (Figure 1) (Kempermann et al., 2004). At first glance it appears as a conundrum to realize these immature cells also express GABA\textsubscript{A}Rs yet receive excitatory, as opposed to inhibitory, input from ambient GABA release from hippocampal interneurons. However, this is in fact due to the type of co-transporters expressed early in neuronal cell development. Specifically, the GABAergic input activates the Na-K-2Cl (NKCC1) co-transporter which functions to increase the cytoplasmic chloride concentration (Song et al., 2012). Over time and as the immature neurons continue to develop their processes and synaptic integration, there is an apparent change from the initial excitatory GABAergic input to the conventional inhibitory effect, as well as increased glutamatergic excitatory connectivity. The reversal of the GABAergic input is the result of an increase in the expression of K-Cl transporter membrane 5 (KCC2) co-transporter relative to the expression of the previously established NKCC1 co-transporter. Consequently, because KCC2 functions to oppose NKCC1’s effect of increasing internal chloride concentration, it decreases the internal chloride concentration upon activation (Jin, 2016). In summary, the maturation of immature granule cells is identifiable by the establishment of the cell’s inhibitory GABAergic response, excitatory glutamatergic inputs, spinal development, and dendritic and axonal extensions (Kempermann et al., 2004). As such, newly developed granule cells eventually become indistinguishable from the resident population upon complete maturation and integration into the hippocampal circuitry (Li et al., 2012). A crucial study performed by Esposito et al. (2005) established a timeline for the development of these morphological and functional attributes of newly formed neuronal cells. Using GFP retroviral labelling and NeuN immunostaining in mice, they targeted the newly developed neuronal cells and measured their input resistance, membrane capacitance, and resting potential between 1 and 29 days post-surgery. They discovered in the first seven days that these cells possessed an irregular shape (i.e. not yet granular) and were silent upon stimulation but began to express GABA receptors. Soon after 14 days they observed spineless apical dendrites penetrating...
through the granule cell layer and reaching the middle molecular layer. Shortly thereafter (approximately 18 days), the neuronal cells began receiving glutamatergic inputs as well as GABAergic inputs. After 28 days, the neuronal cells were reminiscent of mature granule cells; a rounded soma, axonal projections towards the hilus, spinal development on the dendrites extended to the outer molecular layer, and mature excitability with fast GABAergic response. Thus, they conclude, the approximate time for full integration and maturation of newly developed neuronal cells is four weeks.

The unique attributes of immature and to a certain extent, recently matured, neuronal cells go beyond the unusual initial GABAergic response. In fact, these developing neuronal cells demonstrate neural plasticity that is intrinsically different from mature neurons. Specifically, their long-term potentiation (LTP) is enhanced when compared to mature neuronal cells. Furthermore, these cells possess a lower threshold potential and therefore are more responsive to stimuli (Fidaleo, Cavallucci and Pani, 2017). Electrophysiological research into this phenomenon uncovered this difference to be due to the elevated expression of NR2B-containing NMDA receptors (NMDAR2B) in immature neuronal cells, which possess a high affinity for CaMKII and increase LTP levels, as opposed to the typical NR2A-containing NMDA receptors (NMDAR2A). The duration of this enhanced response by immature granule cells has been found to be between four to six weeks, by which point the cells revert to levels of plasticity observed in mature granule cells after the switch to NMDAR2A expression (Toni and Schinder, 2016). It is particularly interesting in the sense that this unusual difference in responsiveness to stimuli between immature and mature neuronal cells results in a dual-response to LTP induction. In a study performed by Marin-Burgin et al. (2012) using calcium imaging and electrophysiology in mouse brain slices, it was found that weak afferent currents applied to the tissue recruits few mature granule cells but activates a vastly greater portion of immature neurons. Since the immature neuronal cells also possess reduced GABA inhibition compared to mature neuronal cells, the immature neurons required even less of an input to excite compared to mature neuronal cells. Ultimately, this finding suggests that the hippocampus encapsulates a heterogeneous population of granule cells which differentially decode incoming messages contingent on the strength of the input that may either be interpreted by immature or mature neuronal granule cells. Moreover, since mature granule cells are under the inhibitory influence of GABA, it suggests a bias towards immature neuronal cell activation upon stimuli acquisition (Marin-Burgin et al.,
2012). Numerous other studies have also further substantiated this feature of newborn neurons by elucidating these type 3 cells’ high input resistance (Esposito et al., 2005), lower inhibition (Li et al., 2012), and greater synaptic plasticity than mature granule neurons (Ge et al., 2007). These experiments highlight a pivotal functional role of cells yet to complete their maturation and oppose the previous dogmatic stance that every cell progressing through neurogenesis is primarily prospective; that their present state is non-functional until furtherance. Though notwithstanding the immature neurons’ incapacity to generate synaptic outputs akin to mature neurons, they should be regarded as neither inferior nor superior to mature neurons, but instead as equally important for creating this heterogeneous population of granule cells in the hippocampus not seen elsewhere in the brain. Still, the dissimilarity between immature and mature neuronal cells in respect to this faculty is something for which we have attained only a modest level of understanding.

The importance of NMDA receptor expression cannot be emphasized enough in the context of newly developed neuronal cell integration. Without this receptor or its sufficient utilization, newly formed granule cells cannot integrate into the hippocampal circuitry and are therefore destined for apoptosis. A pivotal study by Ge et al. (2007) found that newly developed (i.e. one month old) neuronal cells initially possess LTP with high potentiation amplitudes and a low induction threshold, both of which depend on the expression of NR2B-containing NMDA receptors. As is often used to label newly formed cells, they used GFP retroviral labelling and whole-cell patch clamps to measure excitatory post-synaptic potentials in response to low-frequency stimulation (theta-burst stimulation; TBS) of the medial perforant pathway. Their results confirmed the findings of studies before them regarding the synaptic plasticity of newly developed neuronal cells, such that there is an initial low threshold for LTP induction and increased amplitude of response that eventually returns to levels observed in integrated mature granule cells. However, they also used an NMDAR antagonist (APV) and found the LTP to be absent in new and matured granule cells and that the administration of a more specific antagonist to NMDA receptor subtype 2B (NMDAR2B) only reduced this receptor subtype activation. The latter resulted in a decreased excitatory post-synaptic current only in developing one-month old dentate granule cells compared to mature dentate granule cells or two-month old granule cells. Evidently, the unique synaptic plasticity associated with developing dentate granule cells is dependent on NR2B-containing NMDARs and results in enhanced activity compared to mature
dentate granule cells but only for a short period of approximately one month. Ge et al. (2007) also briefly alluded to the possible signalling factors involved in this process, such as BDNF and NK-κB. Therefore, to further understand the complexity of adult hippocampal neurogenesis, it is important to highlight the molecular machinery involved in regulating this phenomenon.

1.5.4 Molecular Mechanisms

The constituent molecular contributors to adult hippocampal neurogenesis are numerous (Table 1). Briefly, they range from growth factors that enhance adult neurogenesis, such as fibroblast growth factor 2 (FGF-2), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF) (Voss et al., 2013); to transcription factors, such as Sex Determining Region Y-box 2 (SOX2), neurogenic differentiation 1 (NeuroD1), and prospero homeobox 1 (Prox1); and regulators such as GABA, glutamate, Wnt proteins, Sonic hedgehog (Shh) protein, bone morphogenic protein (BMP), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (Jessberger and Gage, 2014).

Beginning with the type 1 neural stem cells, numerous studies have identified the transcription factor, Sox2, as an excellent marker to use in identifying such cells. This transcription factor is highly expressed in the type 1 NSCs and type 2a NPCs. Favaro et al. (2009) demonstrated this with an ablation study of Sox2 from the adult hippocampus which resulted in a dramatic reduction of type 1 NSCs and consequently cell proliferation and neurogenesis. The deletion of the Sox2 gene in mouse embryonic brains also resulted in a complete lack of neurogenesis in adulthood. Furthermore, their study highlighted the role of Sox2 in regulating the expression of Shh and Wnt3a, signaling proteins normally present in type 1 NSCs but absent in the Sox2-ablated hippocampus. Even conditional ablation of Sox2 with a Sox2^{loxp} nestin-cre transgenic mouse line was sufficient to result in complete loss of NSCs 7 days post-natal. Furthermore, Favaro et al. (2009) discovered Shh functions to promote cell proliferation since the absence of Sox2 was partially rescued in the proliferative and self-renewal phenotype of NSCs by a Shh agonist (SHH-Ag) (Figure 2).
Sox2 has also been found to have a role in regulating nuclear receptor TLX in addition to Shh. TLX, a transcription regulator, has also been discovered to regulate NSC proliferation. Knockout and conditional ablation studies of TLX have resulted in a loss of NSC proliferative capability in the dentate gyrus and subsequent reintroduction of TLX into these null mice restored this proliferative ability (Beckervordersandforth, Zhang and Lie, 2015). Moreover, Sox2 is a well-established inhibitor of the Wnt signaling pathway known to activate the expression of the pro-neural gene, NeuroD1 (Kuwabara et al., 2009). Kuwabara et al. (2009) demonstrated through Sox2 silencing with siRNA that Wnt signaling triggers expression of NeuroD1 in mice and that NeuroD1-expressing cells are mutually exclusive from Sox2-expressing cells; NeuroD1 co-localizes with nestin, calretinin, and DCX, the latter two being markers of neuronal-fated cells, as well as proliferation markers, Ki67 and BrdU, whereas Sox2 co-localizes with nestin, only. Furthermore, introducing a Wnt3a ligand into NSCs resulted in an increase in NeuroD1 promoter activation, whereas blockade of Wnt signaling prevented transition of Sox2-positive stem cells into immature granule cells. They conclude NeuroD1 expression to therefore be necessary for progression from a proliferating neural progenitor cell into an immature neuronal cell (Kuwabara et al., 2009). It was previously contended that Wnt signaling was associated with neuronal fate determination and immature neuronal cell proliferation. However, this study and others in recent times have provided evidence of Wnt’s role in modulating neural stem cell activity as well (Beckervordersandforth, Zhang and Lie, 2015). Altogether, these studies on Sox2 have provided strong evidence for its role in maintaining NSCs in a state of proliferation and opposing differentiation.

Other regulators of neural stem cell proliferation include the transcription factors, Smad1 and Smad2. Both of these factors form heteromeric complexes with Smad4 and function to transcriptionally regulate BMP2/4 and TGF-β, respectively, and consequently reduce the proliferation of neural stem cells *in vitro* (Johnston and Lim, 2010). *In vivo* studies have subsequently shown that inhibition of BMP2/4 signaling or ablation of Smad4 expression in NSCs results in an increase in their mitotic activity in the dentate gyrus (Beckervordersandforth, Zhang and Lie, 2015). Notch signaling, which plays a central role in promoting cell proliferation, maintenance, and self-renewal in various stem cells, is unsurprisingly found to be present in NPCs. Here, its conditional ablation results in a reduction of NPC proliferation (Breunig et al., 2007). Using an inducible gain and loss-of-function Notch1 mice, Breunig et al. (2007)
discovered by overexpressing Notch1 an increase in NPC proliferation. Conversely, γ-secretase inhibition or genetic ablation of Notch1 resulted in enhanced cell cycle exiting of NPCs, indicating progression into a differentiation phase from proliferation. The Notch1 ablated mice were generated using a tamoxifen-inducible form of Cre recombinase with human GFAP promoter crossed with a loxP-flanked Notch1 mouse. Cell cycle exiting was then measured using iodo-deoxyuridine (IdU) and Ki67 within a 1-day timeframe. Mice genetically ablated for Notch1 had a significantly lower number of proliferating cells, faster cell cycle exiting rate, and, expectedly, greater number of newly developed neuronal cells.

Figure 2. Regulators of Type 1 NSC Proliferation  Schematic diagram of the various protein factors involved in regulating cellular proliferation of type 1 NSCs in adult hippocampal neurogenesis. Sox2 is found to be a major contributor in this regard by its regulation of several integral intracellular pathways and in return is regulated by other major factors. Concurrently, Smad4 dimerizes with Smad1 and/or Smad2 to function in this regulatory process as well. NSCs typically remain in a quiescent state until intrinsic induction by some factors shown here or extrinsic induction. Following this, they enter their symmetric or asymmetric division and in the latter case, enter the neurogenic process by producing a NSC and a NPC from their division.
The transition from neural stem cells to neural progenitor cells recruits new cellular pathways and factors for continuous regulation of neurogenesis. Type 2 NPCs have been shown to transiently express DCX (specifically, type 2b), SoxB2 transcription factor, Sox21, the T-box-containing transcription factor, Tbr2, and the transcriptional co-regulatory protein, Tis21 (Beckervordersandforth, Zhang and Lie, 2015). Sox21 has been found to be highly expressed in type 2a cells with minimal expression in type 1 and 2b cells, and functions to promote the development of cells from a type 2a to a type 2b NPC (Matsuda et al., 2012). Matsuda et al.’s (2012) research on Sox21 demonstrated that loss of Sox21 specifically impaired the transitioning of type 2 NPCs (type 2a to type 2b) and thereby reduced the formation of neuronal cells. Using Sox21 knockout mice, they observed no difference in the pool of NSCs but found a reduction in the DCX and NeuN-positive cells (i.e. neuronal cells). Further analysis led them to identify Hes5 as the target gene of Sox21 binding to regulate this transitional process. Moving forward, Tbr2 has also been identified as highly expressed in type 2a and type 2b cells and similar to Sox21, is responsible for the transition of type 2a to type 2b cells. Finally, Tis21 has been found to be expressed in both NPC subtypes and inhibits the G1-to-S transition and therefore cell proliferation, instead promoting a commitment to differentiation towards a neuronal lineage (Beckervordersandforth, Zhang and Lie, 2015).

Several studies have uncovered immature neuronal cells early in their development expressing two transcriptional targets of Wnt signaling, NeuroD1 and Prox1. However, these protein markers can also sometimes be expressed as early as in type 2b NPCs, indicating their soon-to-be differentiated state of a neuronal cell post-mitotically (Kuwabara et al., 2009). A number of in vitro studies have been performed thus far to demonstrate the necessity of NeuroD1 and Prox1 in promoting neuronal differentiation. In addition to these factors functioning as differentiation mediators are the transcriptional factors, Sox4 and Sox11, which induce neuron-specific gene expression in differentiating NPCs (Beckervordersandforth, Zhang and Lie, 2015). The involvement of Sox proteins, though intriguing, should not be unexpected as we see their involvement in embryonic neurogenesis as well; specifically, the expression of Sox2 and unlike adult hippocampal neurogenesis, Sox3, in the neuronal developmental process. However, it is worth mentioning that adult neurogenesis differs in many ways from embryonic neurogenesis. The research in this comparative field concludes that the period of its infancy when neurogenesis
first takes root to form the brain is later disposed of much of its impressions and mechanisms from the surrounding influences of its own way in adulthood.

Beyond type 3 immature neurons few research projects have examined the integration of newly developed granule cells. At the present moment, the genes involved in regulating neuronal migration, integration, and process extension are not yet entirely resolved. Yet, some insight has been made to identify the involvement of cAMP-response element-binding (CREB) signaling alongside the genes, Disc1 and Cdk5 (Braun and Jessberger, 2014). CREB, together with NeuroD1, are involved in the process of neuronal maturation and functional integration and operates as an essential regulator of this neurogenic process. Like NeuroD1, CREB is also regulated by GABA signaling (Jagasia et al., 2009). Jagasia et al.’s (2009) research into the function of CREB signaling was formative in highlighting its role in the integrative process of neuronal maturation. Using CREB loss-of-function mice they discovered impaired dendritic development, decreased expression of NeuroD1 and DCX, and diminished survival of newly developed neuronal cells. Furthermore, these effects regulated by CREB were mediated by GABA’s excitatory effect on CREB. Using several markers to distinguish neural cells progressing through neurogenesis (i.e. Ki67, DCX, calbindin, pCREB, and NeuroD) as well as retroviral labelling with GFP, they observed pCREB expression present in immature neuronal cells. However, this expression subsequently terminated after 3 weeks which indicates a transient function existing only for the duration of neuronal cell development and integration into hippocampal circuitry. Furthermore, knockdown of CREB with retroviral genetic manipulation resulted in alterations to the normal phenotype of developing neuronal cells; dendritic extensions were parallel to the granule cell layer as opposed to vertically penetrating towards the molecular layer. Finally, activation of CREB via phosphorylation was contingent upon GABA activity demonstrated through a genetic ablation experiment of GABA which resulted in lower pCREB expression.

Altogether, CREB signaling has been shown to be paramount in the neurogenic process. However, despite the complexity unearthed thus far, much of the molecular machinery contributing to the powerhouse of adult hippocampal neurogenesis still requires further discovery and investigation. One additional contributor to this process could be the Raf-1 kinase inhibitory protein (RKIP) and exercise as a potent extrinsic stimuli, as will be discussed in the following chapters.
Table 1: List of growth factors, transcription factors, and other regulators involved in adult hippocampal neurogenesis in type 1, 2, and 3 cells.

<table>
<thead>
<tr>
<th>Growth Factors &amp; Regulators of Neural Cells</th>
<th>Type 1 Neural Stem Cell Regulators</th>
<th>Type 2 Neural Progenitor Cell Regulators</th>
<th>Type 3 Immature Neuronal Cell Regulators</th>
</tr>
</thead>
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<tr>
<td>BDNF</td>
<td>Shh</td>
<td>SoxB2</td>
<td>NeuroD1</td>
</tr>
<tr>
<td>EGF</td>
<td>Wnt3a</td>
<td>Sox21</td>
<td>CREB</td>
</tr>
<tr>
<td>IGF-1</td>
<td>TLX</td>
<td>Tbr2</td>
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<tr>
<td>VEGF</td>
<td>Smad1</td>
<td>Tis21</td>
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<tr>
<td>GABA</td>
<td>Smad2</td>
<td>NeuroD1</td>
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<tr>
<td>Glutamate</td>
<td>Smad4</td>
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<td>IL-6</td>
<td>BMP2</td>
<td>Sox4</td>
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<tr>
<td>TNF-α</td>
<td>BMP4</td>
<td>Sox11</td>
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<tr>
<td>FGF-2</td>
<td>TGF-β</td>
<td>DCX</td>
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<td></td>
<td>Notch</td>
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<td>Sox2</td>
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1.6 Factors Affecting Neurogenesis - Exercise

Neural stem cells are held steadfast by Notch signalling in a state of quiescence and remain aground along the subgranular zone (Fidaleo, Cavallucci and Pani, 2017). The progression of adult hippocampal neurogenesis is a process regulated under the influence of the hippocampal microenvironment and with a strenuous interplay of extrinsic and intrinsic oversight. These cells lie open to the battering of these various stimuli, the former having been covered in Chapter 1.5.4. Here, I will review some of the prominent extrinsic modulators of adult hippocampal neurogenesis in mammals and in particular, the potent effects of voluntary physical exercise.

Research over the years has discovered aging to be a factor involved in diminishing adult hippocampal neurogenesis. Consequently, this perturbs normal functioning of the hippocampus at old age and is likely to promote neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease (Ma et al., 2017). Importantly, studies in mice have intricately detailed their neurogenic capability to decline significantly between the ages of 6 weeks and 5 months (Fidaleo, Cavallucci and Pani, 2017). Other detrimental effects on this process include mood disorders (e.g. prolonged depression), excessive bouts of stress, and chronic epilepsy. These have
been shown to diminish NSC proliferation, promote cell death, and instigate shrinkage of
dendritic extensions (Mahar et al., 2014; Mendonça et al., 2017).

Conversely, other extrinsic factors, such as physical exercise, anti-depressant medications, and
cerebral injuries (e.g. global and focal cerebral ischemia), can promote cell proliferation, mend
damages from physical injuries with newly developed neural circuitry, and increase the pool of
neural progenitor cells in the subgranular zone (Jin, 2016). Additionally, physical exercise has
been linked to increased cerebral blood flow, blood brain barrier permeability, and angiogenesis,
all changes which promote increased neurogenesis and neuroplasticity (Yancey and Overton,
1992; Swain et al., 2003). The impact of exercise on enhancing the vasculature of the
hippocampal area is notably important as this effectively raises the nutrient and growth factor
delivery to the neurogenic niche of the SGZ where NSCs are concentrated (Fidaleo, Cavallucci
and Pani, 2017). Moreover, numerous studies in rodents have highlighted the pro-neurogenic
effects of exercise directly: increased neuronal birth in the hippocampus, enhanced neuronal
spine density and synaptic plasticity, elevated neurotrophin levels, and improved spatial memory
function in the Morris Water Maze task, Y-maze test, and radial arm maze test. Behaviourally,
exercise has also been shown to improve performance in contextual fear conditioning, passive
avoidance learning, spatial pattern separation, and novel object recognition tasks (Voss et al.,
2013). In fact, a prominent study performed by van Praag et al. (2005) demonstrated the
beneficial effects of exercise in mice otherwise kept sedentary until 19 months of age. Using the
Morris Water Maze task, mice engaged in voluntary wheel running for 1 month at such an
elderly age demonstrated improved memory retention and spatial acquisition in conjunction to a
pronounced increase in proliferating neural progenitor cells in the hippocampus when compared
to mice kept under sedentary conditions.

Other studies similar to van Praag’s (2005) that examine the effects of exercise on cell
proliferation add further consensus within the field of adult hippocampal neurogenesis that
exercise approximately doubles the number of proliferating NPC cells (Baek, 2016). The fact
that exercise induces an increase in the sheer number of proliferating NPCs stands as a strong
reason for the observed improvement in hippocampus-related tasks. It therefore warranted
further investigation into the mechanisms behind its effects. Farioli-Veccioli et al. (2014)
discovered that physical exercise procures such an increase in NPCs due to its modulation of cell
cycle kinetics in proliferating neural progenitor cells. Specifically, their study found that
voluntary wheel running in their C57BL/6J wildtype mice resulted in a shortening of the cell cycle in NeuroD1-positive NPCs and an increase in the total number of proliferating cells. This effect of exercise did not impact the Sox2-positive NSCs, however. Specifically, the duration of the S phase of the cell cycle decreased from $12.9 \pm 0.77$ hours under sedentary conditions to $10.25 \pm 0.61$ hours under exercise conditions. Consequently, the total length of the cell cycle of these NPCs also decreased comparatively from $24.95 \pm 0.27$ hours to $22.07 \pm 0.42$ hours. Ultimately, this finding suggests that a shortening of the S-phase and overall cell cycle length is the method by which exercise regulates and enhances NPC proliferation.

Given the overall effects of exercise on neurogenesis, a study performed by Van der Borght et al. (2009) sought to uncover the minimal duration of exercise required in order to observe a significant effect on neurogenesis. In this study, mice voluntarily ran for 1, 3, or 10 days and results showed an increase in Glut1 expression (angiogenesis marker) and $\text{Ki67}^+$ expression in the dentate gyrus after only 3 days of exercise compared to sedentary mice. Furthermore, these effects endure for a period of time after cessation of physical activity. Studies by Nishijima et al. (2017) and Berchtold, Castello and Cotman (2010) on mice find the effects of exercise on increasing NPC proliferation and overall neurogenic progression to persist for up to 2 weeks post-cessation of voluntary wheel running before returning to levels observed under a sedentary state by 3 weeks.

Berchtold, Castello and Cotman (2010) specifically measured the expression level of brain derived neurotrophic levels (BDNF), a neurotrophic growth factor responsive to exercise induction resulting in an increase in its expression, to substantiate their findings. Clearly, exercise is notably potent in regulating adult hippocampal neurogenesis. It is least surprising then that such elevated responses in neurogenesis translate to improved learning and memory, pattern separation, adaptation, and discrimination (Fahimi et al., 2016; Voss et al., 2013). Evidently, the use of exercise as a method of inducing adult hippocampal neurogenesis is strongly supported on both a cellular and behavioural level across several species and constitutes a useful tool to modulate neurogenesis for comparative studies.
2 The Multifunctional RKIP

2.1 Discovery and Localization

In 1984, a soluble and basic 23kDa protein was isolated from bovine brain through purification of isolated grey matter cells. Using high-performance liquid chromatography and SDS-PAGE, this protein was isolated and its amino acid composition delineated. It subsequently came to be known as a phosphatidylethanolamine binding protein (PEBP) for its affinity to bind phosphatidylethanolamines (Bernier and Jollés, 1984). Much later in 1999, Frayne et al. discovered PEBP transcripts and protein in all tissues of the rat, though with varying degrees of expression; analyzing the immunoreactivity of PEBP located its greatest expression in the brain to be within the SGZ of the DG and CA fields with comparatively high expression levels also observed in the adrenal glands, various other brain regions, epididymis, heart, intestine, kidney, liver, and testis. These findings were also confirmed with RT-PCR analysis. In all such tissues, PEBP was isolated from the cytoplasm and inner plasma membrane of the cells. It is particularly interesting that PEBP expression is also widespread among various species, such as in mammals, parasites, worms, flies, and flowering plants, highlighting its evolutionary conservation. Several homologs of PEBP have also been identified across the numerous species that harbour them, including: PEBP1, PEBP2, and PEBP4. In humans and murine species, the genome expresses the PEBP1 homolog which later adopted a new name, Raf-1 kinase inhibitory protein (RKIP), in recognition of its function to inhibit Raf-1 discovered by Yeung et al. (1999). On the other hand, mice also possess the following two other homologs in addition to RKIP: PEBP2 and CORK2, which share some sequence similarity to RKIP (84 and 43%, respectively) (Hagan et al., 2006).

2.2 Role in MAPK Pathway

Yeung et al. (1999) identified the first functional role of PEBP using a yeast two-hybrid screen and co-immunoprecipitation of the protein complex it forms. This allowed for the isolation of a Raf-1 interacting protein (RKIP) bound to Raf-1 and mitogen activated protein kinase kinase (MEK) of the mitogen activated protein kinase (MAPK) pathway. The MAPK pathway is
ubiquitously expressed in all tissues and transmits mitogenic and differentiation signals from the cell membrane to the nucleus. This pathway constitutes the Ras-Raf1-MEK-ERK signal transduction cascade that is responsible for regulating cell growth, differentiation, migration, and apoptosis (Figure 4) (O’Neill and Kolch, 2004). Yeung et al.’s (1999) subsequent overexpression and knockdown experiments of RKIP with transfection vectors and anti-RKIP antibody administration, respectively, further substantiated its role in inhibiting Raf-1 and MEK phosphorylation and activation. Further experiments conducted by Yeung et al. in 2000 using a series of Western blot analyses and anti-RKIP antibodies revealed the exclusive binding of Raf-1 to both MEK and RKIP such that RKIP functions to dissociate the Raf-1-MEK complex to competitively inhibit MEK phosphorylation by Raf-1. Moreover, that RKIP suppression of the MAPK pathway requires suppression of only either MEK or Raf-1 and not both simultaneously, though RKIP seems to have a stronger affinity towards Raf-1. They confirmed the inactivity of Raf-1 bound to RKIP with an assay for MEK phosphorylation and found the kinase activity of Raf-1 was impaired, preventing activation of MEK, due to Raf-1’s direct association with RKIP. Trakul et al. (2005) later identified RKIP’s inhibition of the MAPK pathway due to its physical binding of Raf-1 occurs specifically at the N-region of Raf-1 to prevent phosphorylation at S338 by PAK kinases and at Y340-341 by SRC kinases.

2.3 Role in NF-κB Pathway

Soon thereafter in 2001, Yeung et al. examined other possible pathways under the regulation of RKIP. In this series of experiments they uncovered RKIP’s role in antagonizing a signal transduction pathway mediating the activation of nuclear factor κ-B (NF-κB) (Figure 4). This pathway is understood to regulate genes involved in suppressing apoptosis and mediating inflammation, infections, and other stress stimuli. This signal transduction pathway is activated by tumor necrosis factor-α (TNF-α) or interleukin-1β (IL-1β). Through a series of kinase assays they discovered RKIP’s ability to antagonize activation of IκB kinase-α (IKKα) and IKKβ that are otherwise activated by TNF-α. Moreover, RKIP also functions to inhibit NF-κB-inducing kinase (NIK) and transforming growth factor β-activated kinase 1 (TAK1). Under normal circumstances, NF-κB is localized in the cytoplasm and inhibited by direct affiliation with IκB. Prior studies have shown TAK1 and NIK to functionally activate IKK via phosphorylation which
proceeds to then phosphorylate, and therefore inactivate, IκB and permit activation of NF-κB. Yeung’s (2001) cell culture studies specifically demonstrated through ectopic expression of RKIP a downregulation of NF-κB activity and its upregulation upon endogenous ablation of RKIP. Importantly, Yeung identified RKIP’s ability to physically associate with NIK, TAK1, IKKα, and IKKβ with co-immunoprecipitation assays and specifically block the activation of NF-κB upon TNF-α and IL-1β administration.

2.4 Phosphorylated RKIP

Protein kinase C (PKC) comprises a part of a large family of kinases that regulate numerous factors, including: cell growth, death, differentiation, and transformation and activation of the MAPK pathway. Three classes of PKC exist, namely: the classical PKCs (α, βI, βII, γ) which require Ca^{2+} and diacylglycerol (DAG) for activation, the novel PKCs (η, θ, δ), which only require DAG for activation, and the atypical PKCs (e.g. PKCζ) which require neither Ca^{2+} nor DAG. Interestingly, epithelial growth factor (EGF) and fibroblast growth factor (FGF) (growth factors stimulated by exercise induction) mediate the phosphorylation of Raf-1 by PKCδ and PKCζ activation. However, these factors alone were found to be insufficient for activation of Raf-1 despite its phosphorylation, suggesting the involvement of a mediator to permit PKC activation of Raf-1. Not long after Yeung’s novel findings on RKIP’s regulation of the MAPK and NF-κB pathways was the discovery of PKC’s function to modulate RKIP activity. Corbit’s discovery in 2003 reported direct binding of PKCa, βI, βII, γ, and δ to RKIP at site S153 as a result of in vitro or in vivo PKC administration or in response to EGF (Appendix Figure 4). Ultimately, phosphorylation of RKIP relieves its inhibition of Raf-1 and permits activation of the MAPK pathway. Further evidence to this was the induction of a mutant form of RKIP (RKIP^{S153V}) that prevented release of RKIP from Raf-1 despite PKC activity.

In addition to Corbit’s findings were the investigations of Lorenz, Lohse and Quitterer in 2003 on the interplay between the PKC and RKIP dynamic and the G-protein coupled receptor (GPCR) kinase 2 (GRK2) and GPCR association. Briefly, GRK2 functions to inhibit the β-2 adrenergic receptor (ADRB2) via phosphorylation that results in the uncoupling of the G protein from the receptor and internalization of the GPCR (Figure 3). Lorenz, Lohse and Quitterer
(2003) discovered this novel function of RKIP as an inhibitor of GRK2 upon phosphorylation and activation of RKIP. This was observed since stimulation of the GPCR activates PKC which functions to phosphorylate RKIP, thereby triggering its dissociation from Raf-1 to dimerize with another pRKIP to bind GRK2 instead. In fact, RKIP was found to directly bind and inhibit GRK2 on the amino terminus of the kinase when it is in a phosphorylated state. Furthermore, as Corbit et al. (2003) had discovered, RKIP was necessarily phosphorylated at S153 in order to bind to and inhibit GRK2 since RKIP mutants of this site (RKIP<sup>S153A</sup>) were unable to prevent ADRB2 phosphorylation by GRK2. Therefore, and in conjunction with a series of additional Western blot and co-immunoprecipitation analyses, it was confirmed that phosphorylation of RKIP leads to its dissociation from Raf-1 to bind to and inhibit GRK2, allowing activation of ADRB2 GPCRs. Interestingly, stoichiometric analyses of the RKIP and Raf-1 complex and the pRKIP and GRK2 complex indicate a significantly stronger affinity for the former complex (1:1) compared to the latter (1:0.1) in the brain of mice, suggesting that under basal conditions is a preference for RKIP to associate with Raf-1 which only switches to GRK2 upon PKC-dependent phosphorylation Corbit et al. (2003).

2.5 Role in GSK3β Pathway

Finally, Al-Mulla et al. (2011) identified yet another major signaling pathway under the regulatory control of RKIP: the glycogen synthase kinase 3-β (GSK3β) pathway (Figure 4). GSK3β functions as a tumor suppressing protein by downregulating oncogenic pathways, including Wnt signaling and cyclin D1 activation. Moreover, GSK3β has been identified for having a prominent involvement in various cell functions, including: cell division, proliferation, apoptosis, adhesion, motility, and differentiation (Forde and Dale, 2007). Al-Mulla et al. (2011) showed that although RKIP was found to bind GSK3α and GSK3β in co-immunoprecipitation assays, it was nonetheless only co-localized with GSK3β in the cytoplasm and membrane under confocal microscopy analyses. Using HEK-293 cells transfected with an antisense miRNA vector against RKIP, they discovered a reduction in GSK3β protein levels. They subsequently confirmed RKIP’s role as a positive regulator of GSK3β with a doxycycline-inducible FLAG-RKIP transgene line where RKIP overexpression was found to upregulate GSK3β levels. RKIP
was also found to be responsible for preventing phosphorylation of the inactivating site T390 on GSK3β by p38 MAPK, an inhibitor of GSK3β.

Altogether, the findings of Corbit, Lorenz, Yeung, and Al-Mulla highlight an important and unique phenomenon about RKIP: it is either by attachment to its constituent targets of the MAPK, NF-κB, or GSK3β pathway in an un-phosphorylated state or by dimerization with itself upon phosphorylation by PKC to bind GRK2 that the integrity of RKIP’s structure is transmitted to its function. Accordingly, this duality is brought into an active state to operate at any moment regardless of its phosphorylation status; no single circumstance can direct RKIP’s action towards modulating either pathway alone in a model by the best disposition of other factors involved. Therefore, in an effort to study its role in various models and processes we must be conscious of this fact.

**Figure 3. RKIP Regulation** Schematic diagram of protein kinase C’s (PKC) regulation of the Raf1 kinase inhibitory protein (RKIP) and the downstream effects as a consequence of RKIP’s phosphorylation. Broken lines indicate effects imposed on downstream targets by the activity of an otherwise un-inhibited regulatory factor.
Figure 4. RKIP Cellular Pathway Schematic diagram of the Raf1 kinase inhibitory protein and its downstream regulation of 3 major pathways: NFκB, GSK3β, and MAPK. Included is the in vitro study by Sagisaka et al. (2010) on RKIP and the resulting neural stem cell differentiation profile. Broken lines indicate effects imposed on downstream targets by the activity of an otherwise un-inhibited regulatory factor.
2.6 Adult Hippocampal Neurogenesis

One study has thus far been performed addressing the possibility of RKIP’s role in modulating adult hippocampal neurogenesis. In this novel study by Sagisaka et al. (2010), the function of RKIP on neural stem cell fate was investigated. Prior research had shown hippocampal cholinergic neurostimulating peptide (HCNP), the 10 amino acid-long peptide of RKIP, isolated from rat hippocampus to function to promote differentiation of stem cells into septo-hippocampal cholinergic neurons by stimulating production of choline acetyltransferase. Yet it was unclear whether its full-length 187 amino acid precursor, RKIP, functioned for a comparable purpose (Zeng, Imamoto and Rosner, 2008). Using cultured adult rat hippocampal progenitor cells (AHP cells), Sagisaka et al. (2010) performed immunocytochemical analysis of RKIP expression in Nestin-positive cells and observed high levels of RKIP expression in these precursor cells. Following this, they immunostained AHP cells for RKIP and the following markers of various neural cell types: GFAP for astrocytes, microtubule associated protein 2 (MAP2) for neurons, and receptor interacting protein (RIP) for oligodendrocytes. Over a short period of 2 and 4 days, they observed the differentiation of the AHP cells gravitate towards either a RIP-positive oligodendrocyte or MAP2-positive neuron with undetectable levels of GFAP-positive astrocytes. Furthermore, overexpression of RKIP with an RKIP vector construct conjugated to IRES-GFP under the control of a CMV promoter in AHP cells resulted in an absence of GFAP-positive cells and an increase in RIP and MAP2-positive cells after 4 days. Finally, they confirmed their findings with a downregulation experiment of RKIP using siRNAs in the AHP cells; cells treated with siRNA against RKIP resulted in a greater proportion of GFAP-positive cells compared to controls with no change in the number of MAP2 and RIP-positive cells. They therefore concluded from their in vitro study that RKIP functions to promote the differentiation of AHPs into neurons and oligodendrocytes as opposed to astrocytes (Figure 4). In addition to Sagisaka et al.’s (2010) findings, Hellmann et al. (2010) also examined the role of RKIP on neuronal differentiation in cultured neuroblastoma cells overexpressing RKIP or given shRNA against RKIP expression. Their study found RKIP to function in accelerating neurite growth, promote elaborate neuronal networks, and increase expression of neuronal markers in as early as 7 days after retinoic acid treatment.
Studies such as these suggest that the function of RKIP is much in advance of the neurogenic process; beyond the proliferative phase of progenitor cells. Though it may be tempting to pin RKIP's role as only a mediator of neural progenitor cell differentiation, other studies have brought to light its effects on modulating cell cycle kinetics as well. These suggest yet another possible role for RKIP to regulate neural progenitor cell proliferation. In support of this hypothesis is the finding that RKIP has also been detected in the nucleus of the various cells it resides in in addition to the cytoplasm and inner plasma membrane (Ling et al., 2014). This suggests it may function to regulate some aspect of the mitotic phase of the cell cycle. It is well established that in order to ensure proper progression through mitosis, the kinetochores must necessarily completely attach to the chromosomes of the dividing cell for equal separation of chromosomal DNA in the daughter cells. This orderliness is regulated by Aurora kinases and studies in cultured mammalian cells have shown RKIP depletion to result in MAPK pathway hyper-activation and Aurora B kinase inhibition (Figure 4). Consequently, the appropriate spindle checkpoints by G2/M checkpoint molecules are bypassed resulting in genetic abnormalities (Zeng, Imamoto and Rosner, 2008). Specifically, cells rapidly transition to anaphase from pro-metaphase without proper control from phosphorylated Aurora B kinase which otherwise would ensures proper chromosomal alignment, cell division, and spindle checkpoints. Furthermore, RKIP depleted cells have reduced transition times from nuclear envelope breakdown to anaphase. However, Raf-1 or MEK inhibition effectively reverses this damaging bypass of mitotic checkpoints (Eves et al., 2006). Al-Mulla et al. (2011) corroborated the findings of Eves et al. (2006) later by demonstrating how RKIP silencing leads to an accelerated DNA synthesis and G1/S transition entry as well as a deregulation of 61 cell cycle-related genes, including important regulators that accelerate mitosis (NEK6 and APC11/7) and regulators that delay mitosis (Aurora B, cyclin G1, and Sirtuin). Cumulatively, these experiments highlight the importance of RKIP in cell cycle progression and by extension, cell proliferation, such that cells lacking RKIP modulation become excessively proliferative due to a hastened cell cycle.

Several methods of modulating RKIP expression exist and some have been briefly mentioned in this chapter (e.g. siRNA and miRNA knockdown and anti-RKIP antibodies). However, Theroux et al. (2007) were first to successfully create an RKIP knockout strain using a gene trap vector. Specifically, the embryonic stem cells derived from C57BL/6-Tyr-c-Brd carried a gene trap of
intron 1 of the *RKIP* gene. These cells were grown to a blastocyst and later transfected into pseudo-pregnant females to produce chimeric pups. The chimera mice were then backcrossed with C57BL/6-*Tyr^c-Brd* females to attain the F1 progeny and the *RKIP^1Gr(pGT01xrBetaglo)1Jk1* (hereafter referred to as RKIP knockout) were selected for study. However, this mutation may not affect the expression of HCNP since HCNP is localized to exon 1 of the RKIP gene. Nonetheless, quantitative PCR and Western blot analyses confirmed knockdown of RKIP and X-Gal staining of neural tissues identified the GCL of the DG and surrounding CA3 regions to highly express the inserted β*-geo* marker, indicating these sites as regions of otherwise high RKIP expression. They further documented mice of this strain to be viable for up to 10 months of age with only an olfactory deficit exhibiting onset at approximately 3-4 months of age. This suggests that the RKIP protein is likely not of great need during embryonic neuronal development and may function for other purposes within the hippocampus later in life.
Given the novelty in identifying RKIP as a mediator of several cell signaling pathways, I aim to identify the role of RKIP in regulating adult hippocampal neurogenesis. Specifically, I seek to elucidate the function of RKIP in modulating neural stem cell proliferation and differentiation in the confines of this highly proliferating region of new neuronal cell formation. I also look to highlight any potential role RKIP may undertake to dictate the differentiation and maturation process of neural progenitor cells. To this end, I employed RKIP knockout mice of the same lineage as created by Theroux et al. (2007) in my investigations to observe the impact of an absent RKIP expression on this hippocampal neurogenic process. To ensure knockdown of RKIP expression, we have previously crossed our RKIP knockout mice and confirmed absence of RKIP expression through Western blot analysis. As the wild-type control, I saw fit to use mice of the same genetic background as my RKIP knockout mice (C57BL/6J). Furthermore, I have opted to use voluntary wheel running paradigms as a well-established form of exercise capable of neurogenic induction. We have also previously recorded the distance travelled on the wheels over several days in behavioural cabinets and found no difference in the duration spent or distance travelled by both phenotypes to ensure no difference in physical capability of RKIP knockout and wildtype C57BL/6J mice (Antoun et al., 2012).

Given the prior findings of Sagisaka et al.’s (2010) in vitro study of RKIP on neural progenitor cell differentiation and other studies on cell cycle kinetics, I hypothesize that RKIP functions to reduce neural progenitor cell proliferation via inhibition of the MAPK pathway and modulation of NPC cell cycle kinetics. Furthermore, that RKIP functions to promote differentiation of NPCs towards a neuronal fate. To address these research objectives, I examined the process of adult hippocampal neurogenesis in vivo to identify neural progenitor cell proliferation, differentiation, and maturation phenotypes. As well, I investigated the cell cycle kinetics, with respect to the S-phase and overall duration, of NPCs and the MAPK pathway role with a specific exogenous inhibitor. Altogether, I look to provide novel evidence of the regulatory function of RKIP in adult hippocampal neurogenesis.
Chapter 3
Methods

1 Animals and Exercise Paradigms

All animals and experiments are conducted at the University of Toronto Mississauga in compliance with the Canadian Council on Animal Care. Mice are maintained in housing cages sized for up to five animals and provided with food pellets (Harlan Laboratories) and water ad libitum. RKIP knockout mice are generated via a gene trap in intron 1 of the RKIP gene (Theroux et al., 2007). These mice were backcrossed onto C57BL/6J (The Jackson Laboratory) mice for >10 generations before experimental use. Consequently, C57BL/6J mice were used as controls for comparison (The Jackson Laboratory). Western blot analysis was performed to ensure complete suppression of RKIP expression in RKIP knockout mice (data not shown). For experiments examining mice under sedentary conditions, mice were maintained under their current colony conditions. Mice placed under exercise conditions were either single or pair-housed depending on the cage used to ensure each mouse had access to their own wheel; small cages accommodate a single wheel whereas the largest cage accommodates two wheels.

2 Thymidine Analog Solution Preparation and Injections

Bromo-deoxyuridine (BrdU), chloro-deoxyuridine (CldU), and iodo-deoxyuridine (IdU) are injected intraperitoneally. Given the toxicity of thymidine analog solutions, all precautionary measures were taken as indicated by the University of Toronto’s Division of Comparative Medicine’s Standard Operating Procedure #3.7.1 (Safe Handling of Rodents Exposed to BrdU) and #3.7.3 (Safe Handling of Rodents Treated with BrdU, CldU, EdU and IdU). For BrdU (Sigma-Aldrich), mice are given a dosage of 100mg/kg of body weight using a 10mg/mL solution prepared with 0.9% saline solution. For CldU (Sigma-Aldrich), mice are given a dosage
of 42.5mg/kg of body weight using a 4.25mg/mL solution prepared with 0.9% saline solution. Finally, for IdU, mice are given a dosage of 57.5mg/kg of body weight using a 5.75mg/mL solution prepared with 0.9% saline solution. Mice treated with thymidine analogs are housed in separate cages from mice not injected with the analogs to prevent unnecessary exposure.

3 Tissue Harvesting

For immunofluorescence and immunohistochemistry, mice are killed by cervical dislocation and the brain is immediately extracted from the skull and submerged momentarily in chilled oxygenation media (125mM NaCl, 2.5mM KCl, 2mM CaCl$_2$, 25mM NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 25mM dextrose). The brain is then sectioned using a vibratome (Electron Microscopy Sciences) to produce 800-900µm coronal slices with an intact hippocampus. Sectioned tissues are thereafter incubated and fixed in 4% w/v PFA dissolved in PBS pH 7.4 for six hours at room temperature. Following fixation, sectioned tissues are transferred into a solution of 30% sucrose w/v dissolved in PBS (with added 0.2% v/v NaAz and 0.4% 1M NaF) and refrigerated in 4°C indefinitely. Sectioned tissues are then further sliced into 30µm sections using a Leica SM2010 R sliding microtome (Leica Biosystems).

For SDS-PAGE and subsequent Western blotting experiments, mice are killed in the same manner by cervical dislocation and their brain immediately extracted from the skull to be submerged briefly in chilled oxygenation media. The brain is then sectioned using a vibratome to produce two 500µm coronal slices with an intact hippocampus. The second coronal slice is immersed in cold PBS solution meanwhile attending to the first coronal slice for microdissection of the CA3 and DG of the hippocampus; the tissue is placed on a dry ice-cooled plate underneath a Zeiss Stemi 2000 stereomicroscope and the CA3 and DG regions of the bilateral hippocampal structure are removed with a 26.5 gauge needle (Becton Dickinson). The tissue extracts are then stored in -80°C until further use.
4 Immunofluorescence Staining

Coronal tissue sections of adult mouse brains with the intact hippocampus are selected at a thickness of 30µm. Tissues are washed five times in PBS-T (PBS with 0.1% Triton X-100 (Thermo Fisher Scientific)) pH 7.4 then blocked in blocking solution (10% horse serum in 0.1% PBS-T) for one hour. Tissues are thereafter incubated overnight at 4°C with the primary antibody diluted in the blocking solution. The following day, the primary antibody solution is removed and the tissues are washed in PBS-T five times then incubated for two hours in the dark at room temperature with the secondary antibody fluorophore diluted in blocking solution. The secondary antibody solution is then removed and the tissues are washed again three times for ten minutes with PBS-T. For some experiments, tissues are then incubated in DAPI (1:10,000, Thermo Fisher Scientific) for five minutes. Finally, the tissues are washed twice for five minutes with PBS and thereafter mounted on a gelatin-free glass microscope slide with DAKO (Agilent) applied to cover the slip for imaging.

In experiments utilizing a thymidine analog (i.e. BrdU, IdU, or CldU) for immunofluorescence, a harsher protocol is to be followed which modifies our standard technique for immunofluorescence staining as outlined before. The coronal tissue sections are first washed twice for ten minutes with PBS and then incubated for thirty minutes at 37°C in 2N HCl. Tissues are then washed for five minutes in PBS and then incubated at room temperature for ten or twenty minutes with 0.1M boric acid pH 8.5 for BrdU or IdU/CldU staining, respectively. Sections are then washed five times for five minutes with PBS and blocked in blocking solution (10% horse serum in 0.1% PBS-T) for one hour. For IdU/CldU tissues, 2% BSA and 50mM glycine are added to the blocking solution. Tissues are then incubated overnight at 4°C with the primary antibody diluted in blocking solution. As before, in the following day the primary antibody solution is removed and the tissues are washed in PBS-T five times and then incubated for two hours in the dark at room temperature with the secondary antibody fluorophore diluted in blocking solution. The secondary antibody solution is then removed and the tissues are washed again five times for five minutes with PBS-T. Finally, the tissues are washed twice for five minutes with PBS and thereafter mounted on a gelatin-free glass microscope slide with DAKO (Agilent) applied to cover the slip for imaging.
5 Immunohistochemistry Protocol

For immunohistochemistry, coronal tissue sections of adult mouse brains with the intact hippocampus are selected at a thickness of 30µm. Tissues are washed five times for five minutes with PBS-T (PBS with 0.1% Triton X-100 (Thermo Fisher Scientific)), then incubated for twenty minutes in 0.3% H₂O₂ in PBS. The tissues are then washed again five times for five minutes with PBS-T and blocked for one hour at room temperature in blocking solution (10% horse serum in 0.1% PBS-T). Tissues are then incubated overnight at 4°C with the primary antibody (rat anti-BrdU (1:1,000; Bio-Rad)) diluted in blocking solution. The following day, the antibody is removed and the tissues are washed five times for five minutes in PBS-T then incubated for two hours at room temperature in biotinylated secondary antibody (biotinylated anti-rat IgG) solution diluted in blocking solution. Tissues are then washed five times for five minutes in PBS-T and incubated in Avidin-Biotin Complex reagent using the Vectastain Elite ABC HRP Kit (Vector Laboratories) for forty-five minutes at room temperature. The tissues are then washed five times for five minutes in PBS-T then developed with DAB reagent as per the DAB Peroxidase Substrate Kit (Vector Laboratories) guidelines. Tissues are then mounted on gelatin-free glass microscope slides and cover slipped with Permount (Thermo Fisher Scientific).

6 SDS-PAGE and Western Blot

The micro-dissected CA3 and DG tissues are used for Western blot analyses. The tissues kept at -80°C are homogenized manually with a pestle in RIPA buffer. The following are added to the RIPA buffer as well in anticipation of intending to isolate phosphorylated proteins: 0.2% proteinase inhibitor, 0.1% 1mM Na₃VO₄, 0.1% 1mM β-glycerophosphate, and 0.5% 5mM NaF. The homogenized solution is allowed to incubate on ice for twenty minutes with periodic vortexing every five minutes. The homogenate is then centrifuged at 4°C for twenty minutes and the supernatant is then extracted for quantification. The protein extracted is then quantified using
a spectrophotometer (Bio-Rad Laboratories) against a BSA (Thermo Fisher Scientific) solution standard.

For proteins of 42-44 kDa in size, an 8% polyacrylamide gel is prepared for SDS-PAGE. Each well is loaded with 10µL of 20µg of protein. The sample solution loaded contains Coomassie Brilliant Blue dye, dithiothreitol (DTT), RIPA buffer, and the protein lysate. Alongside the samples loaded is 10µL of a Novex LC5800 ladder (Thermo Fisher Scientific). The gel is run in running buffer solution for one to two hours at 100V. Thereafter it is transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore) in a Western blot apparatus sandwich to run for an additional two hours at 80V in transfer buffer solution at 4°C. The membrane is then removed and blocked with 5% w/v skim milk in TBS-T (TBS with 0.3% Tween-20 (Sigma Aldrich)) blocking solution for one hour. The blocking solution is then removed and the membrane is incubated in the primary antibody diluted in 5% blocking solution overnight in 4°C. The following day, the membrane is washed five times with TBS-T for five minutes and incubated in the secondary antibody diluted in 5% blocking solution for one hour at room temperature. Finally, the membrane is washed five times in TBS-T for five minutes before proceeding to detection.

An autoradiography method is employed for Western blot band detection. First, enhanced chemiluminescence (ECL) solution is prepared as indicated by the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) guideline and poured over the membrane for five minutes. Thereafter, in a dark room using an autoradiograph cassette, the membrane and autoradiograph film overlap and clasp to imprint for thirty seconds to five minutes to produce images of the clearest quality. The film is then immersed in developing solution, followed by a fixative solution, and finally rinsed in water.

The primary and secondary antibodies used in the Western blot experiment include the following: rabbit anti-p44/p42 MAPK (Cell Signalling), rabbit anti-Phospho-p44/p42 MAPK (Cell Signalling), and goat anti-rabbit IgG HRP (Thermo Fisher Scientific).
7 Imaging and Quantification

Imaging of immunofluorescent and immunohistochemical stained-tissues were performed using a Zeiss Axio Observer Z1 inverted microscope platform with a confocal Laser Scanning Microscope 700 and an AxioCam MRm Rev.3 microscopy camera. Imaging and camera settings were setup to be identical for each experiment performed and all images were 1.278mm x 0.3195mm captured at 40x magnification. Each hippocampal image was acquired with 6 z-stacks set apart by 3-5μm intervals.

Thereafter, images from IF, IHC, and Western blot experiments were used for analysis of cell counts over the hippocampal area of interest (i.e. hilus, DG, or SGZ) and protein band intensity comparisons using the software, ImageJ 1.50i. All cell counts were performed with a cell marker intensity threshold established observationally to minimize bias; cells that clearly and fully encapsulated the marker under investigation (e.g. Ki67) were counted as positive for that marker. Cells were not counted as positive for the marker if they were only partially expressing the marker (i.e. faintly blotchy or spotted).

8 Statistical Analyses

Two-way ANOVA, three-way ANOVA, Fisher’s least significant difference (LSD) and Student’s T-Test post-hoc tests were performed using IBM SPSS Statistics 20 software (IBM Corp.) with 95% confidence intervals (α = 0.05).
1 Neural Progenitor Cell Proliferation

Neural progenitor cells proliferate in the subgranular zone of the hippocampus throughout the first 5 days (Gonçalves, Schafer and Gage, 2016). Here, 35-day-old RKIP knockout and C57BL/6J wild-type mice were placed under either sedentary or exercise conditions; mice in the exercise group were housed in pairs with two wheels and mice in the sedentary group were group housed with no wheel access. Food and water were provided ad libitum. After 5 days of habituation and exercise, mice under both conditions were injected with BrdU (100mg/kg; Sigma-Aldrich) and killed 3 hours after injection (Figure 5A, B). Coronal cross sections of tissues containing the hippocampus were attained for immunohistochemistry staining of BrdU to highlight actively proliferating neural progenitor cells within the subgranular zone of the hippocampus (Figure 5C).

The results of the BrdU⁺-cell density measurements in the subgranular zone of the hippocampus shown in Figure 5D indicate an effect of genotype and condition (i.e. sedentary or exercise) on cell proliferation (two-way ANOVA: F = 5, p < 0.05). Further Fisher’s LSD post-hoc analysis revealed a significant difference between wild-type and RKIP knockout mice placed under exercise conditions: under the voluntary exercise paradigm, RKIP knockout mice exhibit a significantly lower BrdU⁺ cell density of neural progenitor cells compared to wild-type mice (p < 0.01). No difference was observed in the proliferation profile of RKIP knockout and wild-type mice placed under sedentary conditions. Moreover, both RKIP knockout and wildtype mice exhibited an induction effect of exercise (two-way ANOVA: F = 44, p < 0.001).
Figure 5. Neural Progenitor Cell Proliferation  Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice under sedentary (A) or exercise (B) conditions for 5 days then injected with BrdU (100mg/kg) and killed 3 hours post-injection. C Sagittal cross section immunohistochemistry stain results of hippocampal tissue. Yellow arrow points to BrdU\(^+\) proliferating NPCs. Left to right: Sedentary-Wildtype (n = 6), Sedentary-Knockout (n = 5), Exercise-Wildtype (n = 8), Exercise-Knockout (n = 6). D Cell density measurements of BrdU-positive cells in the subgranular zone of the hippocampus in mice of both phenotypes under both conditions (#: F = 44, \( p < 0.001 \); *: \( p < 0.01 \)). Data shown as mean ± SD.
My initial findings on the neural progenitor cell proliferation profile of RKIP knockout mice prompted further investigation into the possibility of specific differences between the two subtypes of neural proliferating cells within the subgranular zone: type 2a and type 2b cells. To identify potential differences in these two progenitor cell subtypes in RKIP knockout mice, I performed immunofluorescence labeling to highlight these cellular subtypes. As before in my proliferation phenotype experiment in section 1, 35-day-old RKIP knockout and wild-type mice were placed under either sedentary or exercise conditions; mice in the exercise group were housed in pairs with two wheels and mice in the sedentary group were group housed with no wheel access. Food and water were provided *ad libitum*. Mice were then killed after 5 days of habituation and exercise (Figure 6A, B). Coronal cross sections of tissues containing the hippocampus were attained for immunofluorescence staining of Ki67, Doublecortin (DCX), and DAPI to measure the cell density of type 2a and type 2b cells in the subgranular zone of the hippocampus (Figure 6C, D). Cells expressing Ki67 and not DCX (Ki67+/DCX−) indicate type 2a cells given their expression of a marker for actively proliferating cells (Ki67) and the absence of the early marker for neuronal differentiation (DCX). On the other hand, cells expressing both Ki67 and DCX (Ki67+/DCX+) indicate type 2b cells due to their expression of both the proliferation and differentiation marker. This is indicative of cells that are within the intermediary phase of neurogenic progression between proliferation and entry into differentiation. Figure 6E illustrates the comparison for type 2a cells between RKIP knockout and wild-type mice under sedentary and exercise conditions. Evidently, no significant difference was found between genotypes, however an overall induction effect of exercise was observed in both RKIP knockout and wildtype mice (two-way ANOVA: F = 18, p < 0.001). Figure 6F shows the comparison for type 2b cells and once again, no significant difference was observed between the genotypes but an induction effect of exercise in both RKIP knockout and wild-type mice is evident (two-way ANOVA: F = 48, p < 0.005). Interestingly, the proportion of neural progenitor cell subtype changes significantly upon exercise induction. Under sedentary conditions in RKIP knockout and wild-type mice together, the type 2a and type 2b NPCs constitute an approximately 39-43% and 57-61% composition of the total population of proliferating cells, respectively. This
proportion is not maintained, however, in mice of both genotypes under voluntary exercise conditions where it changes to 29-30% type 2a and 70-71% type 2b (p < 0.001) (Figure 6G).

Figure 6. Neural Progenitor Cell Subtype Proliferation Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice placed under sedentary (A) or exercise (B) conditions for 5 days then killed at 40 days of age. C Sagittal section immunofluorescence stain results of hippocampal tissue. IF stains from left to right: DAPI, Ki67, DCX, and composite image of each marker overlapped. Red arrows indicate type 2a cells (DCX/Ki67+) and yellow arrows indicate type 2b cells (DCX+/Ki67+). D Composite images of DAPI, Ki67, and DCX-stained inner edge of the hippocampal tissue. Left to right: Sedentary-Wildtype (n = 5), Sedentary-Knockout (n = 5), Exercise-Wildtype (n = 6), Exercise-Knockout (n = 6). E Cell density measurements of DCX/Ki67+ (type 2a) cells in the subgranular zone of the hippocampus in mice of both phenotypes under both conditions (#: F = 18, p < 0.001). F Cell density measurements of DCX+/Ki67+ (type 2b) cells in the subgranular zone of the hippocampus in mice of both phenotypes under both conditions (#: F = 48, p < 0.001). G Proportion of type 2a and type 2b neural progenitor cells constituting the entire pool of proliferating cells. Left to right: Wildtype-Sedentary, Knockout-Sedentary, Wildtype-Exercise, and Knockout-Exercise. Data shown as mean ± SD.
3 Type 3 Neural Progenitor Cell Proliferation

As an extension of my findings on type 2a and type 2b NPCs, I profiled the cell density measurements of type 3 immature neuronal cells using the same tissues of mice and images from section 2. To do this, I quantified cells expressing DCX and lacking Ki67; cells that are Ki67-\text{DCX}^+ are type 3 immature neuronal cells which have exited the cell cycle and are on the trajectory to develop into neuronal cells (Figure 7A, B). Two-way ANOVA of the results of this quantification shown in Figure 7C indicates a significant difference between genotype (\(F = 20; p < 0.001\)) and condition (\(F = 73; p < 0.001\)). Further post-hoc analysis indicates that RKIP knockout mice possess a significantly lower Ki67-\text{DCX}^+ cell density profile compared to wild-type mice under both sedentary and exercise conditions (Fisher’s LSD; \(p < 0.001\)). However, the magnitude of this difference between phenotypes under both conditions was not significantly different (\(p > 0.1\)). Furthermore, both RKIP knockout and wild-type mice exhibit a significant increase in Ki67-\text{DCX}^+ cell density under exercise conditions compared to sedentary conditions (i.e. exercise induction) (Fisher’s LSD, \(p < 0.001\)).

Additionally, I consolidated my initial findings of neural progenitor cell proliferation in section 2 by examining the total Ki67+ cell density profile of RKIP knockout and wild-type mice under both conditions. This quantification constitutes the entire pool of proliferating neural progenitor cells within the subgranular zone. Two-way ANOVA of the results shown in Figure 7D indicate a significant difference within the conditions (\(F = 51; p < 0.001\)) but not between genotypes. Further post-hoc analysis confirmed both wild-type and RKIP knockout mice exhibit a significantly greater Ki67+ cell density profile when placed under voluntary exercise conditions than under sedentary conditions (Fisher’s LSD; \(p < 0.001\)). However, no significant difference between RKIP knockout and wildtype mice within either sedentary or exercise conditions was observed.
Figure 7. Neural Progenitor Cell Differentiation

Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice under sedentary or exercise conditions outlined in Figure 6A and Figure 6B. A Sagittal cross section immunofluorescence stain results of hippocampal tissue. IF stains from left to right: DAPI, Ki67, DCX, composite image of DAPI and Ki67, and composite image of DAPI and DCX. Red arrow points to Ki67+ cells. Yellow arrow points to DCX+ cells. B Composite images of DAPI and Ki67 and DAPI and DCX. Left to right: Wildtype Ki67+ cells, Wildtype DCX+ cells, RKIP Knockout Ki67+ cells, RKIP Knockout DCX+ cells. C Cell density measurements of DCX+/Ki67+ (type 3) cells in the SGZ of RKIP knockout and C57BL/6J wildtype mice under sedentary and exercise conditions (#: F = 73, p < 0.001; *: F = 20, p < 0.001). D Cell density measurements of total Ki67+ cells (type 2a and type 2b cells) in the SGZ of RKIP knockout and C57BL/6J wildtype mice under sedentary and exercise conditions (#: F = 51, p < 0.001). Data shown as mean ± SD.
4  Cell Cycle Kinetics

Prior in vitro studies demonstrate that RKIP silencing results in an accelerated cell cycle via hastened DNA synthesis, G1/S transition entry, and progression from prometaphase to anaphase (Eves et al., 2006; Al-Mulla et al., 2011). Here, I performed in vivo experiments to further elucidate RKIP’s regulation of cell cycle kinetics in proliferating neural stem cells. As before, 35-day-old RKIP knockout and C57BL/6J wildtype mice were placed under either sedentary or exercise conditions; mice in the exercise group were single housed with one wheel and mice in the sedentary group were group housed with no wheel access. Food and water were provided ad libitum. After 5 days of habituation and exercise, mice were injected with iododeoxyuridine (IdU) (57.5mg/kg; Sigma-Aldrich) followed by an injection of chlorodeoxyuridine (CldU) (42.5mg/kg; Sigma-Aldrich) either 4 hours or 18 hours after IdU injection and killed 45 minutes post-CldU injection (Figure 8A, B). IdU and CldU are thymidine analogs that function in the same manner as BrdU by labelling cells in the S-phase of the cell cycle to highlight proliferating cells. Injection of both analogs spaced either 4 or 18 hours apart is a double labelling method that allows for the identification of cells transitioning between phases of the cell cycle or exiting the cell cycle. Coronal cross sections containing the hippocampal tissue were attained for immunofluorescence staining of IdU and CldU (Figure 8C). Quantitative comparisons between phenotypes under both conditions were made using a protocol established by Brandt, Hübner and Storch (2012). They report the following method to calculate the length of both the S-phase and full length of the cell cycle based on a ratio of cells incorporating either one or both of the thymidine analogs:

A. The S-Phase ($T_s$):

To measure the length of the S-phase of the cell cycle, mice are injected with IdU followed by an injection of CldU 4 hours later. The length of the S phase can then be calculated using the following equation:

$$T_s = 4hrs \times \frac{Number\ of\ IdU^+\ cells}{Number\ of\ IdU^+/CldU^-\ cells}.$$
where IdU+/CldU− cells are those that have exited the S-phase in between injections and IdU+ cells encompasses both IdU+/CldU− cells and cells in the S phase during both injections (IdU+/CldU+) (Figure 8D).

B. *The Total Cell Cycle Length (Tc):*

To measure the total length of the cell cycle, mice are injected with IdU followed by a CldU injection 18 hours later. The length of the total cell cycle can then be calculated using the following equation:

\[ T_c = 18\text{ hrs} + T_s \times \frac{\text{Number of CldU}^+/\text{IdU}^- \text{cells}}{\text{Number of CldU}^+ \text{ cells}}, \]

where CldU+/IdU− cells are those in the S-phase during the second injection, and CldU+ cells encompasses both CldU+/IdU− cells and cells that re-entered the cell cycle and were in the S-phase during both injections (IdU+/CldU+) (Figure 8E).

Figure 8F and Figure 8G show the results of the cell cycle kinetics quantification. Initial two-way ANOVA analysis uncovered a significant difference between genotypes in Tₙ (F = 5; \( p < 0.05 \)) and Tₖ (F = 10; \( p < 0.01 \)) duration. Further post-hoc analysis indicated RKIP knockout NPCs to have a significantly shorter S-phase (7.1 ± 0.2 hours) than wild-type mice (8.7 ± 0.1 hours) under exercise conditions (Fisher’s LSD; \( p < 0.01 \)). No difference in S-phase length between RKIP knockout and C57BL/6J wildtype mice was observed under sedentary conditions. Likewise, RKIP knockout mice had a significantly shorter overall cell cycle length (21.7 ± 0.3 hours) than wild-type mice (23.6 ± 0.6 hours) under exercise conditions (Fisher’s LSD; \( p < 0.01 \)) (Figure 8I). Once again, no difference in total cell cycle length between mice of both phenotypes was observed under sedentary conditions.
Figure 8. Cell Cycle Kinetics Experiment outline for 35-day-old RKIP knockout and C57BL/6J wildtype mice under sedentary or exercise conditions. A Mice in this cohort were used to measure the S-phase of the cell cycle (T_s) with IdU and CldU injections given 4 hours apart. Mice were then killed 45 minutes after the last thymidine injection (CldU). Groups: Wildtype-Sedentary (n = 5), Wildtype-Exercise (n = 6), Knockout-Sedentary (n = 6), Knockout-Exercise (n = 5). B Mice in this cohort were used to measure the full length of the cell cycle (T_c) with IdU and CldU injections given 18 hours apart. Mice were then killed 45 minutes after the last thymidine injection (CldU). Groups: Wildtype-Sedentary (n = 5), Wildtype-Exercise (n = 5), Knockout-Sedentary (n = 5), Knockout-Exercise (n = 6). C Coronal cross-section immunofluorescence stain results of hippocampal tissue. IF stains from left to right: IdU, CldU, and composite image of IdU and CldU. Green arrows indicate IdU+CldU cells. Red arrows indicate IdU+CldU cells. Yellow arrows indicate IdU+CldU cells. D Composite images of IdU and CldU stain in inner hippocampus for T_s measurement. Left: Sedentary-Wildtype. Right: Sedentary-RKIP Knockout. E Composite images of IdU and CldU stain in inner hippocampus for T_c measurement. Left: Exercise-Wildtype. Right: Exercise-RKIP Knockout. F Duration of the S-phase of the cell cycle (T_s) in RKIP knockout and wildtype mice under sedentary and exercise conditions reported in hours (#: F = 16, p < 0.01; *: p < 0.01). G Duration of the entire cell cycle (T_c) in RKIP knockout and wildtype mice under sedentary and exercise conditions reported in hours (#: F = 10, p < 0.01; *: p < 0.01). H Average length of the S-phase and G1, G2, and M phase of the cell cycle of both wildtype and RKIP knockout mice under sedentary conditions combined. I Length of the S-phase and G1, G2, and M phase of the cell cycle (hours) of wildtype (Left) and RKIP knockout (Right) mice under exercise conditions. Data shown as mean ± SD.
5 Mechanism of Action: MAPK Pathway

Following the characterized differences in RKIP knockout and wild-type mice regarding the differentiation and cell cycle kinetic profiles is an examination of the potentially underlying mechanisms involved. Of particular interest is the MAPK pathway and to elucidate its potential role in modulating these phenotypic differences observed, I utilized a MAPK inhibitor, SL-327 (ApexBio Technology). 35-day-old RKIP knockout and wild-type mice were placed under sedentary and exercise conditions for this study; mice in the exercise group were single-housed with one wheel and mice in the sedentary group were group-housed with no wheel access. Food and water were provided ad libitum. After 5 days of habitation and exercise, mice were given 3 injections of either SL-327 (30mg/kg suspended in dimethyl sulfoxide (DMSO)) or DMSO (vehicle; Sigma-Aldrich) every 2 hours beginning at 6A.M. until 10A.M. Mice in the control group were administered DMSO at the same dosage as mice given SL-327. We previously tested SL-327 administration at various times and found early morning injections to be more effective at suppressing phosphorylated ERK (pERK) expression in the suprachiasmatic nuclei, a region of high pERK expression. I therefore inferred SL-327’s efficacy of morning administration to be equally as effective in suppressing pERK expression in the hippocampus. Selcher et al. (1999) indicated SL-327’s efficacy of suppressing pERK expression to be limited to between 30 minutes and 3 hours after injection with the prescribed dosage of 30mg/kg. As a result, and concurrent with the third injection of SL-327 at 10A.M., a single injection of BrdU (100mg/kg; Sigma-Aldrich) was also administered and mice were killed 3 hours post-injection (Figure 9A, B). Coronal cross sections containing the hippocampus were attained for the following: immunohistochemistry fluorescence microscopy to measure BrdU⁺-cell density in the subgranular zone of the hippocampus (Figure 9C, D); and immunofluorescence microscopy to measure Ki67⁺-cell density for type 2a, type 2b, and total NPCs in the subgranular zone of the hippocampus (Figure 10). Both experiments highlighted actively proliferating cells.

A three-way ANOVA of the results of the immunohistochemistry experiment shown in Figure 9E and Figure 9F indicate a significant difference between conditions (F = 93; p < 0.001) and injection paradigm (F = 11; p < 0.01). Further post-hoc analysis confirmed that under both sedentary and exercise conditions, RKIP knockout mice demonstrated a significant increase in
BrdU$^+$ cell density when given SL-327 injections compared to vehicle injections (Fisher’s LSD, $p < 0.05$). However, this effect was not observed in wild-type mice which showed no significant difference in BrdU$^+$ cell density when given either injection under either sedentary or exercise conditions (Fisher’s LSD, $p > 0.05$). Nonetheless, an overall induction effect of exercise was observed in both RKIP knockout and wild-type mice given either SL-327 or DMSO injections (Fisher’s LSD, $p < 0.001$).

**Figure 9. RKIP Mechanism of Action: the MAPK Pathway (IHC)** Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice under sedentary (A) or exercise (B) conditions for 5 days. Beginning at 6A.M. at 40 days of age, mice were injected with either SL-327 (30mg/kg) or DMSO every 2 hours for 6 hours. In addition to the final injection, a single injection of BrdU (100mg/kg) was administered and mice were killed 3 hours post-injection. C Sagittal cross-section BrdU immunohistochemistry stain results of hippocampal tissue from mice placed under sedentary conditions. From left to right: Wildtype-DMSO ($n = 5$), Knockout-DMSO ($n = 5$), Wildtype-SL-327 ($n = 5$), Knockout-SL-327 ($n = 5$). D Sagittal cross-section BrdU immunohistochemistry stain results of hippocampal tissue from mice placed under exercise conditions. From left to right: Wildtype-DMSO ($n = 4$), Knockout-DMSO ($n = 6$), Wildtype-SL-327 ($n = 5$), Knockout-SL-327 ($n = 5$). E Cell density measurements of BrdU-positive cells in the subgranular zone of the hippocampus in mice of both phenotypes given either vehicle (DMSO) or SL-327 injections under sedentary and (F) exercise conditions ($*: p < 0.01$). Data shown as mean ± SD.
Looking to the results of the immunofluorescence microscopy experiment shown in Figure 10C and Figure 10D, a three-way ANOVA analysis indicates a significant difference only between conditions ($F = 85, p < 0.001$). Further post-hoc analysis confirmed that the only observable significant difference in Ki67$^+$ cell density profile was between mice placed under sedentary conditions and those under the voluntary exercise conditions; RKIP knockout and wild-type mice under exercise conditions exhibited a significantly greater Ki67$^+$ cell density in the SGZ than mice under sedentary conditions for both SL-327 and DMSO injection cohorts (Fisher’s LSD; $p < 0.001$).
I next examined the constituent subtypes of the proliferating cells highlighted by Ki67. As before in section 2, Ki67+ cells comprise type 2a and type 2b cells. Therefore, I teased apart cells that were Ki67+/DCX−, indicating type 2a cells, from Ki67+/DCX+, indicating type 2b cells, using the
same IF-stained tissues shown in Figure 10 to identify any potential differences amongst the two proliferating cell types in RKIP knockout and wildtype mice under exercise and sedentary conditions. Three-way ANOVA of the results shown in Figure 11B-E indicates a significant difference between mice under sedentary and exercise conditions, only, for both type 2a \((F = 85, p < 0.001)\) and type 2b \((F = 30, p < 0.001)\) cells under investigation. For both cell types, no difference was observed in mice given SL-327 injections compared to DMSO injections or between RKIP knockout and wildtype mice within respective conditions. Post-hoc analyses confirmed, as before, that both RKIP knockout and wild-type mice given either SL-327 or DMSO exhibited a significantly greater type 2a and type 2b cell density when placed under a voluntary exercise paradigm compared to mice under sedentary conditions (Fisher’s LSD; \(p < 0.001\)).
Figure 11. RKIP Mechanism of Action: the MAPK Pathway (IF) and NPC Subtypes

A composite image of DCX, Ki67, and DAPI. Red arrows indicate Ki67+/DCX−/type 2a cells. Yellow arrows indicate Ki67+/DCX+/type 2b cells.

B Sedentary – Type 2a Cells

C Exercise – Type 2a Cells

D Sedentary – Type 2b Cells

E Exercise – Type 2b Cells

Ki67+/DCX+/DAPI

DG

SGZ

Hilus

Wildtype

Knockout

Vehicle

SL-327

Vehicle

SL-327

Figure 11. RKIP Mechanism of Action: the MAPK Pathway (IF) and NPC Subtypes Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice under sedentary or exercise conditions outlined in Figure 9A and Figure 9B. A composite image of DCX, Ki67, and DAPI. Red arrows indicate Ki67+/DCX− (type 2a) cells. Yellow arrows indicate Ki67+/DCX+ (type 2b) cells. B Ki67+/DCX− cell density in the subgranular zone of the hippocampus in mice of both genotypes given either vehicle (DMSO) or SL-327 injections under sedentary and (C) exercise conditions. D Ki67+/DCX+ cell density in the subgranular zone of the hippocampus in mice of both genotypes given either vehicle (DMSO) or SL-327 injections under sedentary and (E) exercise conditions. Data shown as mean ± SD.
Finally, I proceeded to confirm SL-327’s suppression of pERK expression with a Western blot experiment. For this, I examined the efficacy of the drug in RKIP knockout and wild-type mice under sedentary conditions. Mice used in this experiment were between the ages of 21 and 40 days and were group housed. Food and water were provided ad libitum. Mice were then given a single injection of either SL-327 (50mg/kg) or DMSO at 10A.M. and killed 1 hour post-injection. Mice in the control group were administered DMSO at the same dosage as mice given SL-327. Immunoblotting for phosphorylated ERK (pERK) was performed with anti-pERK (anti-Phospho-p44/p42 MAPK; Cell Signalling) to quantify pERK expression levels (Figure 12A, C). Subsequent immunoblotting for total ERK was performed with anti-ERK (anti-p44/p42 MAPK; Cell Signalling) to quantify ERK expression levels as control (Figure 12B, D). Figure 12E, F shows the results of the pERK and total ERK Western blots in the dentate gyrus and CA3 of the hippocampus. As indicated by the immunoblot results, pERK expression levels (pp44 and pp42) were significantly reduced in the dentate gyrus and CA3 of both wild-type and RKIP knockout mice given an injection of SL-327 compared to mice given vehicle injections (Student’s T-Test; p < 0.05).
Finally, I proceeded to examine the role of RKIP in the maturation of hippocampal neuronal and glial cells. Here, 35-day-old RKIP knockout and C57BL/6J wildtype mice were placed under
either exercise or sedentary conditions; mice under exercise conditions were housed in pairs with two wheels and mice in sedentary conditions were group housed with no wheel access. Food and water were provided *ad libitum*. After 5 days of habituation, mice were injected with BrdU (100mg/kg; Sigma-Aldrich) every morning at 10 A.M. for 7 days and killed 30 days after the first injection (Figure 13A, B). Coronal cross sections containing the hippocampus were attained and immunofluorescence microscopy was performed by co-staining tissues with BrdU, S100β, and NeuN. S100β, a cytoplasmic and nuclear protein involved in regulating numerous cellular processes, is expressed by mature astrocytes (Wang and Bordey, 2008). NeuN, a protein resident of the nuclei and perinuclear cytoplasm, is found in and used to detect mature neuronal cells (Gusel’nikova and Korzhevskiy, 2015). Comparisons between phenotypes and conditions were made with cell density measurements of co-localized BrdU+/S100β+ cells (newly developed glial cells; Appendix Figure 14) or BrdU+/NeuN+ cells (newly developed neuronal cells; Figure 13C, D) within the dentate gyrus. The results of the newly developed mature neuronal cells shown in Figure 13E indicate a significant difference between conditions, only (*F* = 32, *p* < 0.001). Further post-hoc analysis showed an increase in BrdU+/NeuN+ cell density in both RKIP knockout and wildtype mice under exercise conditions when compared to mice under sedentary conditions (Fisher’s LSD, *p* < 0.05). Similarly, the results of the newly developed mature glial cells shown in Appendix Figure 14F indicate a significant difference between conditions, such that mice placed under exercise conditions exhibit a significantly greater BrdU+/S100 β+ cell density when compared to mice placed under sedentary conditions (*F* = 11, *p* < 0.05). Ultimately, no difference in mature neuronal and glial cell formation was observed between RKIP knockout and wildtype mice within either condition.
Figure 13. NPC Neuronal Cell Maturation Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice under sedentary (A) or exercise (B) conditions given 7 daily injections of BrdU (100mg/kg) at 40 days of age and then killed at 70 days of age. C Sagittal cross section immunofluorescence stain results of hippocampal tissue. IF stains from left to right: NeuN, BrdU, and composite image of both. Red arrow indicates BrdU⁺/NeuN⁺ cell. D Composite images of BrdU and NeuN-stained hippocampal tissue. Left to right: Wildtype-Sedentary (n = 7), Knockout-Sedentary (n = 8), Wildtype-Exercise (n = 7), Knockout-Exercise (n = 7). E Cell density measurements of NeuN⁺/BrdU⁺ cells in the dentate gyrus of the hippocampus of RKIP knockout and C57BL/6J wildtype mice under sedentary and exercise conditions. Co-localized NeuN⁺/BrdU⁺ cells indicate newly matured neuronal cells (#: F = 11, p < 0.01). Data shown as mean ± SD.
Chapter 5
Discussion

My experiments here have provided novel evidence elucidating the role of RKIP in regulating adult hippocampal neurogenesis in mice. Specifically, and as hypothesized, these findings highlight RKIP’s function to regulate neural progenitor cell proliferation and maintain NPC cell cycle kinetics in duration. However, these effects may not be due in entirety to the MAPK pathway as originally predicted. Furthermore, my results regarding the role of RKIP in the maturation of NPCs do not corroborate with current literature findings of RKIP’s function as a driver of NPC neuronal fate. Instead, here I show that while RKIP initially promotes a neuronal fate in NPCs to differentiate into type 3 immature neuronal cells, it does not see through this decision to the extent of full maturation. However, much work remains to be completed on this end.

1 Neural Progenitor Cell Proliferation

The results shown in Figure 5D were unexpected considering prior investigations into RKIP’s role in modulating cellular proliferation. Given RKIP’s ability to suppress the MAPK pathway, I anticipated a significant increase in cellular proliferation in RKIP knockout mice upon exercise induction when compared to wildtype mice under similar conditions. In fact, Yeung et al.’s (1999) original study into RKIP’s role in regulating cellular proliferation concluded RKIP as functioning to elevate the activation threshold of the MAPK pathway, thereby reducing the rate of cellular proliferation. In other fields, such as in cancer, prominent studies such as one by Hellmann et al. (2010) further alluded to this effect; they demonstrated in human neuroblastoma cells the reduction of cancer cell proliferation upon RKIP overexpression and, conversely, the increase in neuroblastoma cell proliferation upon RKIP silencing. Instead, here I discovered a reduction in the induction effect and moreover, a significantly lower cellular proliferation of NPCs in RKIP knockout mice compared to wildtype mice under exercise conditions. This discrepancy could not be due in part to errors of my exercise paradigm used since, as expected
and in accordance with numerous studies, there was a significant increase in NPC proliferation in RKIP knockout and wildtype mice under exercise conditions compared to sedentary conditions (Ma et al., 2017).

Still, the similarity in the NPC proliferation profile between both genotypes under sedentary conditions was anticipated. In fact, RKIP’s role as a regulatory protein does not function in likeness to an on-off switch; rather, it functions to modulate the intensity of the response to an intrinsic or extrinsic input, in this case being exercise. Thus, the absence of a functional RKIP protein in RKIP knockout mice under sedentary conditions does not necessarily initiate or prevent activation of the numerous pathways it regulates (Trakul et al., 2005). Therefore, the reduced induction effect of exercise on the number of proliferative NPCs in RKIP knockout mice prompted further investigation into the possibility of differences between the subtypes of neural progenitor cells: type 2a and type 2b NPCs. It is also important to bear in mind the limitations of BrdU as a marker of cellular proliferation. As an exogenous marker, BrdU is restricted to labelling cells at the time of injection within the S-phase of the cell cycle, only (Zhang et al., 2015). Thus, by natural limits imposed by a cell’s cell cycle, labelling of NPCs is constrained to those within a specific portion of their mitotic phase. Should differences exist in the length of the S-phase of the NPC cell cycle in RKIP knockout mice it would also shed light on the results seen here.

In looking to tease apart potential differences in NPC subtypes, the use of immunofluorescence microscopy was necessary to differentiate such closely progressive cells. The results shown in Figure 6E and Figure 6F indicate a similar proliferation phenotype of type 2a and type 2b cells between RKIP knockout and wild-type mice under either condition. Furthermore, there was an apparent induction effect observed in both RKIP knockout and wild-type mice for both type 2a and type 2b cells. These results were further corroborated when examining the total NPC proliferation phenotype (i.e. sum of type 2a and type 2b NPC densities) shown in Figure 7D. Cumulatively, this may suggest that the proliferation of type 2a and type 2b NPCs are independent of a functional RKIP protein. In relation to my initial experiment using BrdU as a marker for cellular proliferation, these results differ in the apparent absence of a significant difference in NPC proliferation between RKIP knockout and wildtype mice under exercise conditions. I propose this difference observed is due to the proliferation marker of choice used in this experiment, Ki67, and that these results further substantiate my initial suspicion that RKIP
affects certain phases of the cell cycle, namely, the S-phase. This discrepancy could also be due to apoptotic regulations or cell stasis but I contend it is a matter of faster progression through the cell cycle (Eves et al., 2006). Ki67 is an endogenous marker which labels cells in all phases of the cell cycle at any given time. A drawback to its use is its labeling of cells that become quiescent at G1 and remain terminally undifferentiated in addition to cells actively in the cell cycle. This may also explain the wider deviations observed in the results despite using a reasonable sample size per group which can be addressed with more mice and an additional proliferation marker used for the study. Nonetheless, here I find the total pool of proliferating NPCs to be identical between RKIP knockout and wild-type mice under either conditions as indicated by Ki67 labelling but significantly lower when using an S-phase marker in RKIP knockout mice compared to wild-type mice under exercise conditions. This suggests the possibility of a difference in the cell cycle dynamics which may be hindered or accelerated in certain phases in RKIP knockout mice. I propose that these effects are unlikely due to apoptotic regulations at this point in the neurogenic process since previous studies have provided evidence of no alteration in the rate of apoptosis in RKIP-depleted HEK499 cells (Al-Mulla et al., 2011). Instead, flow cytometry experiments have demonstrated fewer RKIP-silenced cells in the G2/M phase and S-phase of the cell cycle and more in the G0/G1 phase. Additionally, these colonies of RKIP-silenced cells have significantly lower mitotic indices (i.e. ratio of mitotic to non-mitotic cells) than normal cells (Al-Mulla et al., 2011). Overall, this suggests that in the absence of RKIP expression, cells progress through the mitotic phase faster and remain highly proliferative as opposed to beginning their differentiating phase.

Figure 6G brought to light an important effect of exercise on cellular proliferation: the increase in the proliferation of NPCs is not proportionally equal amongst its type 2a and type 2b constituents. In fact, there was a significantly greater increase in the type 2b cell density than there was in the type 2a cell density in mice placed under exercise conditions compared to sedentary conditions; the margin of difference due to exercise induction resulted in over 3 times the increase in type 2b cells compared to type 2a cells. Consequently, the pool of type 2 NPCs collectively changed from constituting approximately 39-43% type 2a cells and 57-61% type 2b cells under sedentary conditions to 29-30% type 2a cells and 70-71% type 2b cells under exercise conditions. This may suggest that type 2b NPCs proliferate at a faster rate than type 2a NPCs under exercise induction or perhaps that exercise induces a faster progression from a type
2a NPC to a type 2b NPC. Indeed, a study by Brandt, Hübner and Storch (2012) examined C57BL/6J mice given access to a wheel for 7 days of exercise to measure the cell cycle length of type 1, 2a, and 2b cells with various cellular markers; NeuroD1, a marker of developing neuronal cells (type 2b), GFAP, a marker of type 1 neural stem cells, and CldU, a thymidine analog akin to BrdU used as a proliferative marker. Their results identified the cell cycle length of type 1 cells as 22.8 ± 0.5 hours, type 2a cells as 27.0 ± 0.5 hours, and type 2b cells as 22.6 ± 0.1 hours. This provided preliminary evidence that cells involved in the adult hippocampal neurogenic process possess differences in the cell cycle length which may explain the increase in the proportion of type 2b cells compared to type 2a cells in Figure 6 upon exercise induction. Consequently, the results found in my proliferation experiments and prior research prompted subsequent experiments examining the cell cycle kinetics of RKIP knockout and wildtype NPCs.

2 Cell Cycle Kinetics

Quantification and calculation of the S-phase of the cell cycle (T_s) (Figure 8F) in wild-type mice under sedentary conditions (control) was found to be 9.0 ± 0.4 hours. This was comparable to several studies of NPCs that have concluded a 6-13 hour timeframe for this phase of the cell cycle (Overall et al., 2016). Furthermore, the total length of the cell cycle (T_c) (Figure 8G) for this group of mice was comparable as well: 23.7 ± 0.3 hours to the 22-27 hour timeframe reported in previous studies (Overall et al., 2016). A study by Farioli-Vecchioli (2014) demonstrated in C57BL/6J wild-type mice that a short period of voluntary wheel running (12 days) resulted in a 23% increase in proliferating NPCs in the hippocampus and a shortening of the S-phase, and by extension the overall length, of the cell cycle from 12.9 ± 0.77 hours under sedentary conditions to 10.25 ± 0.61 hours under the exercise paradigm. However, this effect of exercise reducing the duration of the S-phase of the cell cycle shown by Farioli-Vecchioli was not observed in this experiment. Instead, my results suggest that exercise had no effect on cell cycle kinetics in these mice and support the findings of Fischer et al. (2014) who performed a study using C57BL/6J mice placed under a 5-day exercise paradigm. Fischer et al. (2014) found that the NPCs of mice placed under sedentary conditions had a T_s of 11.0 ± 0.7 hours and a T_c of 22.7 hours which were not significantly different from the NPCs of mice placed under exercise conditions which had a T_s of 10.8 ± 0.3 hours and a T_c of 20.9 hours. This suggests that there
may be more mechanisms involved in the process of regulating the cell cycle kinetics of NPCs under exercise conditions which may depend on the duration of exercise.

 Nonetheless, under similar exercise conditions, RKIP knockout mice did in fact exhibit a significantly shorter S-phase by approximately 1.6 hours when compared to wildtype mice. This difference was observed to carry through in total cell cycle length where the difference between these mouse groups was approximately 1.9 hours and the magnitude of this difference was not significantly different from that of the S-phase length difference (Figure 8I) (two-way ANOVA; F = 0.7, p > 0.1). Therefore, it is clear that under the induction effects of exercise, RKIP knockout mice exhibit a shorter S-phase and cell cycle length compared to wild-type mice, suggesting a quicker progression through the cell cycle. This finding may suggest a possible reasoning for the results seen in Figure 5D; the apparent reduction in induction effect in RKIP knockout mice under exercise conditions as measured by BrdU+ cell density could be due to an accelerated S-phase of the cell cycle. Thus, RKIP knockout NPCs may be rapidly progressing through to the subsequent phases of the cell cycle from the S-phase. Yet, this begs the question of whether these RKIP knockout NPCs subsequently exit their proliferative phase quicker to begin differentiating or simply re-enter the cell cycle to undergo further proliferation.

3 Neural Progenitor Cell Differentiation

My initial contention of the point that RKIP knockout NPCs re-enter the cell cycle at a greater rate than wild-type NPCs stems from my quantification of the pool of differentiating immature neuronal cells in the SGZ. Figure 7C shows the results of the DCX+/Ki67− cell density measurements which represent the pool of immature neuronal cells. These cells have effectively exited the cell cycle and begun their trajectory to become neuronal cells which will require several more weeks of maturation and integration into the hippocampal circuitry. The data here indicates that RKIP knockout mice possess significantly less immature neuronal cells at any given time point than wild-type mice under either sedentary or exercise conditions. Furthermore, both wild-type and RKIP knockout mice exhibited an induction effect of exercise which resulted in a significant increase in the immature neuronal cell density of the SGZ. The difference between phenotypes under exercise conditions was expected and further alludes to evidence that
RKIP knockout NPCs re-enter the cell cycle more frequently than wild-type NPCs. Interestingly though, the difference between phenotypes under sedentary conditions was not anticipated given the absence of an induction. It is possible then that while RKIP expression may not induce significant changes to the cell cycle kinetics in the absence of an induction, it still functions to regulate the decision for re-entry or exit of the cell cycle in NPCs. Furthermore, because the magnitude of the difference between genotypes in the DCX⁺ cell density was not different under sedentary and exercise conditions (two-way ANOVA; p > 0.1), it suggests that the difference seen in immature neuronal cell density is not due to the conditions in which mice were placed (i.e. exercise or sedentary). Rather, this difference in immature neuronal cell density between wild-type and RKIP knockout mice is due to the absence or presence of RKIP alone. Alternatively, these results may be a consequence of RKIP knockout NPCs having a propensity to complete their cell cycle but enter the G₀ phase indefinitely, thereby rendering them terminally undifferentiated and incapable of being labelled with Ki67. As a result, it is possible that fewer NPCs are being identified with Ki67 labelling than truly exist in RKIP knockout mice. As such, these possibilities require further investigation into differences in the propensity of NPCs exiting or remaining in the proliferative phase and the use of a marker capable of labelling NPCs at all phases of the cell cycle in RKIP knockout and wild-type mice.

4 Mechanism – MAPK Pathway

The role of RKIP in regulating the S-phase of the NPC cell cycle suggests the likelihood of a mechanistic process involving the MAPK pathway given its role in regulating various mitotic and spindle checkpoints (Al-Mulla et al., 2013). Therefore, I looked to confirm the involvement of this signaling pathway with selective inhibition of ERK, a constituent MAPK kinase, with SL-327. For this experiment, I administered 3 intraperitoneal injections of SL-327 spaced 2 hours apart to ensure inhibition of phosphorylated ERK in accordance to Selcher et al.’s (1999) identification of SL-327’s inhibitory function being limited to between 30 minutes and 3 hours post-injection and given the 3-hour delay between BrdU injection and kill. I also confirmed SL-327’s effectiveness of pERK suppression with a series of Western blot experiments (Fig.12). Clearly, SL-327 was potent in reducing pERK1/2 expression in both the CA3 and DG of the hippocampus by upwards of 90% (Fig. 12E, F).
Importantly, Figure 9E and Figure 9F show RKIP knockout mice placed under either sedentary or exercise conditions exhibiting a significant increase in NPC proliferation when administered SL-327 compared to vehicle injections. In fact, this increase was sufficient to raise the BrdU+ cell density of RKIP knockout mice to levels similar to that of wildtype mice under both conditions. These results were unexpected given the MAPK pathway’s role in inducing cellular proliferation and the administration of an inhibitor of MEK should therefore have resulted in a reduction of NPC proliferation. Instead, here I found the opposite and consequently, I proceeded to conduct an immunofluorescence assay for Ki67+ cell density to shed light on this discrepancy. Figure 10C and Figure 10D indicate similar findings as my earlier experiments regarding overall Ki67+ cell density measurements: the total pool of proliferating cells neither differed between genotypes nor, in this case, between injection paradigms. Additionally, quantification of type 2a (Fig 11B, C) and type 2b (Fig 11D,E) NPCs showed similar findings in this regard; no difference between phenotypes under either injection paradigms in NPC proliferation. It is important to note, however, that additional mice should be added to this experiment in order to minimize the relatively broad deviation seen in Figure 10 and Figure 11.

Taken together, the IF data suggests that SL-327 administration does not affect the total pool of proliferating NPCs in wildtype and RKIP knockout mice. This was further substantiated by the IHC data for the wildtype mice cohort. Indeed, a prior study by Maękowiak, Dudys and Wędzony (2009) also examined the proliferation of Ki67-IR cells using IHC and found no difference in the number of such cells when mice were administered SL-327 (30mg/kg) compared to vehicle control injections (DMSO). Therefore, the MAPK pathway may not be the sole effector in regulating the proliferation of NPCs as initially anticipated. Interestingly though, it may play a role in modulating the cell cycle kinetics since IHC results of the RKIP knockout mice demonstrated the unexpected response to SL-327 with an increase in the BrdU+ cell density when compared to vehicle injections. As such, it would be worthwhile to repeat earlier experiments of cell cycle kinetics analysis in mice given DMSO and SL-327 injections to observe any variances in cell cycle length. Specifically, if the MAPK pathway has been shown to hasten progression through the cell cycle by bypassing mitotic and spindle checkpoints in the G1/S and G2/M transition periods, then perhaps inhibition of MAPK would result in a significant reduction in this accelerated transition to maintain cells in their S-phase longer for probing and visualization (Al-Mulla et al., 2011).
It is also worthwhile to highlight the clear induction effect of exercise in mice of both genotypes under both injection paradigms in the IHC and IF experiments performed here. The results of this cohort resonate with earlier findings of exercise effects in mice shown in Figure 5D. As stated before, Figure 5D results could possibly be due to an accelerated S-phase of the cell cycle upon exercise induction in RKIP knockout mice, resulting in fewer identified proliferating NPCs. Here, it appears as though this may not be the case. In fact, three-way ANOVA of the results indicate that the magnitude of the difference in NPC proliferation between sedentary and exercise conditions in mice of either genotypes given either injections are not significant (p > 0.1). Therefore, this suggests that the NPC proliferation profile differences between mice under sedentary and exercise conditions are not due to the genotype or injection paradigm of the mouse. Instead, they depend on the conditions in which they are placed in. As such, to elucidate the discrepancy between these findings, additional experiments examining the overall NPC proliferation profile must be performed. In particular, immunofluorescence microscopy of co-localized BrdU and Ki67 may provide a more accurate observation and quantification of such cells. Overall, these experiments demonstrate that while the MAPK pathway may not play a central role in regulating NPC proliferation as anticipated, there is evidence of a potential effect on cell cycle kinetics which deserves further investigation.

5 Maturation of Neuronal and Glial Cells

Lastly, I proceeded to examine the effects of RKIP on potentially promoting a neuronal fate in adult hippocampal NPCs. Previous in vitro findings concluded RKIP to promote neuronal differentiation of NPCs (Sagisaka et al., 2010). As originally hypothesized, I anticipated similar results here in my in vivo model such that RKIP knockout mice would exhibit a decrease in newly developed neuronal cells in the DG compared to wildtype mice. This contention was further supported by my initial findings in Figure 7C which show a significant decrease in immature neuronal cells in RKIP knockout mice compared to wildtype mice under both sedentary and exercise conditions. I therefore looked to observe whether such phenotypic differences carried through to the age of neuronal maturity and integration into hippocampal circuitry. Therefore, a 30-day period between the time of initial BrdU injection and death of the mouse is sufficient for newly developing cells to proliferate, differentiate, integrate, and mature
to adult neuronal cells. Surprisingly, no apparent difference was found in the cell density of newly developed neuronal cells in RKIP knockout and wildtype mice under either sedentary or exercise conditions (Figure 13E). At the same time, I examined the formation of newly developed astrocyte cells in the dentate gyrus as well since absence of RKIP expression may elevate astrocyte cell formation at the expense of neuronal cell development (Sagisaka et al., 2010). However, there I discovered no significant difference between genotypes as well (Appendix Figure 14). The latter results were not entirely unexpected, however, given the minimal production of new glial cells in the DG (typically between 1 to 4 cells) which also resulted in the wide deviations in cell density measurements among samples. Nonetheless, it is also apparent and important to note the affinity of this neurogenic region to produce neuronal cells as opposed to glial cells; on average, the newly developed neuronal cell density is approximately 50 times greater than that of the respective newly developed glial cell density. Furthermore, an induction effect of exercise was apparent in the generation of both newly developed neuronal and glial cells in RKIP knockout and wild-type mice. Overall, these results do not support prior in vitro findings that RKIP promotes a neuronal fate in NPCs. Rather, it appears as though the DG remains largely neuronal-inclined, possibly due to microenvironment influences in modulating the differentiation phenotype. Furthermore, while exercise increases the overall newly developed neuronal cell density several folds, it does not induce a difference between the neuronal cell density profile of RKIP knockout and wild-type mice.

The use of BrdU offers a unique insight into the progression of newly developed cells over an extended period of time. Because it labels actively dividing cells and the subsequent daughter cells arising from the single cell labelled at the time of injection, it allows us to follow the neurogenic process with a high degree of accuracy (Zhang et al., 2015). With this, a timeline of the adult hippocampal neurogenic process can be constructed which will require a series of experiments over a broad length of time (beginning at 1 hour up to 30 days) to map out this dynamic balance between cellular proliferation and apoptosis. Doing so will clearly highlight where the turnaround of cell death might begin. I do not anticipate, however, that exercise would impact the survival of NPCs or neuronal cells. It has been clearly established in previous studies, and to a great extent here, that exercise impacts the expansion phase of NPCs by increasing the number of proliferative cells. On the other hand, other forms of extrinsic stimuli, such as enriched environments, have been shown to promote neuronal cell survival (Kempermann, Song
and Gage, 2015). At this point, it is not clear at which specific time point an apoptotic mechanism is recruited to regulate cell death or the exact process by which the decision to maintain or eliminate a neuron is made (Fidaleo, Cavallucci and Pani, 2017). One thing is clear, however: the survival of newly formed cells requires their continuous use and activity. This is achieved through strong synaptic integration and routine LTP inputs and respective outputs to other cells. RKIP has been found to be involved in this process via modulation of granule cell electrophysiological properties. Specifically, it has been discovered in the cerebellum that the PKC-MAPK positive feedback loop is required for long term depression (LTD). LTD is an activity-dependent process that selectively weakens neuronal synapses in a long-lasting fashion (minutes to hours). RKIP is a phosphorylation target of PKC that consequently regulates the MAPK pathway. By quantifying the strength of LTD using a paired electrical stimulation protocol, RKIP mutant Purkinje cells were found to have a reduced amount of LTD when compared to wildtype cells (Ling et al., 2014). Thus, phosphorylation of RKIP is important for LTD formation because it relieves itself from MEK and Raf1. As a result, structurally, the weakening of synapses under increased LTD induction by RKIP expression may impact neuronal cell survival.
Some experiments are currently underway and others planned for the near future to continue uncovering the details of RKIP’s role in regulating adult hippocampal neurogenesis. Specifically, I will be examining the potential role of RKIP in NPC cell cycle entry to determine whether RKIP affects the rate in which neural stem cells enter the proliferative state as NPCs. This will be determined by using the early stem cells markers, GFAP and Sox2, to calculate the cell cycle entrance ratio of the number of cells that have entered the cell cycle within 1 hour of a BrdU injection compared to the total number of non-proliferating neural stem cells. Concurrently, in vitro experiments will also be performed with neurosphere granule cells to examine some of the cell cycle genes involved in G\textsubscript{i}/S and G\textsubscript{2}/M transition checkpoints, such as: Cyclin D1 and SKP2 since these are regulated by GSK3β and therefore by extension, RKIP (Al-Mulla et al., 2011). Furthermore, the completion of a neuronal timeline survival curve, as mentioned briefly before, to carve out the timeframe of neurogenesis from proliferating NPC up to maturation will provide a clear overview of RKIP’s role in regulating apoptotic processes. In furtherance to my proliferation experiments, we also plan to look to examining the role of RKIP in neuronal differentiation and maturation, specifically in granule cell morphology (i.e. spinal density) with Golgi staining assays and possibly electrophysiological experiments with LTP and LTD inductions at the medial perforant pathway (MPP) or lateral perforant pathway (LPP) to measure neuronal activity of RKIP knockout granule cells.

The addition of these proposed experiments will provide a comprehensive overview of RKIP’s role in modulating adult hippocampal neurogenesis. Moving from the cellular and physiological impact are many opportunities to study the effects of this potent regulator of multiple cellular pathways in behavioural experiments. Various forms of stimuli exist to regulate the neurogenic process and the NSCs hold their dominion in the SGZ through unending replenishment with a propensity to create new cells. As previously mentioned, neurogenesis is induced by intrinsic and extrinsic modulators, such as exercise and enriched environments, but the process stagnates without due outlet from other factors, such as depression. Nonetheless, the end results of this process are appreciable in distant gain; the constant renewal of neuronal cells to replace and
replenish resident cells suggests constant contributions and modifications to the neural circuitry. This likely translates to improved behavioural and cognitive performance in tasks involving memory and spatial separation (Fidaleo, Cavallucci and Pani, 2017). Therefore, performing tests such as the Morris Water Maze, Y-maze, and radial arm maze can uncover potential differences in task performance between RKIP knockout and C57BL/6J wild-type mice, thereby implicating RKIP’s impact on neurogenesis to carry through on a behavioural level.

In conclusion, I have provided evidence here of the role of RKIP in regulating adult hippocampal neurogenesis using a gene-trapped knockout of the RKIP gene in C57BL/6J mice. The data presented clearly shows that by utilizing voluntary exercise as an extrinsic stimulus, RKIP plays a central role in maintaining the proliferation of NPCs and the duration of their S-phase, and by extension the entire length, of the cell cycle. Finally, while RKIP may promote the differentiation of NPCs towards a neuronal fate initially, there may be other processes involved in determining the full maturation of such neuronal-fated cells.

When we look to the hippocampus, we find this seemingly small area appear to stretch to the dimensions of a larger tissue; the incredible complexity of the fibre tracts move in succession from one region of the Cornu Ammonis to the next until protruding to far reaches of the cortex. In this hub of the brain is a unique property not seen in many other regions: the potential to create new cells that appear to be the continuation of the resident pool of neuronal cells into new conditions, though more or less propitious. Throughout the extensive process of neurogenesis and period of time, the neural progenitor cells here ingress and egress from a mitotic phase according to the influence of their microenvironment and proceed forward in respective manner a symmetric or asymmetric division. These new cells then settle in the region, mature, and integrate with temporary adhesiveness. Thus in this manner do the intricacies of the function and mechanisms of neurogenesis create an evergreen state of neuronal replenishment which speaks to the evolutionary conservation of this phenomenon; we see it in all mammals, species which share in the likeness a function of memory and in humans, a preservation of individual history. If ever we are tempted to reduce this process to but a few principles, we should be swiftly reminded that the deterioration of this function and by extension, the structure of the hippocampus, can bear unfortunate consequences in the form of dementia-related diseases; some, such as Alzheimer’s disease, bring about forgetfulness and leave us to wander alone in our empty memories before untimely death. To unravel the cellular mechanisms and machinery of the neurogenic process
will then equip us with the tools necessary to combat such diseases. The role of RKIP, though perhaps a small participant in the grand scheme of this process, is nonetheless an important cog in this machinery. To this end do the findings presented here and those to come in the near future move us towards the providence of a greater understanding of a complex process that is, adult hippocampal neurogenesis.
Bibliography


## Table 2. List of Antibodies

Compiled primary and associated secondary antibodies used in all IF experiments. \( \lambda \) indicates emitted wavelength of light detected with confocal microscope.

<table>
<thead>
<tr>
<th>Primary</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat anti-Ki67 (Thermo Fisher Scientific)</td>
<td>Donkey anti-Rat (( \lambda = 488 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Goat anti-DCX (Santa Cruz Biotechnology)</td>
<td>Donkey anti-Goat (( \lambda = 594 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Rat anti-BrdU (Bio-Rad)</td>
<td>Donkey anti-Rat (( \lambda = 488 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Rabbit anti-S100( \beta ) (Abcam)</td>
<td>Donkey anti-Rabbit (( \lambda = 647 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Mouse anti-NeuN (EMD Millipore)</td>
<td>Donkey anti-Mouse (( \lambda = 594 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Mouse anti-BrdU (BD Biosciences)</td>
<td>Donkey anti-Mouse (( \lambda = 594 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Mouse anti-IdU (Novus Biologicals)</td>
<td>Donkey anti-Mouse (( \lambda = 594 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Mouse anti-GFAP (Thermo Fisher Scientific)</td>
<td>Donkey anti-Mouse (( \lambda = 594 ); Thermo Fisher Scientific)</td>
</tr>
<tr>
<td>Goat anti-Sox2 (Santa Cruz Biotechnology)</td>
<td>Donkey anti-Goat (( \lambda = 647 ); Thermo Fisher Scientific)</td>
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</table>
Figure 14. NPC Astrocyte Cell Maturation

Experiment outline for 35-day-old RKIP knockout and C57BL/6J mice under sedentary and exercise conditions shown in Figure 13A and Figure 13B. A Sagittal cross section immunofluorescence stain results of hippocampal tissue. IF stains from left to right: NeuN, S100β, BrdU, and composite image of all three. Yellow arrow indicates BrdU⁺/S100β⁺ cell. B Composite images of BrdU, NeuN, and S100β-stained hippocampal tissue. Left to right: Wildtype-SEDentary (n = 7), Wildtype-Exercise (n = 7), Knockout-SEDentary (n = 8), Knockout-Exercise (n = 7). C Cell density measurements of S100β⁺/BrdU⁺ cells in the dentate gyrus of the hippocampus of RKIP knockout and C57BL/6J wildtype mice under sedentary and exercise conditions. Co-localized S100β⁺/BrdU⁺ cells indicate newly matured glial cells (#: F = 11, p < 0.01). Data shown as mean ± SD.