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The regulation of adult hippocampal neurogenesis by wheel running and environmental enrichment

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The regulation of adult hippocampal neurogenesis by wheel running and environmental enrichment

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DEDICATION

I would like to dedicate the academic process that is this thesis to James Randi, Martin Gardner, Rachel Maddow, Doug Stanhope and the late greats, Lenny Bruce, George Carlin and Bill Hicks.

For teaching (or encouraging) me to question everything, to be curious, to speak freely and to not cow-tow to the status quo, I thank them.

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It goes without saying that I thank my parents for encouraging me to pursue my every dream and ambition.

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LIST OF ABBREVIATIONS

5-HT: Serotonin (5-Hydroxytryptamine)
BDNF: Brain-Derived Neurotrophic Factor
BLBP: Brain Lipid Binding Protein
CA: Cornu Ammonis (Ammon's Horn)
CNS: Central Nervous System
CSF: Cerebrospinal Fluid
CTL: Control Animals
DAB: Diaminobenzidine
DCX: Doublecortin (Protein)
DG: Dentate Gyrus
EGF: Epidermal Growth Factor
FACS: Fluorescence-Assisted Cell Sorting
FGF2: Fibroblast Growth Factor-2
fMRI: Functional Magnetic Resonance Imaging
GAD: Glutamate Acetyl Decarboxylase
GFAP: Glial Fibrillary Acidic Protein
GFP: Green Fluorescent Protein
GZ: Granular Zone
IGF-1: Insulin-like Growth Factor-1
KA: Kainic Acid (Kainate)
LOC: Locked Running Wheel Animals
LTD: Long-Term Depression
LTP: Long-Term Potentiation
NeuN: Neuronal Nuclei (Protein)
NPC: Neural Precursor Cell
OB: Olfactory Bulb
PSA-NCAM: Polysialic Acid-Neural Cell Adhesion Molecule (Protein)
RMS: Rostral Migratory Stream
RUN: Running Wheel Animals
SGZ: Subgranular Zone
SOX2: (Sex Determining Region Y)-Box 2 (Transcription Factor)
SVZ: Subventricular Zone
VEGF: Vascular Endothelial Growth Factor
VTA: Ventral Tegmental Area

SUMMARY

Introduction: In mammals, new neurons continue to be produced throughout the adulthood in two brain regions: 1) the hippocampus and 2) the forebrain subventricular zone. Adult neurogenesis is not a stable process, and changes in response to diverse factors such as age and pathology. Furthermore, because changes in neurogenesis may in fact underlie pathogenesis, regulating or restoring neurogenesis is seen as an important therapeutic objective. In healthy and diseased mice, hippocampal neurogenesis can be robustly regulated by environmental enrichment. However, while physical activity and environmental enrichment are potentially important in the treatment of some pathologies, comparatively little is known about the molecular and physiological mechanisms underlying activity/environment-dependent changes in neurogenesis.

Objectives and hypotheses: The primary objectives of this study are to characterize the neurogenesis-mediating effects of external stimuli and, in doing so, to elucidate the mechanisms that underlie observed changes. Using voluntary wheel running as a model, this study addresses two hypotheses: 1) that extended periods of physical activity can influence adult neurogenesis in the forebrain and the hippocampus and 2) that voluntary wheel running mediates neurogenesis through both running-dependent and running-independent stimuli.

Methods: To address the first hypothesis, we used a prolonged six-week voluntary paradigm and immunohistochemical analyses to characterize neural precursor activity in the subventricular

zone and hippocampus. To address the second hypothesis, we used a modified version of the above paradigm, where an additional group of mice were housed in cages with a locked running wheel.

Results: With respect to the first hypothesis, prolonged voluntary wheel running was found to increase neural precursor proliferation and neurogenesis in the hippocampal dentate gyrus relative to control animals, confirming the results of previous studies. More importantly, in this paradigm, proliferation in the forebrain subventricular zone was also found to be increased. In keeping with the second hypothesis, mice that were housed in locked-running wheel cages showed an increase in hippocampal neural precursor proliferation comparable to that of running animals. However, only running animals displayed increased hippocampal neurogenesis.

Conclusions: These results allow us to draw two novel conclusions regarding the effects of running on neurogenesis. First, proliferation in the forebrain subventricular zone, in addition to proliferation and neurogenesis in the hippocampus, is subject to regulation by wheel-running. Second, the wheel-running environment contains diverse stimuli which can influence some aspects of hippocampal neurogenesis in the absence of wheel running.

Keywords: Neurogenesis, Hippocampus, SVZ, Subventricular Zone, Running, Exercise, Environmental Enrichment

RÉSUMÉ

Introduction: Chez les mammifères, la naissance de nouveaux neurones se poursuit à l'âge adulte dans deux régions du cerveau: 1) l'hippocampe et 2) la zone sous-ventriculaire du prosencéphale. La neurogenèse adulte n'est pas un processus stable et peut être affectée par divers facteurs tels que l'âge et la maladie. De plus, les modifications de la neurogenèse peuvent être à l'origine des maladies de sorte que la régulation ainsi que le rétablissement de la neurogenèse adulte doivent être considérés comme d'importants objectifs thérapeutiques. Chez la souris saine ou malade, la neurogenèse hippocampale peut être fortement régulée par l'enrichissement environnemental ainsi que par l'activité physique. Cependant, lors même que l'activité physique et l'enrichissement environnemental pourraient contribuer au traitement de certaines maladies, très peu d'études porte sur les mécanismes moléculaires et physiologiques responsables des changements qui sont en lien avec ces stimuli.

Objectifs et hypothèses: Les principaux objectifs de cette étude sont de caractériser les effets de stimuli externes sur la neurogenèse et, par le fait même, d'élucider les mécanismes sous-jacents aux changements observés. En utilisant le modèle d'activité physique volontaire sur roue, cette étude teste les deux hypothèses suivantes: tout d'abord 1) qu'une période prolongée d'activité physique peut influencer la neurogenèse adulte dans le prosencéphale et l'hippocampe, et 2) que l'activité volontaire sur roue peut favoriser la neurogenèse à travers des stimuli dépendants ou indépendants de la course.

Méthodes: Afin de valider la première hypothèse, nous avons utilisé un paradigme incluant une activité physique volontaire prolongée sur une durée de six semaines, ainsi que des analyses immunohistochimiques permettant de caractériser l'activité de précurseurs neuronaux dans la zone sous-ventriculaire et l'hippocampe. Ensuite, pour valider la seconde hypothèse, nous avons utlisé une version modifiée du paradigme ci-dessous, en plaçant les animaux (souris) soit dans des cages traditionnelles, soit dans des cages munies d'une roue bloquée soit dans des cages munies d'une roue fonctionnelle.

Résultats: En accord avec la première hypothèse, l'activité physique prolongée volontaire a augmenté la prolifération des précurseurs neuronaux ainsi que la neurogenèse dans le gyrus dentelé de l'hippocampe comparativement aux animaux témoins, confirmant les résultats d'études antérieures. Par ailleurs, dans ce paradigme, nous avons aussi observé de la prolifération acrue au sein de la zone sous-ventriculaire du prosencéphale. De plus, en accord avec la seconde hypothèse, les souris placées dans une cage à roue bloquée ont montré une augmentation de la prolifération des précurseurs neuronaux dans l'hippocampe comparable à celle observée chez les souris ayant accès à une roue fonctionnelle (coureurs). Cependant, seuls les animaux coureurs ont présenté une augmentation de la neurogenèse hippocampale.

Conclusions: Ces résultats nous ont permis de tirer deux conclusions nouvelles concernant les effets de l'activité physique (course) sur la neurogenèse. Premièrement, en plus de la prolifération et de la neurogenèse dans le gyrus dentelé de l'hippocampe, la prolifération dans la zone sous-ventriculaire du prosencéphale peut être augmentée par l'activité physique sur roue.

Deuxièmement, l'environnement dans lequel l'activité physique a lieu contient différents stimuli qui peuvent influencer certains aspects de la neurogenèse hippocampale en l'absence d'activité physique sur roue (course).

Mots Clés: neurogenèse, hippocampe, ZSV, zone sous-ventriculaire, exercice, enrichissement environemental

INTRODUCTION

1. Plasticity in the central nervous system

One of the fundamental capacities of the central nervous system is to adapt and change in response to its environment and to any pathology or injury. This phenomenon is broadly known as plasticity. While early neuroanatomists and neuropsychologists favored a static or pavlovian brain (one where every process can be effectively reduced to a conditioned "reflex"), recent research has provided many examples of brain plasticity. Without plasticity, commonplace functions of the CNS such as the formation and consolidation of memories would not be possible. Furthermore, it is because of adaptive capacities that the CNS that the brain can maintain or recover function in cases of pathology or injury. Simply put, without plasticity, there would be no thought, no cognition and no adaptation. Mechanistically, the dynamic processes of the CNS (i.e., plasticity) can be roughly categorized into three basic types: 1) synaptic, 2) connectional and 3) neocellular.

The first breakthrough with respect to CNS plasticity came about because of the conceptual work of Donald Hebb. In his seminal work, "The Organization of Behavior," Hebb postulated that neurons that repeatedly exchange information with one another (via firing) will strengthen or improve the efficiency of said communication (Hebb, 1949). In other words, "if neurons fire together, they wire together." The implications of this postulate were enormous because Hebb linked the strengthening and weakening of synapses to behaviors like memory and

learning. Effectively, the behaviorist viewpoint had been challenged, and the concept of CNS plasticity was born.

Confirmation of Hebb's postulate about synaptic plasticity came in the 1970s, with the work of Bliss and Lomo. In their 1973 study, Bliss and Lomo discovered long-term potentiation (LTP), i.e., the strengthening of a synapse in terms of post-synaptic evoked potential (Bliss and Lomo, 1973). Since this discovery, LTP has been recognized as the major physiological mechanism underlying learning and the formation of memories. However, a dynamic brain must also be able to "un-learn" or weaken connections at some synapses, and this is accomplished by long-term depression (LTD) which is conceptually similar to LTP. LTD was first described in the late 1970s by Lynch and colleagues, as a process occurring in parallel with LTP (Lynch et al., 1977). Together, LTP and LTD permit changes in the strength of synapses throughout the brain. Thus, connections from one system or set of neurons can be potentiated to reflect changes in external stimuli and cognitive processes. This forms the mechanistic basis for how the brain learns and remembers.

Another form of plasticity that is central to normal CNS function is connectional plasticity. The first evidence for this phenomenon came about in the early 1960s via the work of Bach-y-Rita, Merzenich and Kaas (Allard et al., 1991; Bach-y-Rita, 1967; Bach-y-Rita et al., 1969a; Bach-y-Rita et al., 1969b; Bach-y-Rita et al., 1969c; Buonomano and Merzenich, 1998; Guic et al., 2008; Hickmott and Merzenich, 2002; Jenkins and Merzenich, 1987; Kaas et al., 1983; Merzenich et al., 1987; Merzenich et al., 1984; Merzenich and Sameshima, 1993). The various ways in which these researchers discovered this phenomenon goes beyond the scope of this thesis. Suffice to say that, in response to CNS and peripheral injury, and in normal CNS

function, neurons can modify the distribution of their axonal projections to innervate different neurons or areas. For example, in the case of Bach-y-Rita's early studies (Bach-y-Rita et al., 1969a), blind persons could be taught to "see" via tactile stimuli. In this case, cortical structures that would have normally been responsible for processing visual cues were recruited by the somatosensory pathways of the brain. Another example came from the work of Ramachandran, who described how phantom sensations were produced in amputated limbs via some re-organization of the somatosensory and motor cortices (Ramachandran, 1993; Ramachandran et al., 1992). In this manner, extant structures of the brain can facilitate or replace the functions of damaged structures, and this has also been termed as axonal or cortical plasticity.

The third type of plasticity that is important for CNS function is neocellular plasticity. This refers specifically to the genesis of new cells in the mature or adult brain. Adult gliogenesis has been described and studied for some time, and it contributes to the maintenance of normal CNS function and the repair of lesioned or diseased CNS tissue. However, it has since been shown that the mature brain is capable of forming new neurons in specific anatomical regions and that these cells contribute to CNS plasticity. Adult neurogenesis and the neocellular plasticity of the CNS is the primary focus of this thesis, and will be discussed in greater detail throughout.

2. Adult neurogenesis

2.1 Neurogenesis: How are neurons formed?

Through years of experimentation and observation, researchers have learned and described how the diverse cells of the brain are produced. Simply stated, neurons do not divide symmetrically, as might be the case for cells in other biological systems. Instead, they originate from a complex system of multipotent progenitors and stem cells.

The system of stem cells, progenitors and neuroblasts in the brain is akin to other stem cell systems, such as the hematopoietic system, and can be broken down into several distinct stages. In the developing brain, all neuronal tissue arises from the neuroepithelium, which is induced from embryonic stem cells. Once the framework of the neural axis is laid out, multipotent stem cells in the neural tube will form all the remaining neurons and glia of the cerebrum and spinal cord. The principal, or earliest-lineage stem cells in the developing CNS are the radial glia (Cameron and Rakic, 1991; Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002), which have two main roles, both dividing to produce other progenitors/daughter cells and providing scaffolding for subsequent migration and integration of newly formed cells. The daughter cells, or transit-amplifying progenitors, will then migrate to the appropriate regions or layers and can expand the local population through symmetrical division. Following this, neural progenitors differentiate into neuroblasts or glioblasts, from which all of the diverse neurons and glia of the fully formed CNS will be produced.

2.2 The discovery of adult neurogenesis

Adult neurogenesis is a term used to describe the process by which new neurons are formed in the mature brain forms new neurons. While there was always some speculation that the brain can form new neurons throughout adulthood, it is only within the last 20 years that the dogma of "no new neurons" has been successfully challenged. Prominent and pioneering neuroanatomists like Ramon y Cajal championed the fixed and non-regenerating CNS largely because of the fact that neurons were never observed to be mitotic. To quote Ramon y Cajal's "Degeneration and Regeneration of the Nervous System" (Ramon y Cajal, 1928): "In the adult centers [of the brain], the nerve paths are something fixed and immutable: everything may die, nothing may be regenerated."

Surprisingly, some of Ramon y Cajal's contemporaries made observations that suggested that adult cell genesis in the brain, and even ongoing neurogenesis, might be a possibility. In 1889, Wilhelm His was among the first to describe what we now call stem or germinal cells in the neural tube of the developing CNS (His, 1889), which spawned additional research into the genesis of neurons and glia by the likes of Penfield (Penfield, 1932). In 1912, Ezra Allen showed that, in rodents, cells surrounding the ventricular system, in the region we now call the subventricular zone, were undergoing mitosis well into adulthood (Allen, 1912). Further studies of this subventricular zone in humans (Kershman, 1938; Rydberg, 1932) suggested that this region could produce different types of glia well into adulthood and contribute to tumor formation. In 1958, Messier, Leblond and Smart were among the first to use tritiated thymidine to label and identify mitotic cells in the central nervous system (Messier et al., 1958).

Confirming previous findings, they found large numbers of labeled, dividing subependymal cells and, to some surprise, occasional labeled neuron-like cell in the cortex. However, studies by these groups had failed to demonstrate whether any of the dividing cells were or were becoming true neurons, and the dogma persisted.

The first real breakthrough in the discovery of adult neurogenesis came about through the work of Joseph Altman in the 1960s. Altman began his studies with tritiated thymidine by studying and describing cell genesis in response to brain injury (Altman, 1962a; Altman, 1962b; Altman and Altman, 1962). He was then able to show that some labeled cells in the brain had a neuronal morphology or phenotype, which suggested that adult neurogenesis was a possibility. Altman pursued this line of research and went on to provide some of the first characterization of adult neurogenesis in the subventricular zone and the hippocampus, which had not been hitherto considered as a possible germinal zone of adult neurons. In his seminal studies, Altman demonstrated with autoradiographic techniques that new cells , which appeared to be neurons, were being produced in the germinal zone of the dentate gyrus and the subventricular zone and even identified their candidate precursor or progenitor cells (Altman, 1963; Altman and Das, 1965a; Altman and Das, 1965b). These studies also showed that the mitotic activity in the germinal zones of the SVZ and the hippocampus was not stable, but decreased with age. In spite of this convincing evidence, his studies were met with skepticism and some hostility. Several years later, Michael Kaplan built on Altman's findings and confirmed, via electron microscopy, that the cells labeled with thymidine were in fact neurons (Kaplan, 1985; Kaplan and Hinds, 1977; Kaplan et al., 1985). The last of the definitive confirmations came in 1985 from Fernando Nottebohm (Nottebohm, 1985; Paton et al., 1985). Using songbirds as a model organism, he was

able to show, with a combination of techniques, that new cells were being produced in the brain and that these cells had electrophysiological properties of neurons. He was also the first to co-label thymidine labeled cells with another cytological marker, which in this case was horseradish peroxidase that had been injected into the neurons following electrophysiological recordings.

In the years since these groundbreaking studies, several other researchers have contributed to transforming the field of neurogenesis from "anti-dogmatic" and "esoteric" to being widely accepted. Arturo Alvarez-Bullya, a student of Nottebohm, was able to describe the morphology and behavior of candidate, radial glia-like stem cells in mammals in vivo (Alvarez-Buylla et al., 1990), while Sam Weiss used a cell culture assay to establish the existence of neural stem cells isolated from the ventricular system in vitro (Reynolds and Weiss, 1992). Several important methodological strategies were also developed during this period, which enabled more accurate measurement of adult neurogenesis. Elizabeth Gould and Heather Cameron were the first to co-label thymidine-labeled cells with an immunohistochemical marker, namely neuron-specific enolase (Cameron et al., 1993). Fred Gage and colleagues improved on the labeling of dividing cells by first substituting the thymidine analog bromodeoxyuridine (BrdU) for tritiated thymidine (Kuhn et al., 1996) and then by using retroviral labeling of live cells via green fluorescent protein (GFP) (van Praag et al., 2002). Finally, confirmation of neurogenesis in primates came from diverse groups (Gould et al., 1999b; Kornack and Rakic, 1999) and definitive evidence of human hippocampal neurogenesis was provided by a landmark study by Eriksson and Gage (Eriksson et al., 1998).

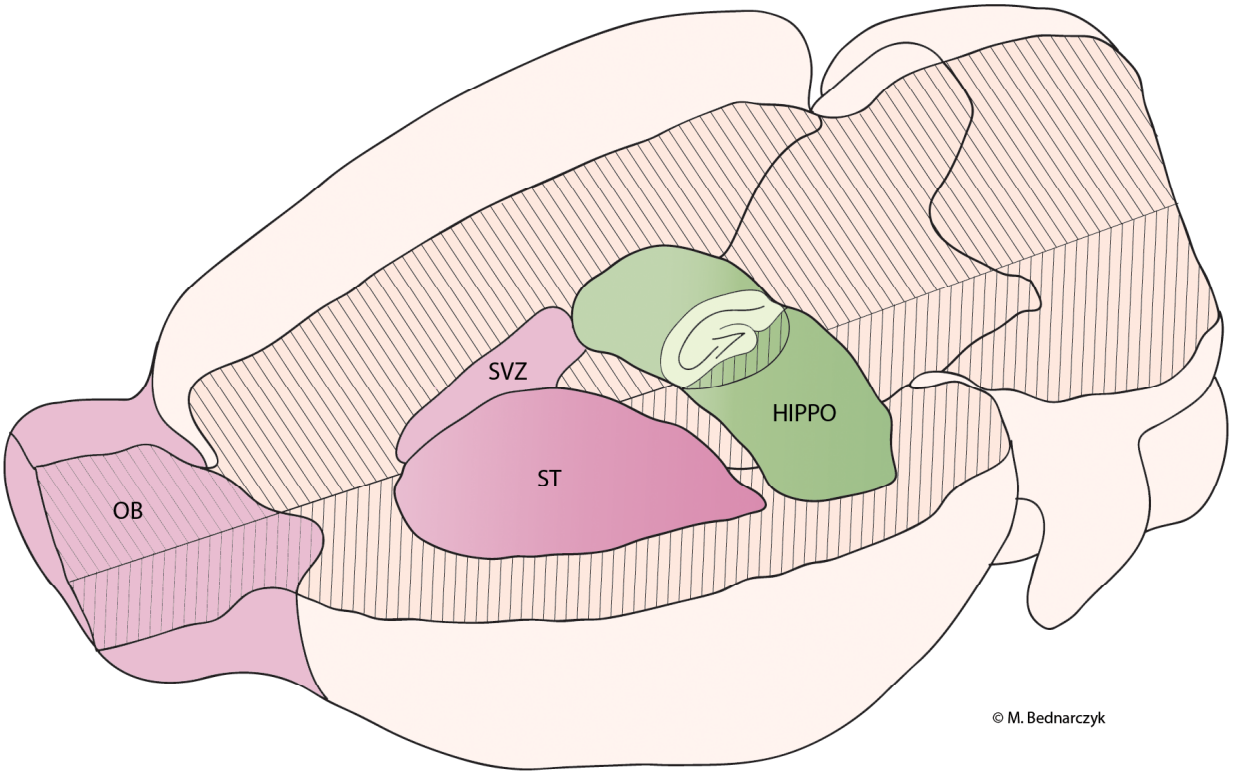


FIGURE 2.1 Three-dimensional schematic view of the primary neurogenic regions in the adult mouse brain. The SVZ-Olfactory Bulb region is outlined in dark pink. The SVZ is shown adjacent to the contralateral lateral ventricle. The striatum is included in the SVZ-OB system not because it is a neurogenic region per se, but because the SVZ is found on the ventricular surface of this structure. The hippocampus is outlined in green, with a cutaway view of the Cornu Ammonis and dentate gyrus structures in situ. Abbreviations: HIPPO = Hippocampus, OB = Olfactory Bulb, ST = Striatum, SVZ = Subventricular Zone. © M. Bednarczyk

3. The subventricular zone (SVZ)

3.1 Structure of the subventricular zone

Current studies and literature on the SVZ have defined its basic anatomical structure. In humans, the SVZ consists of four layers, each of which is populated by distinct types of cells and each of which serves different functions (Quinones-Hinojosa and Chaichana, 2007; Quinones-Hinojosa et al., 2007; Quinones-Hinojosa et al., 2006). The outermost, periventricular layer (Layer I) of the human SVZ is formed by ependymal cells. These cells have a ciliated apical surface and are involved in the production and circulation of the cerebrospinal fluid (CSF). Consequently, this layer forms the main barrier between the ventricular system and the other cells of the SVZ. Proximal to the basal surface of the ependymal cell layer is the hypocellular layer (Layer II). As its name implies, this layer is devoid of cell bodies and significant numbers of resident cells. Conversely, it consists primarily of cellular processes originating from other layers and cells of the SVZ, such as the astrocyte-like stem cells. Adjacent to and below the hypocellular layer is the principal layer of constituent SVZ cells (Layer III). Astrocytes, astrocyte-like stem cells, neural precursors and neuroblasts can all be found in this layer. The final layer (Layer IV) forms the interface between the cells of the SVZ and the adjacent brain parenchyma.

The rodent SVZ, by comparison, differs slightly in its structure and cellular make-up. Of greatest importance is the fact that there are only two clearly demarcated “layers” of cells, unlike the human SVZ. The rodent SVZ is composed of an outer layer of ependymal cells, proximal to the lateral ventricles, and a subependymal layer that contains all of the various neural precursors (Doetsch et al., 1997).

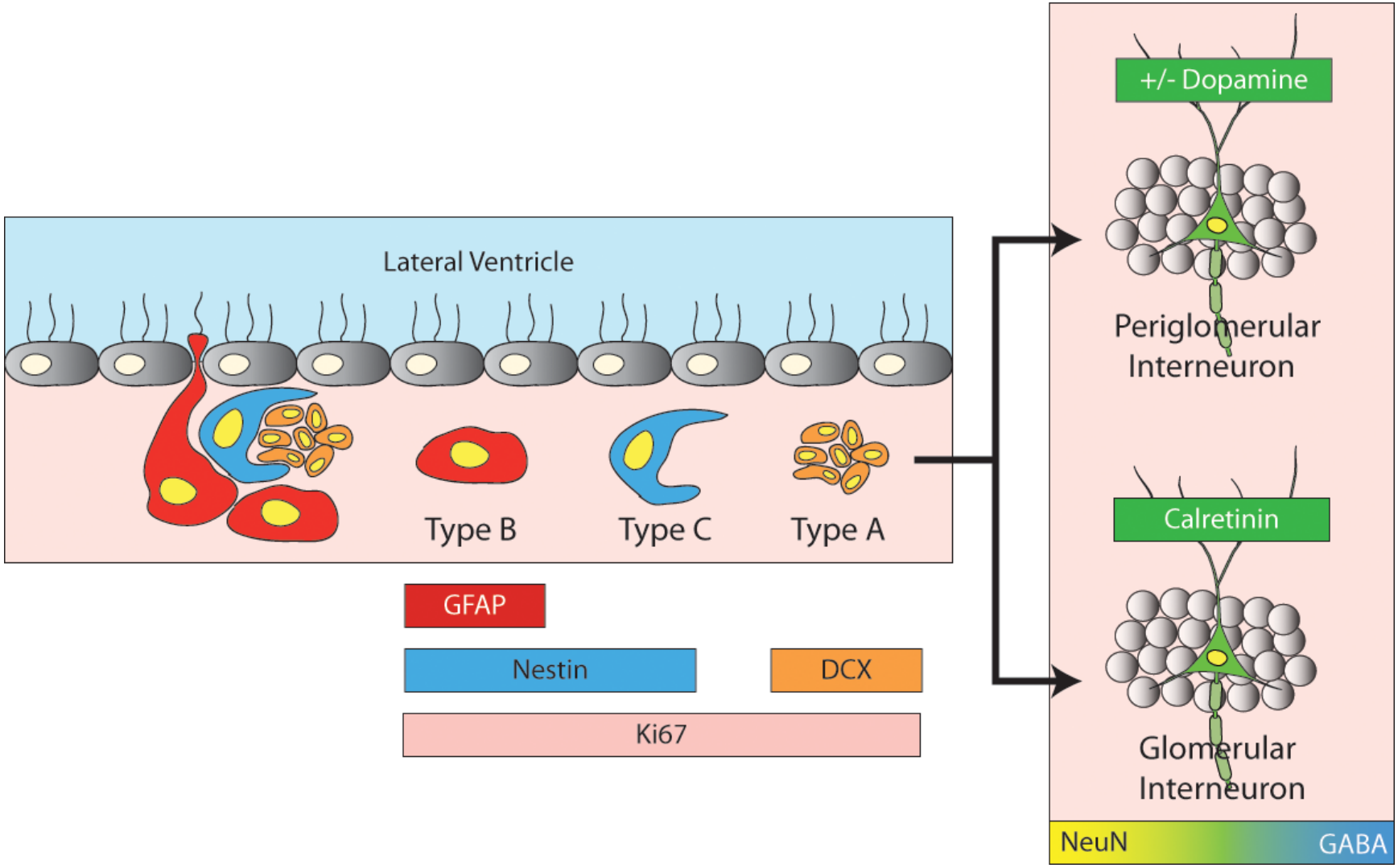

Lateral Ventricle
Type B
Type C
Type A
GFAP
Nestin
DCX
Ki67
+/- Dopamine
Periglomerular
Interneuron
Calretinin
Glomerular
Interneuron
NeuN
GABA

FIGURE 3.1 (PRECEDING PAGE) Schematic representation of the SVZ-OB neurogenic lineage. From left to right are shown a cluster of neural precursors in situ followed by the individual cells and their lineage with corresponding immunohistochemical markers. Arrows represent the rostral migratory stream (RMS) and neurons correspond to mature interneurons in the Olfactory Bulb. © M. Bednarczyk

3.2 Subventricular zone neurogenesis

The subventricular zone is a vestige of the embryonic germinal zone that is derived from the circumventricular neurogenic areas. Consequently, the manner by which new neurons are produced in this structure is comparable to the manner by which new neurons are produced embryonically. The prevailing model of SVZ neurogenesis was first outlined in the late 1990s by Doetsch and Alvarez-Buylla (Doetsch et al., 1997). Using histological techniques and electron microscopy, these authors showed that there are is an organized cellular structure to the SVZ neurogenic niche, comprising four principal types of cells, which they categorized as follows: 1) Type A cells (neuroblasts), 2) Type B cells (astrocytes), 2) Type C cells (transit-amplifying progenitor cells) and 4) Type E cells (ependymal cells). In their 1999 study, this same group outlined the lineage, or order in which these cells are produced: Type B cells divide to produce Type C cells which then divide to produce Type A cells (FIGURE 3.1) (Doetsch et al., 1999a; Doetsch et al., 1999b). In contrast, Type E cells do not divide and are not directly involved in the neurogenic process, although there is some evidence to the contrary originating from Frisen and colleagues (Johansson et al., 1999). It is generally accepted, however, that Type E cells serve important functions in the regulation of SVZ neurogenesis.

The principal stem cell in the SVZ is the Type B cell, which bears several of the morphological and functional hallmarks of astrocytes and radial glia. These cells reside in the subependymal layers of the SVZ and have processes that ensheath migrating type A cells and make contact with the CSF of the ventricular system, permitting the free exchange and uptake of CSF-borne trophic factors. Type B cells are easily identifiable via immunohistochemistry for markers like GFAP, Nestin, BLBP and Pax6. The multipotent and stem cell-like nature of these cells was first confirmed by Doetsch and colleagues in the late 1990s. In their 1999 studies, Doetsch and Alvarez-Buylla selectively ablated most neurogenesis in the SVZ through cytostatic treatments, which spared some of the slowly-dividing B cells. After termination of the cytostatic treatments, the B cells were able to regenerate themselves and to completely repopulate the progenitor population of the SVZ (Doetsch et al., 1999a; Doetsch et al., 1999b). Moreover, Type B cells have the potential to form neurospheres in vitro which can subsequently be differentiated into neurons and glia, all hallmarks of a neural stem cell phenotype (Chiasson et al., 1999; Laywell et al., 2000; Morshead et al., 1998; Morshead et al., 1994; Reynolds and Weiss, 1992).

The next cell in the neurogenic lineage of the SVZ is the Type C cell, or transiently-amplifying progenitor that is produced as a result of the asymmetric division of a B cell. Type C cells can be identified by their highly proliferative nature and by diverse markers of neural precursors, such as Pax6 (Hack et al., 2004), Mash, Ki67 and the transcription factor Dlx2 (Doetsch et al., 2002). Consequently, Type C cells are largely responsible for the expansion of proliferating and still-undifferentiated neural precursors. As such, they form the largest pool of dividing cells in the forebrain SVZ (Doetsch, 2003b).These cells were identified morphologically in the same Doetsch and Alvarez-Buylla study (1997) that identified B cells.

Migrating neuroblasts, or Type A cells, are the penultimate cells in the SVZ neurogenic lineage and are produced from Type C cells. They are relatively small cells with two prominent equatorial processes and can be detected via immunohistochemistry for markers of immature neurons, such as DCX and PSA-NCAM.

Type E cells, or ependymocytes, do not actually contribute directly to the neurogenic lineage according to the prevailing model. However, they do form an important interface with the CSF of the adjacent lateral ventricles, and could be responsible, in part, for regulating the activity of neural precursors in the SVZ.

Type B, C, A and E cells are often grouped in close proximity and in small clusters throughout the SVZ. Together, they form the constituents of the SVZ neurogenic niche and interact with themselves and with the environment to regulate the rate of neurogenesis (Doetsch, 2003a; Lim and Alvarez-Buylla, 1999). It should also be noted that, according to the prevailing model, all of the aforementioned cells are capable of self-renewal to some degree. The Type A cells will only become post-mitotic and fully differentiated once they have left the SVZ proper, in the final step of the SVZ neurogenic process.

Type A cells, after being produced, will move tangentially across the SVZ towards the rostral migratory stream, in which they continue to mature and differentiate and through which they eventually reach the olfactory bulb (OB). Here, they become post-mitotic and migrate out radially into the glomerular or periglomerular layers to finally integrate into existing OB networks as fully functional neurons. The Type A cells can be identified by immunohistochemistry for NeuN, GAD and Calretinin. Regardless of where they migrate to in

the OB, the vast majority of SVZ-derived new neurons become GABAergic interneurons (FIGURE 3.1).

Interestingly, some of the periglomerular interneurons are also immunoreactive for tyrosine hydroxylase and use dopamine in addition to GABA as their neurotransmitter. Of note, the vast majority of incoming, newly-formed neurons will eventually die, with only a small proportion being conserved for a longer period of time. Because most of the cell death is accomplished in the OB proper, it is assumed that the successful integration of incoming neurons into OB networks determines whether or not the cells survive. Because the integration of new neurons into the OB occurs concomitantly with the death of existing OB interneurons, this whole neurogenic process mostly serves in the lifelong turnover of the latter.

3.3 Functions of subventricular zone neurogenesis

The primary function of the SVZ is to supply the forebrain with neural precursors and neuroblasts. As such, the vast majority of these nascent cells will be directed towards the OB, where they will participate in a life-long turnover of OB interneurons. A strong body of research demonstrates that this neurogenic process is crucial for the normal function of the OB, including tasks such as fine odor discrimination (Breton-Provencher et al., 2009; Grubb et al., 2008; Nissant et al., 2009; Whitman and Greer, 2009).

It should also be noted that in the case of injury to other regions of the forebrain, neuroblasts (and potentially glioblasts) that are produced in the SVZ can migrate out of the rostral migratory stream and contribute to repairing damaged structures (Kim and Szele, 2008).

This is particularly evident with injuries or diseases where entire cortical structures are ablated, such as stroke (Arvidsson et al., 2002; Faiz et al., 2008; Gotts and Chesselet, 2005a; Gotts and Chesselet, 2005b; Gotts and Chesselet, 2005c; Kojima et al., 2010; Massouh and Saghatelyan, 2010; Ohab et al., 2006; Yamashita et al., 2006) or traumatic brain injury (Goings et al., 2004; Ramaswamy et al., 2005; Richardson et al., 2007; Salman et al., 2004). Because of this fact, finding ways to increase or modulate forebrain neurogenesis after injury is an important research and therapeutic objective (Alvarez-Buylla et al., 2000).

However, while the SVZ neurogenic pool does provide some regenerative capacity to the brain, in the vast majority of cases, newly formed "replacement" structures cannot fully approximate the function of the structures they replace. In cases where studies have shown a restoration of function (Kolb et al., 2007), it has mostly been through indirect behavioral analyses, and could be attributed to connectional plasticity in the remaining healthy structures.

4. The hippocampus

4.1 Structure of the hippocampal formation

The hippocampus is a distinct, crescent-shaped structure located on the floor of the middle horn of the lateral ventricle. It is a bilaminar, submerged gyrus, comprising two main parts. The most voluminous part is the Cornu Ammonis, or "ram's horn," which is situated dorsally in rodents, proximal to the surfaces of the caudal ventricular system. The Cornu Ammonis is populated primarily by pyramidal cells, organized into a single, curved layer. This layer is subdivided along its axis into three distinct regions: CA1, CA2 and CA3 (FIGURE 4.1). The CA3 region extends around the hippocampal sulcus into the second, smaller hippocampal "gyrus," the dentate gyrus.

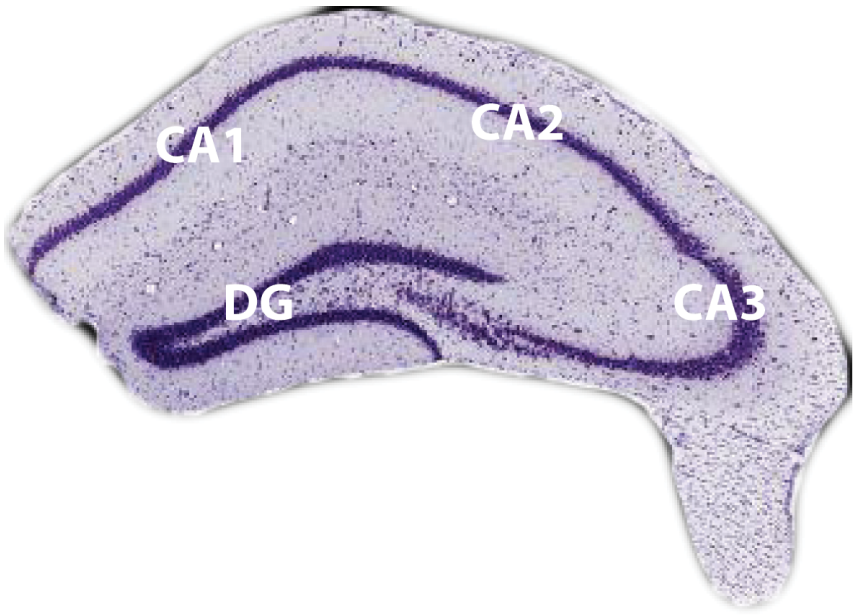
The dentate gyrus is situated just below, or ventral to the Cornu Ammonis, proximal to the surfaces of the midbrain. The largest population of neurons in this structure forms the granule cell layer of the dentate gyrus. The granule cell layer is surrounded on either side by the molecular layer, which consists primarily of innervating axons from the perforant path and from other CNS structures (FIGURE 4.1). In between the two laminae of the granule cell layer is the hilus, or polymorphic layer. At the interface of the hilus and granule cell layer is the subgranular zone, which is the primary germinal region in the hippocampus. The structure, function and constituent cells in this region will be discussed further in sections 4.2 and 4.3.

The subiculum forms part of the parahippocampal gyrus and projects caudally from the Cornu Ammonis. The subiculum is composed of loosely clustered pyramidal cells that receive

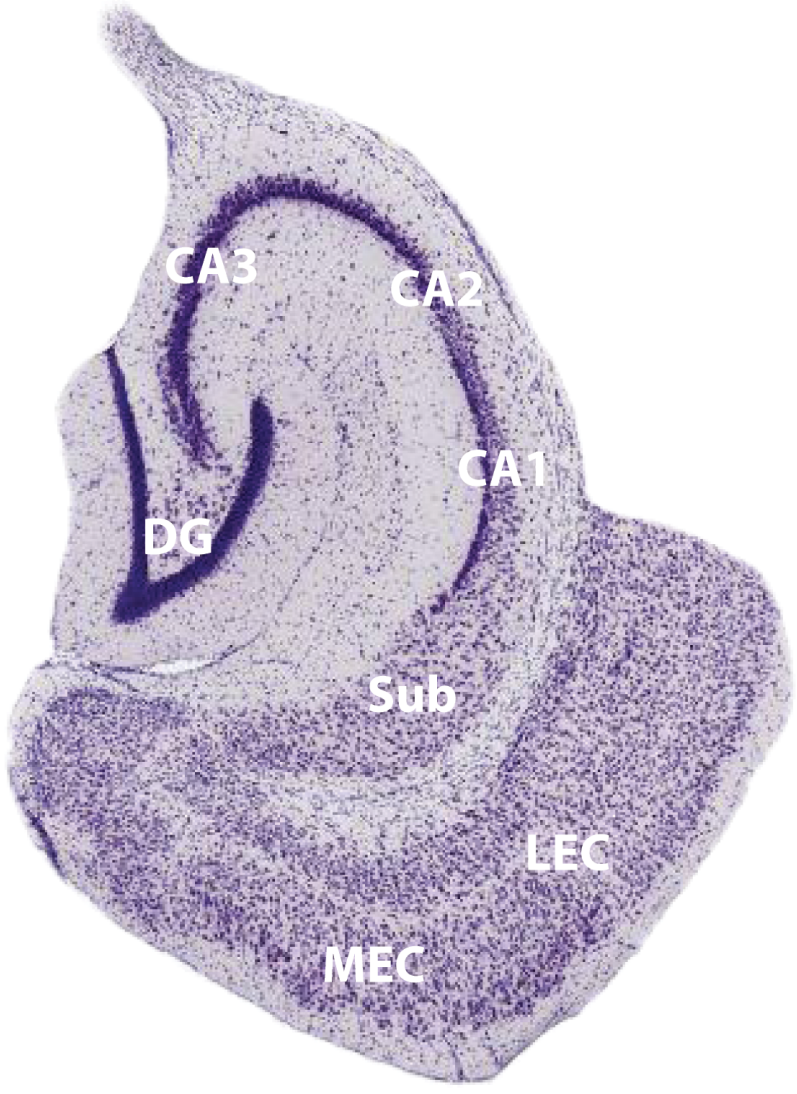
their input primarily from the CA1 cell layer of the hippocampus proper (Andersen, 2007; Duvernoy, 2005; Shepherd, 2004). Consequently, the subiculum is the primary output of the hippocampus, and its efferent fibers project out to diverse structures of the limbic system, midbrain and forebrain, such as the nucleus accumbens, amygdala, hypothalamus and the prefrontal cortex (in humans).

The subiculum also forms important connections with the entorhinal cortex, which is located ventro-caudal to the subiculum in rodents. The entorhinal cortex is composed of several layers of neurons and serves as the primary interface between the structures of the cerebrum and the hippocampus. Its superficial layers (II and III) project to the granule cell layer of the dentate gyrus, to the CA3 and CA1 pyramidal cells and to the subiculum (Andersen, 2007; Duvernoy, 2005; Shepherd, 2004). Together, this forms the main input to the hippocampus, or the Perforant Path. The superficial layers of the entorhinal cortex also receive the primary inputs from other regions of the cerebrum. The deep layers of the entorhinal cortex (i.e., V) also receive important inputs from the hippocampus (FIGURE 4.1).

FIGURE 4.1 (FOLLOWING PAGE) Hippocampal anatomy in the rodent. Shown are coronal and horizontal views of the hippocampus and parahippocampal structures. Note that both representations are oriented in the same rostro-caudal direction. Abbreviations: DG = Dentate Gyrus, Sub = Subiculum, LEC = Lateral Entorhinal Cortex, MEC = Medial Entorhinal Cortex. © M. Bednarczyk



CORONAL VIEW



HORIZONTAL VIEW

The circuitry of the hippocampus is unique in the CNS in terms of its complexity and the functions it supports. While it comprises many different inhibitory, extrinsic and intrinsic components, its basic excitatory (glutamatergic) circuitry can be summarized as follows: 1) The dentate gyrus receives its input from the entorhinal cortex via the perforant path. 2) The dentate gyrus granule cells, in turn, innervate the CA3 pyramidal cell layer. 3) The CA3 pyramidal cells innervate CA1 pyramidal cells which project their axons to the subiculum. 4) Neurons in the subiculum project their axons to the entorhinal cortex (FIGURE 4.2) (Andersen, 2007; Duvernoy, 2005; Shepherd, 2004).

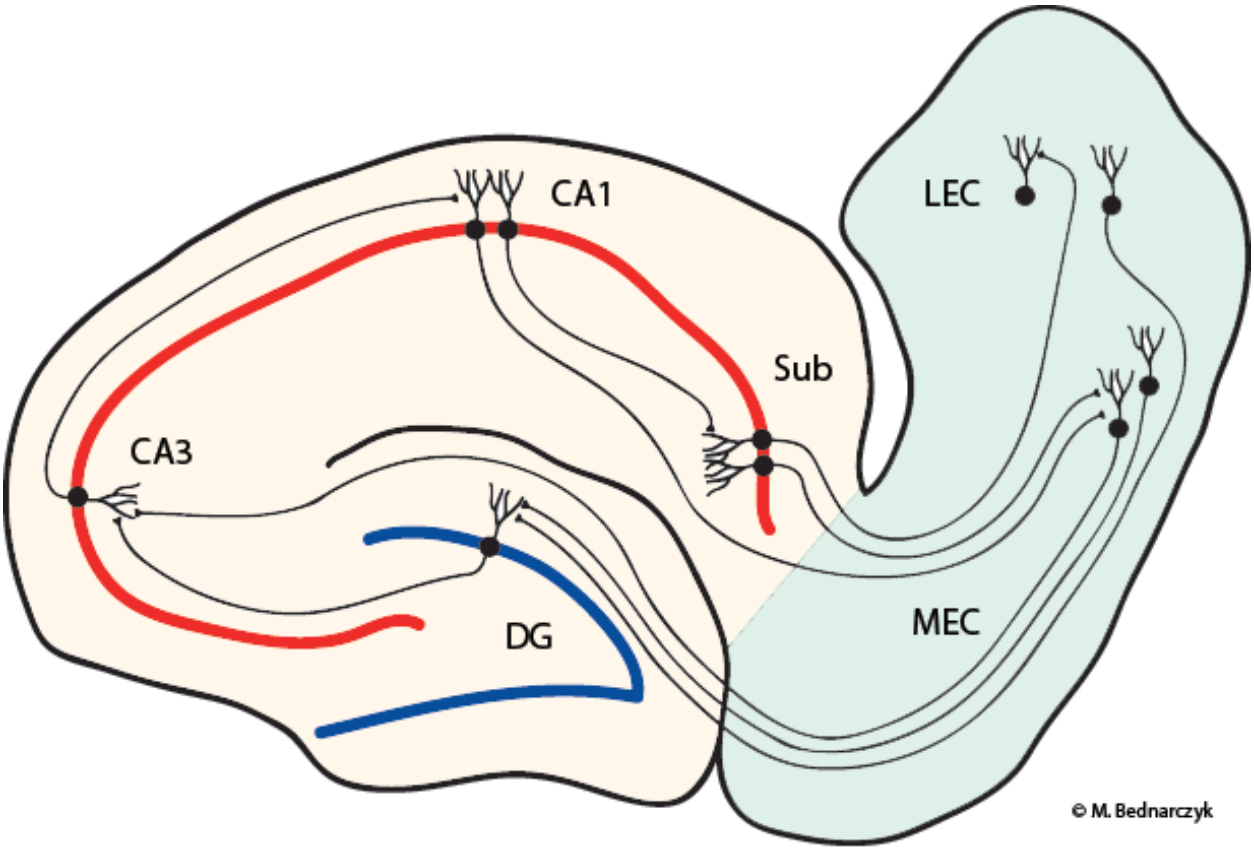
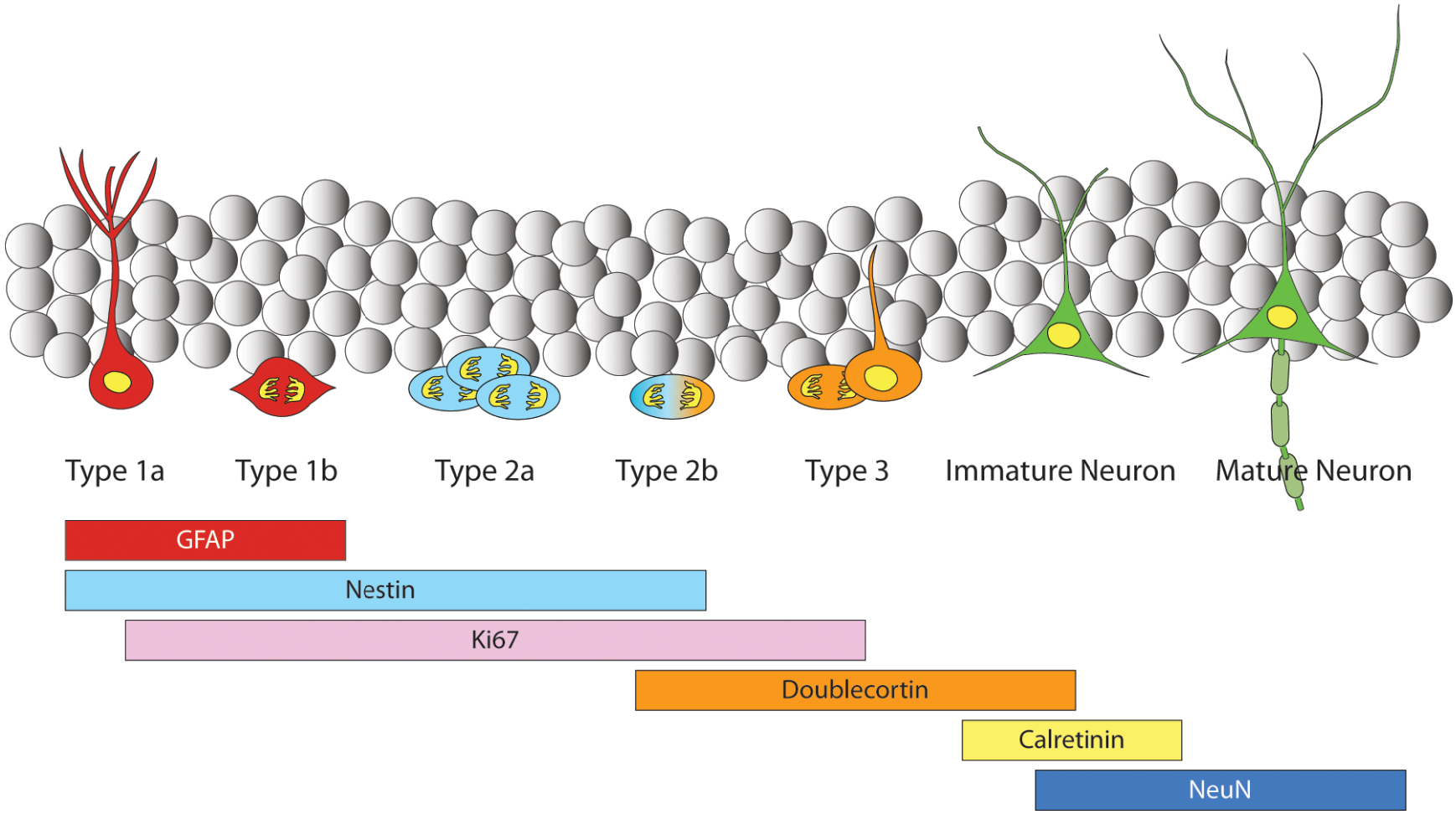


FIGURE 4.2 (above) Schematic of the basic excitatory circuitry in the hippocampal formation. The hippocampus proper is colored in light beige, while the entorhinal cortex/parahippocampal gyrus is colored in light green. Abbreviations: CA1 = CA1 pyramidal cells, CA3 = CA3 pyramidal cells, DG = dentate gyrus, LEC = Lateral entorhinal cortex, MEC = Medial entorhinal cortex, Sub = Subiculum. © M. Bednarczyk

FIGURE 4.3. (FOLLOWING PAGE) Schematic representation of the hippocampal neurogenic lineage. Cell types are presented in the general order in which they are produced, from left to right. Boxes below show the immunohistochemical markers that can be used to identify the specific cells. © M. Bednarczyk


Type 1a
Type 1b
Type 2a
Type 2b
Type 3
Immature Neuron
Mature Neuron
GFAP
Nestin
Ki67
Doublecortin
Calretinin
NeuN

4.2 Hippocampal neurogenesis

In the healthy CNS, hippocampal neurogenesis occurs in the subgranular zone of the dentate gyrus. This area is defined as a 2 to 3 cell thick region below the granule cell layer, in which several different types of neural precursors contribute to the formation of new granule cell neurons.

The basic plan of the hippocampal neurogenic system and lineage is comparable to that of the subventricular zone, in that the newborn neurons produced in the hippocampus derive from stem-like cells and progenitors in an almost linear fashion. However, unlike in the SVZ, there is some contention about specific nomenclature and the precise identity of constituent cells. Moreover, while the basic plan of hippocampal neurogenesis is comparable to that of the SVZ, there are several notable differences between these two systems, ranging from the morphology of precursor cells to the function of newly-formed cells.

The current, widely-accepted model is derived from the research of many different groups, working to address all manner of questions about the hippocampal neurogenic lineage. In its simplest form, the neurogenic lineage of progenitors and precursors in the hippocampus can be summarized as follows: 1) Radial glia-like astrocytes (Type 1 cells), which may or may not self-renew, divide slowly and asymmetrically to produce rapidly proliferating progenitors (Type 2 cells). 2) Type 2 progenitors, multiplying at a relatively fast rate, expand in number and, in the latter stages, begin to differentiate into their respective lineages. Consequently, the vast majority of these cells will differentiate into the neuronal lineage. 3) Neuroblasts (Type 3 cells) result from the differentiation of Type 2 cells and will slowly migrate out of the germinal zone and

become post-mitotic. 4) Finally, after developing all of the phenotypic and morphological hallmarks of granule cell neurons, newly-formed neurons will mature fully and will integrate into existing granule cell structures and networks (FIGURE 4.3) (Kempermann et al., 2004a; Seri et al., 2004).

In the hippocampal subgranular germinal zone, Type 1 cells (which roughly correspond to SVZ Type B cells) are considered to be the earliest progenitor or stem cells (Palmer et al., 1997). These cells display many of the hallmarks of radial glia and, unlike in the SVZ, have a distinct radial glia-like morphology (Filippov et al., 2003; Fukuda et al., 2003; Mignone et al., 2004; Seri et al., 2004). The early progenitor identity of Type 1 cells was first established via the work of the Seri and colleagues in 2001. Using retroviral labeling of GFAP+ astrocytes, they showed that new neurons in the granule cell layer originated from a GFAP+ progenitor, thus establishing that the radial-glia like astrocyte is the likely cell of origin in the hippocampal neurogenic lineage (Seri et al., 2001).

In terms of morphology, the triangular soma of Type 1 cells is located in the SGZ and the cells project a long, apical process up into the upper granule cell layer and molecular layer, where it branches out. Much as with SVZ Type B cells, the Type 1 cells are immunoreactive for Nestin, BLBP and GFAP (Fukuda et al., 2003; Kempermann et al., 2004a; Mignone et al., 2004). In the hippocampus, they also co-express the transcription factor SOX2, and are negative for S100Beta, which is another independent marker of astrocytes. Type 1 cells proliferate slowly, accounting for roughly 5% of proliferating cells at any given time (Filippov et al., 2003; Kronenberg et al., 2003). In some studies and models, there is even an additional breakdown of the Type 1 cell, into the Type “1a” and “1b”, the latter representing a more active and

proliferative state and having a shortened horizontal, rather than radial, orientation (Seri et al., 2004; Suh et al., 2007).

There is some controversy as to whether or not Type 1 cells are in fact stem cells, because their ability to self-renew has not been accurately ascertained. In support of their stem cell-like nature, a study by Mignogne and colleagues used FACS to isolate Nestin-positive cells from the dentate gyrus, and these were capable of forming primary neurospheres (Mignone et al., 2004). However, no clonal analyses or second passages were performed and it is likely that the neurospheres merely represent the expansion of unipotent precursor cells. In another study by Suh and colleagues (Suh et al., 2007), SOX2+ cells were shown to be capable of self-renewal in vivo, but, to date, this is the only study showing this in vivo potential. Indeed, some researchers, including van der Kooy (Seaberg and van der Kooy, 2002), even suggest that any favorable findings regarding in vitro culture of hippocampal stem cells may result from cellular contaminants (i.e., stem cells) originating from the SVZ.

What is generally accepted is that Type 1 cells will divide to produce a more-rapidly proliferating daughter cell, the Type 2 cell of the hippocampal neurogenic lineage. The first identification of type 2 cells came about via the work of Fukuda and colleagues (2003), in an attempt to differentiate between the various types of Nestin-expressing cells in the SGZ (Fukuda et al., 2003). Indeed, this study showed that the large number of Nestin+ cells in the subgranular zone can be subdivided into those that express GFAP and have radial glia-like morphology and those that do not. Thus, cells that are immunoreactive for Nestin but do not co-express radial glia-like markers were designated “Type 2” cells. These same Nestin+/GFAP- cells will divide more quickly than their GFAP+ counterparts, and are generally more numerous than Type 1 cells

in the SGZ (Filippov et al., 2003; Kronenberg et al., 2003). Moreover, the Fukuda et al. study (2003) demonstrated that the electrophysiological properties of these two types of cells differ. Type 1 cells have astrocyte-like electrophysiological properties, whereas Type 2 cells have a much higher input resistance and display basic sodium currents, which is indicative of some neuronal-lineage differentiation.

Morphologically, Type 2 cells are small, rapidly dividing cells with short, tangential processes. Immunohistochemically, they can be identified by markers such as Nestin and SOX2, and are basically analogous to the transiently-amplifying progenitors, or Type C cells, in the SVZ. Type 2 cells can be further subdivided into Type 2a and Type 2b cells (Kronenberg et al., 2003). While both of these subtypes are morphologically similar, they differ in their expression of certain neural precursor markers. For example, Type 2b cells express markers characteristic of neuroblasts, including DCX, PSA-NCAM and NeuroD, which is indicative of a specification for a neuronal phenotype (Brandt et al., 2003; Brown et al., 2003b; Filippov et al., 2003; Francis et al., 1999; Kempermann et al., 2004a; Kronenberg et al., 2003; Rao and Shetty, 2004; Seki, 2002).

The final type of neural precursor in the hippocampus is the Type 3 cell. It derives from the Type 2 cell and is analogous to the proliferating Type A cell in the SVZ. The primary difference between Type 2a and Type 3 cells is that Type 3 cells no longer express Nestin. However, they can still be identified by their expression of DCX, PSA-NCAM and NeuroD. It is during the Type 3 stage that cells become increasingly differentiated and begin to migrate from the germinal zone into the inner third of the granule cell layer (Brown et al., 2003b; Kempermann et al., 2004a).

Cells up to and including the Type 3 cell all have a limited capacity for self-renewal and will proliferate to some degree. Moreover, while somewhat lineage-determined, the Type 1, 2 and 3 cells can change their lineage and eventual phenotype. At a certain point, the majority of Type 3 cells will exit the cell cycle, will become post mitotic and will begin maturing into granule cell neurons (Ambrogini et al., 2004). This period is marked by continuing expression of DCX and, towards its later stages, expression of Calretinin (Brandt et al., 2003; Brown et al., 2003b). It is hypothesized that, during this period, newly-formed cells send out their axons and will begin making functional connections with neurons in the CA3 layers (Hastings and Gould, 1999; Kempermann et al., 2004a). Consequently, it is during this process of maturation and functional integration that the cells will be recruited for survival, which eventually leads to a process of terminal differentiation and elimination of unnecessary or surplus cells (Biebl et al., 2000; Kempermann et al., 2003; Young et al., 1999).

Two to three weeks after becoming post-mitotic, newly-formed cells will stop expressing PSA-NCAM, NeuroD and DCX, and will begin expressing NeuN. This, along with the substitution of Calbindin for Calretinin, marks their transition to more or less mature neurons (Ambrogini et al., 2004). Over a period of four to seven weeks, these new cells will begin to integrate functionally into the hippocampal network and with the population of local neurons, and will become almost entirely indistinguishable from other glutamatergic granule cells (Carlen et al., 2002; Jessberger and Kempermann, 2003; van Praag et al., 2002). Notably, newly-formed neurons of the granule cell layer have a lower threshold for LTP and display a higher propensity for synaptic plasticity than their older, existing counterparts (Schmidt-Hieber et al., 2004; Wang et al., 2000).

4.3 Functions of adult neurogenesis in the hippocampus

Given that the dentate gyrus forms an important interface between parahippocampal structures and the hippocampus, the fact that new neurons are continuously being added to the DG has important implications for the function of the hippocampus. As such, neurogenesis in the dentate gyrus has been linked to diverse cognitive and functional properties of the hippocampus, including a role in learning, short-term memory formation/consolidation, in the processing of spatial memory and in diverse behaviors such as anxiety and fear conditioning. Research into the functions of hippocampal neurogenesis is still a nascent field of study and so little is known about the actual functions of hippocampal neurogenesis. Many of the studies on this subject employ behavioral tests or computational modeling, both of which have their limitations. However, some important progress has been made and some insight has been provided on the functional implications of this phenomenon (Deng et al., 2010; Kempermann, 2008; Kempermann et al., 2004b; Schinder and Gage, 2004).

With respect to the physiological functions of the hippocampus, hippocampus-dependent learning (e.g., learning and remembering where objects are located) is positively correlated with increased survival and maturation of neuroblasts and new neurons, but does not seem to affect proliferation (Epp et al., 2007; Gould et al., 1999a; Gould et al., 1999c; Leuner et al., 2004). Interestingly, some tasks that depend on spatial learning, such as the Morris water maze, are associated with increased neurogenesis and simultaneously increased apoptosis, which suggests that some turnover of neurons is required in the normal spatial learning process (Dobrossy et al., 2003; Dupret et al., 2007; Dupret et al., 2008). Moreover, an increased pool of new neurons

(produced by a voluntary wheel running paradigm) is correlated with improved performance on the Morris water maze (van Praag et al., 1999a; van Praag et al., 2005). In support of these findings, simply activating the local hippocampal networks via stimulation of the excitatory perforant path (which mimics normal processes that occur with hippocampal activation) is sufficient to drive an increase in proliferation and in neurogenesis (Bruel-Jungerman et al., 2006; Chun et al., 2006; Madsen et al., 2000; Malberg et al., 2000).

Some additional, but more controversial information comes from studies that ablate neurogenesis to determine its behavioral and cognitive functions. The principal controversy arises because of the variability and mixed specificity of using biochemical or transgenic approaches to ablate neurogenesis, and also because of differences in assessing behavioral or cognitive effects. Consequently, there are mixed and sometimes contradicting data about the behavioral/cognitive consequences of decreased or ablated neurogenesis. However, some conclusions can still be drawn from these studies, namely that hippocampal neurogenesis may be important for contextual fear conditioning (Hernandez-Rabaza et al., 2009; Snyder et al., 2009; Warner-Schmidt et al., 2008; Winocur et al., 2006), spatial discrimination (Clelland et al., 2009) , object recognition memory (Jessberger et al., 2009) and spatial memory (Dupret et al., 2008; Farioli-Vecchioli et al., 2008; Garthe et al., 2009; Jessberger et al., 2009; Zhang et al., 2008).

Because of the unique architecture of the hippocampus, researchers have long sought to understand its computational function in cognition (Marr, 1971). As an example, there is a growing body of research on the computational role of the hippocampus in pattern separation (i.e., distinguishing between single and overlapping stimuli) (Bakker et al., 2008; Leutgeb et al., 2007; Leutgeb and Leutgeb, 2007; McHugh et al., 2007; O'Reilly and McClelland, 1994; Rolls,

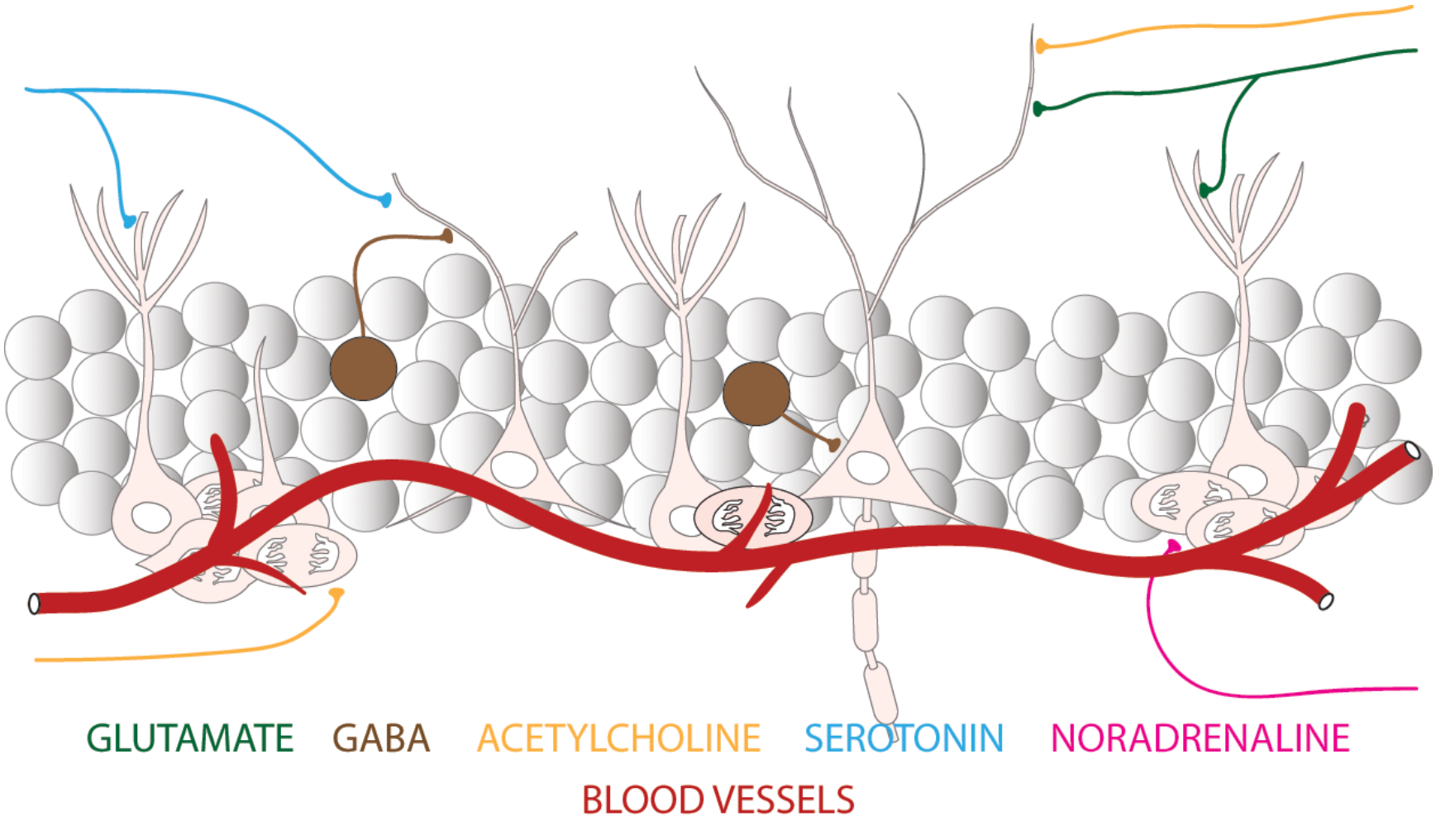
1996; Treves and Rolls, 1992). With the advent of more powerful computers and software platforms, accurate computational modeling of hippocampal function has become possible. Consequently, using computational modeling can provide important insight into the theoretical functions of hippocampal neurogenesis. By integrating hippocampal neurogenesis into computational hippocampal models, recent research has shown that neurogenesis is an important contributor to or facilitator of such functions as pattern separation, memory formation and its related temporal information (Aimone et al., 2006; Aimone et al., 2009; Becker, 2005; Becker and Wojtowicz, 2007; Chambers et al., 2004; Crick and Miranker, 2006; Weisz and Argibay, 2009; Wiskott et al., 2006).

5. Molecular and physiological mediators of adult neurogenesis

Adult neurogenesis changes readily in response to external stimuli and and pathology. Consequently, there must be a system of biochemical and/or physiological mediators that govern the dynamics of neurogenesis itself. Because the field of adult neurogenesis is still in its infancy, these mechanisms are not yet fully characterized and remains poorly understood. However, there is a sizable volume of work on the regulation of neurogenesis that provides some insight into its possible mechanisms (Balu and Lucki, 2009).

The niche, or microenvironment, in which stem cells, neural progenitors and neuroblasts reside is of utmost importance to the regulation of their activity. In fact, under the appropriate conditions, cells from most of the CNS can be stimulated to divide and form neurons in vitro (Palmer et al., 1999; Palmer et al., 1995). However, only those cells residing within a special microenvironment will divide and form neurons in vivo. Conceptually, the niche forms the interface between the extrinsic and intrinsic mediators of neurogenesis and the neural precursors themselves. For the purposes of this thesis, it is important to outline how vasculature, innervation, secreted factors and neurotransmitters - all constituents of the "niche" - contribute to the regulation of neurogenesis, as they may have important implications in the activity-dependent mediation of neurogenesis (FIGURE 5.1).

FIGURE 5.1 (FOLLOWING PAGE) A schematic representation of the regulatory components of the hippocampal neurogenic niche. Shown are the diverse innervating axons and neurotransmitter systems as well as extant and developing vasculature. Note the clustering of proliferating neural precursors in close proximity to vasculature as well as the concomitant budding and development of new vasculature. © M. Bednarczyk


GLUTAMATE
GABA
ACETYLCHOLINE
SEROTONIN
NORADRENALINE
BLOOD VESSELS

Vasculature and secreted factors

One can use the example of CNS neoplasia to illustrate how important vasculature is to maintaining proliferation and the growth of new structures. Indeed, CNS tumors are highly (perhaps "overly") vascularized and some treatment strategies target their vasculature in an attempt to mitigate their growth. Similarly, neural stem cells and precursors in both the hippocampus and SVZ depend on the influence of vasculature for their proliferation, growth and survival.

Neural precursors often reside in close proximity to blood vessels and capillaries (Fabel et al., 2003b; Palmer et al., 2000; Shen et al., 2008). Many of the early progenitor or stem cells in both neurogenic regions will directly contact the vasculature, forming an interface between vascular factors and the process of neurogenesis via the neurogenic lineage. Interestingly, the proliferation of neural precursors and of vasculature often occurs concomitantly (Hellsten et al., 2004; Palmer et al., 2000), suggesting that elaboration of existing vascular networks is required to support increased neural precursor proliferation. This is further evidenced by the fact that Vascular Endothelial Growth Factor (VEGF) promotes not only angiogenesis but also promotes neural precursor proliferation in neurogenic systems (Cao et al., 2004; Jin et al., 2002; Schanzer et al., 2004). Importantly, the vasculature provides a means of entry for diverse secreted factors into the neurogenic niche. These secreted factors can then influence diverse aspects of the neurogenic process, ranging from proliferation to the survival of newly-formed cells.

In addition to VEGF, other trophic factors directly influence adult neurogenesis, such as EGF, FGF2, IGF-1 and BDNF. It is important to consider how these diverse factors affect

neurogenesis because they are produced in response to different intrinsic and external stimuli and, therefore, might be directly responsible for the changes in neurogenesis seen with physical activity.

The influence of epidermal growth factor (EGF) was first demonstrated by Reynolds and Weiss in being essential for the growth of neurospheres in vitro (Reynolds and Weiss, 1992). The actions of EGF, however, are not similar in the SVZ and hippocampus. For example, EGF does not appear to stimulate hippocampal precursors in vivo and in vitro (Kuhn et al., 1997; Palmer et al., 1995), while neural precursors in the SVZ and olfactory system react favorably to EGF (Gonzalez-Perez and Quinones-Hinojosa, 2010; Gonzalez-Perez et al., 2009; Kuhn et al., 1997). Fibroblast Growth Factor 2 (FGF2) is another mitogenic factor whose activity parallels or approximates that of EGF. However, FGF2 is a potent stimulator of proliferation in both the SVZ and Hippocampus (Kuhn et al., 1997; Palmer et al., 1999; Palmer et al., 1995; Rai et al., 2007; Zhao et al., 2007). Unlike EGF and FGF2, which are secreted primarily by cells in the germinal CNS microenvironments, Insulin-like Growth Factor-1 (IGF-1) is a systemic growth factor that arrives in the CNS via the vasculature. IGF-1 has been shown to potently increase neurogenesis in the SVZ and hippocampus, via either intracranial or peripheral administration (Aberg, 2010; Aberg et al., 2000; Aberg et al., 2003; Hurtado-Chong et al., 2009; Lichtenwalner et al., 2001; Trejo et al., 2008). Moreover, because systemic secretion of IGF-1 depends on growth hormone (GH), administration of GH into the periphery will also stimulate neurogenesis (Aberg et al., 2009; Barlind et al., 2010; Johansson et al., 2008). IGF-1 may act principally by stimulating Brain-Derived Neurotrophic Factor (BDNF), which, on its own, has neurogenesis-stimulating properties in some neurogenic regions (Galvao et al., 2008; Lee et al., 2002; Pencea et al., 2001;

Sairanen et al., 2005; Scharfman et al., 2005). It is also interesting to note that the actions of BDNF may underlie diverse processes in neuroplasticity, such as the induction of LTP (Korte et al., 1996a; Korte et al., 1996b; Minichiello et al., 2002).

There are other secreted factors that modulate neurogenesis that do not belong to the trophic or neurotrophic families. The two most influential types are the corticosteroids and the sex hormones (i.e., estrogen and testosterone). Corticosteroids are secreted systemically in response to stress by the adrenal cortex. As such, they have been implicated in stress-mediated changes in neurogenesis in the SVZ and the hippocampus (Lau et al., 2007; Qiu et al., 2007). For example, by performing an adrenalectomy, it is possible to potently increase neurogenesis in the hippocampus (Cameron and Gould, 1994; Cameron and McKay, 1999; Cameron et al., 1998; Montaron et al., 1999; Montaron et al., 2003). Conversely, stimulating corticosteroid secretion via stress results in decreased hippocampal neurogenesis (Ekstrand et al., 2008b; Gould et al., 1997). Sex hormones also have profound effects on adult neurogenesis. For example, both higher levels of endogenous estrogen and systemic administration of estradiol have been shown to modulate proliferation and neurogenesis in the hippocampus of rats (Barha et al., 2009; Barker and Galea, 2008; Lagace et al., 2007; Ormerod et al., 2003; Tanapat et al., 1999). Interestingly, factors whose secretion is mediated by estrogen levels, such as prolactin, also directly influence neurogenesis independent of estrogen activity (Shingo et al., 2003). In a manner similar to estrogen, both high endogenous levels and peripheral administration of testosterone have been shown to potentiate hippocampal neurogenesis (Brannvall et al., 2005; Spritzer and Galea, 2007).

Innervating axons and neurotransmitter systems

The neurogenic niche of the hippocampus is densely innervated by axons originating from diverse CNS structures and neurotransmitter systems, namely the glutamatergic, GABAergic, acetylcholinergic, serotonergic, noradrenergic and, to a lesser extent, the dopaminergic (Kempermann, 2006). The SVZ niche, on the other hand, is only sparsely innervated when compared to the hippocampus. It is primarily innervated (although indirectly) by dopaminergic neurons of the mesostriatal system (Freundlieb et al., 2006). There is some evidence, however, that other neurotransmitter systems, such as the serotonergic, may influence neural precursors in the RMS (Diaz et al., 2009).

It is important to underline that different innervating axons and neurotransmitter system form an integral part of the regulatory mechanisms of the neurogenic niches and contribute to mediating or stimulating neurogenesis. The fact that neurogenesis is subject to regulation by innervating neurons and neurotransmitter systems provides a mechanism by which environmental, cognitive or behavioral stimuli can influence neurogenesis.

Glutamate

The principal excitatory input to the dentate gyrus comes from the perforant path originating in the entorhinal cortex. It is composed almost entirely of the axons of glutamatergic neurons, whose synapses contact dendrites and processes in the molecular layer. Glutamate acts directly on NMDA receptors to induce depolarization in post-synaptic cells and, consequently,

influences many forms of neuroplasticity, including neurogenesis. For example, when the perforant path is lesioned, or when NMDA receptors are blocked, glutamatergic influence is decreased and there is a marked increase in hippocampal proliferation and, sometimes, neurogenesis (Arvidsson et al., 2001; Bernabeu and Sharp, 2000; Cameron et al., 1995; Cameron et al., 1998; Gama Sosa et al., 2004; Gould et al., 1994; Nacher et al., 2001; Okuyama et al., 2004). Kainic acid (KA) receptors are another class of glutamatergic receptors whose activity influences adult neurogenesis. However, unlike with glutamate and NMDA receptors, activation of KA receptors induces large increases in proliferation and neurogenesis (Gray and Sundstrom, 1998; Parent et al., 1997). Interestingly, KA stimulation is often used as a form of kindling when studying temporal-lobe epilepsy and its epileptogenesis. Although KA increases proliferation and neurogenesis, the majority of newly-formed neurons and neuroblasts then become ectopic both in their localization and in their orientation (Parent et al., 1997), which might be one of the factors that contributes to epileptogenesis (Parent, 2002).

GABA

It stands to reason that the system of inhibitory (GABAergic) neurons in the hippocampal dentate gyrus could also serve in the regulation of neurogenesis. Newly-formed neurons in the hippocampus use GABA as an excitatory neurotransmitter, and GABA signaling could mediate their activity-dependent integration into functional hippocampal networks (Deisseroth et al., 2004; Ge et al., 2006; Markwardt and Overstreet-Wadiche, 2008; Owens and Kriegstein, 2002). However, comparatively little is known about the influence of GABA in the neurogenic process, which is largely due to the complexity of GABAergic innervation. Because GABAergic

interneurons are located throughout the dentate gyrus and adjacent structures, it is impossible to perform lesion studies to evaluate the effects of GABA suppression on neurogenesis. Consequently, the few extant studies that address the question of whether or not GABA neurotransmission influences neurogenesis rely on blockade or suppression of GABA receptors via pharmaceuticals or transgenic animals, which is, at best, only an indirect measure of the influence of GABA. Still, use of a GABA antagonist has been shown to increase proliferation (and seizure activity) in the dentate gyrus (Jiang et al., 2003), while heterozygous deletion of the gamma-2 subunit of the GABA receptor (which weakens the response of the receptor) results in decreased survival and differentiation of newly-formed cells (Earnheart et al., 2007).

Acetylcholine

Acetylcholine is the principal modulatory neurotransmitter in the CNS. The dentate gyrus is innervated by acetylcholinergic neurons located in the septum and the nucleus basalis of Meynert, whose neurons form synapses primarily with dendrites and processes in the molecular layer of the dentate gyrus. Consequently, acetylcholine is implicated in diverse plasticity-related phenomena in the hippocampus, such as synaptic plasticity and, potentially, neurogenesis. Lesioning of the septo-hippocampal tract (the primary bundle of afferent acetylcholinergic axons to the dentate gyrus) does not seem to affect hippocampal proliferation but decreases overall neurogenesis and cell survival (Cooper-Kuhn et al., 2004). This finding is supported by the fact that administration of donepezil, an acetylcholine esterase inhibitor (which increases levels of

acetylcholine), improves the survival of newly-formed neurons (Kaneko et al., 2006; Kotani et al., 2006).

Serotonin

Serotonin is another important neurotransmitter that has been implicated in the regulation of neurogenesis. Serotonergic innervation to the hippocampus originates in the raphe nuclei, and pervades structures in the molecular layer as well as at the interface of the hilus and the granule cell layer. Thus, serotonin can directly influence neural precursors in the germinal subgranular zone. It is also possible, though still unproven, that serotonin can influence forebrain neurogenesis, as there is some serotonergic innervation in the RMS (Diaz et al., 2009) though not in the SVZ proper. Consequently, increasing the concentration of serotonin in the hippocampus, via inhibition of serotonin re-uptake transporters, greatly increases levels of neural precursor proliferation in the hippocampus, but does not positively impact cell survival or differentiation (Banasr et al., 2004; Santarelli et al., 2003; Schmitt et al., 2007). This finding is particularly important because pharmaceuticals that block the re-uptake of serotonin are used for the treatment of depression, whose etiology might include perturbations in hippocampal neurogenesis. Conversely, when serotonin is depleted in the hippocampus, via lesioning of serotonergic afferents or via the administration of 5-HT receptor antagonists, there is a decrease in the proliferation of neural precursors in the dentate gyrus and the SVZ (Brezun and Daszuta, 1999; Jha et al., 2006; Radley and Jacobs, 2002).

Noradrenaline and Dopamine

The dopaminergic innervation of the hippocampus originates from the substantia nigra and the ventral tegmental area. In the forebrain, the striatum is a major target of the dopaminergic innervation from the substantia nigra. For this reason, it is likely that the adjacent SVZ also receives some dopaminergic innervation (Freundlieb et al., 2006). By comparison, the dopaminergic innervation of the dentate gyrus proper is relatively weak. However, manipulation of dopaminergic neurotransmission can still influence neurogenesis in both the hippocampus and SVZ. Depletion or blockade of the action of dopamine, via dopaminergic lesioning or cocaine administration, has been shown to decrease levels of proliferation in the hippocampus as well as the SVZ (Dominguez-Escriba et al., 2006; Hoglinger et al., 2004). Moreover, treatment with dopamine receptor agonists has been shown to upregulate neurogenesis in the SVZ (O'Keeffe et al., 2009a; O'Keeffe et al., 2009b). The noradrenergic innervation of the hippocampus, originating from the locus coeruleus, is not well understood in terms of its contribution to the regulation of adult neurogenesis. However, a number of recent studies have demonstrated that noradrenaline can activate, and may be required for activity-dependent increases in the activity of hippocampal neural precursors (Jhaveri et al., 2010; Kulkarni et al., 2002; Veyrac et al., 2009).

6. Physical activity, environmental enrichment and adult neurogenesis

The greatest contribution of research into the different forms of plasticity is that it provides a mechanism to explain how the environment can directly influence the form and function of the CNS. Interestingly, Hebb was among the first to consider and study the effects of environmental enrichment on CNS function and plasticity. Briefly, by raising rats in different environments, Hebb was able to show that the environment itself had measurable effects on learning and memory (Hebb, 1947; Hymovitch, 1952). Hebb's early work was subsequently validated by a flurry of studies from the group of Ronsenzweig and Bennett in the 1960s, who showed that environmental enrichment directly influences CNS anatomy and physiology (Diamond et al., 1964; Diamond et al., 1972; Krech et al., 1960; Krech et al., 1962; Rosenzweig, 1966; Rosenzweig and Bennett, 1969; Rosenzweig et al., 1967; Rosenzweig et al., 1964; Rosenzweig et al., 1962a; Rosenzweig et al., 1962b).

The first evidence that adult neurogenesis could be stimulated by an animal's physical environment and the activities performed in it came from the work of Fred Gage and colleagues at the Salk Institute in the late 1990s. The idea that external stimuli could influence neurogenesis was largely based on older studies demonstrating that environmental complexity and the like could influence structural dynamics and plasticity in the hippocampus of rodents (Altman and Das, 1964; Cummins et al., 1977; Cummins et al., 1973; Greenough, 1975; Juraska et al., 1985; Juraska et al., 1989; Rosenzweig et al., 1978; Rosenzweig et al., 1962a; Rosenzweig et al., 1962b; Walsh et al., 1969; Walsh et al., 1973). In light of these data, Gage and colleagues used BrdU labeling to assess proliferation in mice living in an "enriched environment," comprising

toys, tunnels, nesting material and, importantly, a running wheel. In their landmark study, (Kempermann et al., 1997) showed that animals that were exposed to such an environment had higher rates of neurogenesis (via survival of newly-formed cells) in the germinal zone of the dentate gyrus, an increased overall number of dentate gyrus granule cells increased and performed better in a simple Morris water maze task. Consequently, the same group expanded their efforts and showed that environmental enrichment could also "rescue" neurogenesis in senescent animals (Kempermann et al., 1998).

Because the increases in neurogenesis that were reported were attributable to the survival of newly-formed cells, it was hypothesized that the actions of trophic factors could account for the observed effects (Craig et al., 1996; Gensburger et al., 1987; Kuhn et al., 1997; Palmer et al., 1995; Ray et al., 1993; Reynolds et al., 1992; Tao et al., 1996). Moreover, the enriched environment used in their previous studies contained a running wheel, which mice were allowed to use *ad libitum*, and running or *exercise* has been associated with increased production, action and effect of circulating factors (Gomez-Pinilla et al., 1997; Gomez-Pinilla et al., 1998; Neeper et al., 1995). Given these facts, in a subsequent study, Gage and colleagues compared the neurogenesis-promoting effects of diverse components of the enriched environment: broad enrichment (as per their previous study), voluntary wheel running, yoked swimming (as a model of forced physical activity), and learning via the Morris water maze task (van Praag et al., 1999b). Remarkably, they found that swimming and learning tasks had no effect on neurogenesis while the enriched environment and wheel running robustly increased neurogenesis. Moreover, unlike environmental enrichment, wheel running was also found to increase proliferation of neural precursors in the germinal zone of the dentate gyrus. In a follow-up study, published later

in the same year, the authors also showed that running animals faired better at learning tasks than their controls and that dentate gyrus granule cells (including newborn neurons) in these animals could produce LTP more readily (van Praag et al., 1999a). In further research into the electrophysiological and functional properties of newly-formed neurons, Gage's group also used retroviral GFP labeling to identify new neurons and assess their electrophysiological properties in control and running mice (van Praag et al., 2002). They found that new neurons in both the control and running groups displayed morphological and electrophysiological properties similar to other, mature granule cells.

In 2003, using tissues that were obtained from one of their more recent studies (van Praag et al., 1999a), Gage's group published another landmark article in the field of environment-mediated neurogenesis. The specific goal of the study was to determine how an enriched environment and voluntary wheel running contributed to the modulation of neurogenesis in the hippocampus and in the SVZ/olfactory system (Brown et al., 2003a). Paralleling their other studies, they found that physical activity (wheel-running) increased proliferation, cell-survival and neurogenesis, while environmental enrichment only increased the survival of newly-formed cells and neurogenesis. Surprisingly, the effects of running and environmental enrichment, which increased neurogenesis robustly in the hippocampus, had no apparent effect in the SVZ. The running paradigm did not increase SVZ proliferation, nor did it result in increased numbers of newly-formed neurons in the olfactory bulb.

The influence of Gage's group on this subfield of research into hippocampal neurogenesis cannot be understated. As a result of their experiments and findings, the modulation of neurogenesis in the hippocampus by physical activity and external stimuli has become well

established and, to some degree, canonized. In the years since these publications, many additional studies have been produced that have elucidated some of the finer or more controversial questions about the subject. That being said, the field is still very much in its infancy and there is much research to be done in order to find the underlying mechanisms and functions.

OBJECTIVES

The primary, or global, objective of my research is to elucidate how external stimuli contribute to the regulation of hippocampal and forebrain neurogenesis. More specifically, my main focus is on the stimulus to neurogenesis that is provided by physical activity, i.e., wheel-running. Cognizant of the existing body of research on this subject, this thesis aims to address two fundamental problems or questions, namely:

1. Can forebrain neurogenesis, under the appropriate conditions, be stimulated by voluntary wheel running?
    - Hypothesis: Extended periods of physical activity can influence adult neurogenesis in the forebrain and the hippocampus.

2. Does the running environment contribute to running-mediated changes in hippocampal neurogenesis?
    - Hypothesis: Voluntary wheel running mediates neurogenesis through both running-dependent and running-independent stimuli

These two question are addressed by two independent publications which I have co-authored, and are presented here in their entirety. The implications of and finer points of these studies will be addressed in a general discussion that follows the articles.

ARTICLE 1 : Prolonged Voluntary Wheel Running Stimulates Neural Precursors in the Hippocampus and Forebrain of CD1 Mice.

Matthew R. Bednarczyk, Anne Aumont, Simon Décary, Raynald Bergeron and Karl J.L. Fernandes

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PREFACE

As outlined previously, hippocampal neurogenesis is subject to important regulation by both environmental enrichment and wheel-running. In contrast, from one extant study on the matter (Brown et al., 2003a), it would seem that neither wheel running nor environmental enrichment have any influence on SVZ/OB neurogenesis. However, in the Brown et al. study, SVZ proliferation was assessed using a running paradigm of two-week's duration. Given that the effects of exercise, such as increased vasculature, might take longer than two weeks to manifest, it is reasonable to hypothesize that a longer running paradigm could produce changes in forebrain proliferation or neurogenesis.

In order to test this hypothesis, we devised an extended, six-week running paradigm utilizing individually-housed experimental animals. As such, this extended running paradigm would allow us not only to compare running performance with neural precursor activity, but to see if an extended running period has any additional influence on both hippocampal and SVZ neurogenesis.

AUTHOR CONTRIBUTIONS

Experimental conception: M.R.B., K.F., R.B. and A.A.

Tissue Processing: K.F., A.A., M.R.B.

Immunohistochemistry experiments: M.R.B. and A.A.

Running paradigm setup: A.A., S.D. and R.B.

Manuscript: M.R.B. and K.F.

AUTHOR AND EDITOR AUTHORIZATION IS PRESENTED IN ANNEX I

ABSTRACT

Voluntary wheel-running induces a rapid increase in proliferation and neurogenesis by neural precursors present in the adult rodent hippocampus. In contrast, the responses of hippocampal and other central nervous system neural precursors following longer periods of voluntary physical activity are unclear and are an issue of potential relevance to physical rehabilitation programs. We investigated the effects of a prolonged, 6-week voluntary wheel-running paradigm on neural precursors of the CD1 mouse hippocampus and forebrain. Examination of the hippocampus following 6 weeks of running revealed two to three times as many newly born neurons and 60% more proliferating cells when compared with standard-housed control mice. Among running mice, the number of newly born neurons correlated with the total running distance. To establish the effects of wheel-running on hippocampal precursors dividing during later stages of the prolonged running regime, BrdU was administered after 3 weeks of running and the BrdU-retaining cells were analyzed 18 days later. Quantifications revealed that the effects of wheel-running were maintained in late-stage proliferating cells, as running mice had two to three times as many BrdU-retaining cells within the hippocampal dentate gyrus, and these yielded greater proportions of both mature neurons and proliferative cells. The effects of prolonged wheel-running were also detected beyond the hippocampus. Unlike short-term wheel-running, prolonged wheel-running was associated with higher numbers of proliferating cells within the ventral forebrain subventricular region, a site of age-associated decreases in neural precursor proliferation and neurogenesis. Collectively, these findings indicate that (i) prolonged voluntary wheel-running maintains an increased level of hippocampal neurogenesis whose magnitude is linked to total running performance, and (ii) that it influences multiple neural precursor populations of the adult mouse brain

INTRODUCTION

Physical activity has diverse and widespread effects in the adult mammalian central nervous system (CNS). In both human and rodent experimental models, physical activity is associated with antidepressive alterations in mood, improved rehabilitation following physical trauma, prevention of age-related disease and pathology, and enhanced cognitive function involving various forms of learning and memory (Duman et al., 2008; Fabel and Kempermann, 2008; Lazarov et al., 2005; Luo et al., 2007; Ma, 2008; van Praag, 2008). Given the growing incidence of disorders related to mood, age, and cognition, uncovering the mediators and molecular substrates of exercise-induced effects has become an increasingly important therapeutic objective.

Multiple physiological mechanisms may mediate the effects of physical exercise on the CNS. These mechanisms include exercise-induced alterations in circulating hormones and other blood-borne factors (Koehl et al., 2008), increases in tissue vascularization (Ekstrand et al., 2008a), enhanced recruitment and activation of immune cells (Ziv et al., 2006), changes in dendritic structure (Redila and Christie, 2006; Stranahan et al., 2007) and neurophysiological alterations in neurons and their synapses (Farmer et al., 2004), local increases in neurotrophic factor expression (Fabel et al., 2003a; Gomez-Pinilla et al., 1997; Rossi et al., 2006), and the production of new neurons via activation of neural precursor proliferation and neurogenesis (Brown et al., 2003a; Kempermann et al., 2002; Kronenberg et al., 2006; Rhodes et al., 2003; van Praag, 2008; van Praag et al., 1999b; van Praag et al., 2005). With respect to neural precursor activity, adult mice given voluntary access to a running wheel for as little as 3 days show highly significant increases in the total number of proliferating cells and newly born

neurons within the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Kronenberg et al., 2006; van Praag et al., 1999b). These newly born neurons can survive over long periods and will eventually integrate electrophysiologically into the hippocampal network (Toni et al., 2008).

Although the forebrain subventricular zone (SVZ) is the principal reservoir of neural precursors in the adult rodent CNS, the neurogenic effects of short-term wheel-running on neural precursors appears to be specific to the hippocampus (Brown et al., 2003a). In contrast, recent work has shown that inactivity, via hindlimb suspension, can reduce endogenous SVZ proliferation in rats (Yasuhara et al., 2007), while neural precursors transplanted into the SVZ display increased proliferation and migration in response to 4 weeks of enriched environmental conditions (Hicks et al., 2007). These findings suggest that neural precursors outside of the hippocampus may be susceptible to the effects of longer periods of physical activity.

Finding ways of stimulating endogenous CNS neural precursors is an important therapeutic objective. The present study explores the impact of an extended voluntary wheel-running period on neural precursors of the hippocampus and forebrain. In this study, we modified several aspects of a commonly used physical exercise paradigm (voluntary wheel-running), including the mouse strain, the exercise and housing conditions, and the BrdU incorporation strategy. These modifications allowed us to make several relevant observations, including (i) an association between long-term wheel-running performance and the level of hippocampal neurogenesis, and (ii) a detectable effect of prolonged wheel-running on cell proliferation within the ventral forebrain SVZ stem cell niche.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the institutional animal care committee. Twenty adult male CD1 mice (Charles River, Quebec) at 2 months of age were used in the evaluation of the exercise paradigm. Ten mice were used in each experimental series (Series A and B), separated into groups of five running and five control animals. Mice were housed individually in cages equipped only with running wheels and odometers (‘‘running’’ or ‘‘RUN’’ mice) or in cages of comparable dimensions that lacked a running wheel or any other form of environmental enrichment (‘‘control’’ or ‘‘CTL’’ mice). Both series of experimental mice ran voluntarily for a period of 6 weeks (40–41 days) and were supplied with food and water ad libitum. For the BrdU incorporation experiments, mice from Series A received three intraperitoneal injections of BrdU (Sigma, 50 mg/kg) at 3-h intervals 24 h prior to sacrifice. Mice from Series B received a single intraperitoneal injection of BrdU (50 mg/kg) following 23 days of exercise, i.e., 18 days prior to sacrifice.

Tissue Preparation

Mice received a lethal dose of chloral hydrate (7%) and were then perfused transcardially with 25 ml of phosphate-buffered saline (PBS) followed by 40 ml of 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde for 2 h, and then kept in PBS at 4°C until sectioning. The entire forebrain and hippocampus of each animal was cut into 40-µm

coronal sections using a vibrating microtome (Leica VT1000S) and the tissue sections were stored at -20°C in an antifreeze solution (glycerol:ethylene glycol:PBS, 3:3:4).

Immunohistochemistry

The primary antibodies used in these experiments were mouse antihuman Ki67 (1:200, BD Pharmingen), goat antihuman doublecortin (1:250, Santa Cruz Biotechnology, CA), mouse antimouse NeuN (1:200, Chemicon), and rat anti-BrdU (1:200, AbD Serotec, Oxford, UK). For immunohistochemical labeling of Ki67, NeuN, and doublecortin (DCX), free-floating 40-lm sections were washed in PBS (pH 7.4) and blocked in 10% normal goat serum (NGS) in 0.1% Triton-X for 2 h. Sections were then incubated at room temperature overnight in primary antibodies diluted in 5% NGS/0.1% Triton-X in PBS. For BrdU immunohistochemistry, sections were washed in PBS, rinsed in double-distilled water to remove excess buffer, and then treated with 2 N HCl for 40 min at 37°C to denature the DNA strands. The HCl was subsequently washed out in PBS and the sections then blocked for 2 h in 4% bovine serine albumin (BSA) in 0.1% Triton-X in PBS and incubated at room temperature overnight in the rat anti-BrdU antibody alone or in combination with other primary antibodies.

For diaminobenzine (DAB)-based detection of primary antibodies, sections were washed in PBS and then incubated in the appropriate biotinylated secondary antibody (Jackson Immuno Research, PA) for 1.5 h at room temperature, diluted in either 5% NGS (for Ki67, NeuN, DCX) or 2% BSA (BrdU) in 0.1% Triton- X. Following washes, the signal was amplified using the avidin–biotin–peroxidase system (VectaStain ABC Kit, Vector Laboratories) for 1 h and 30 min, and then detected using a DAB-containing solution (0.5 mg/ml DAB, 0.015% H_2O_2, 0.040%

NiCl2 in PBS) for 1–2 min. Sections were mounted onto glass slides, dried overnight, and then dehydrated in a graded series of alcohol baths and coverslipped with Permount (Fischer Scientific). For fluorescence detection of primary antibodies, sections were rinsed and incubated in appropriate secondary antibodies conjugated to either CY3 (1:400, Jackson ImmunoResearch, PA) or Alexa 488 (1:1,000, Molecular Probes, OR) for 1 h at room temperature. They were then washed, mounted on glass slides, and coverslipped with Mowiol antifade solution [13% (w/v) polyvinyl alcohol and 2% (w/v) DABCO in 2:1 Tris–HCl (pH 8.5):Glycerol].

Cell Counts and Statistical Analyses

When using DAB immunohistochemistry, quantifications were performed on every sixth section through the hippocampus from Bregma -1.00 mm (the approximate first appearance of the DG) to Bregma -3.50 mm, thus encompassing a rostro-caudal distance of 2.5 mm. Tissue sections processed using DAB were digitized at 40X magnification using an automated system (NDP Scan, Hamamatsu Photonics K.K.). Digitized images were sorted according to stereotactic coordinates for quantification of spatial distribution. Counts were performed manually using the digitized images and were limited to positive cells within the SGZ/GZ of the DG. Fluorescence signals were imaged in z-stacks at 1-µm intervals using a motorized Olympus IX81 microscope and manually counted from the digital images. Quantifications were performed by an individual who did not have access to the running distance data.

DAB-labeled cells, counted on every sixth section through the hippocampus (10 sections total), were transformed into stereological data by multiplying by six. All counts are expressed as a total number of cells in both hippocampi of each animal, except where otherwise indicated.

For fluorescence labeling, cells were quantified from two to four sections at comparable stereotactic levels across the hippocampus. The total number of BrdU-labeled cells that differentiated into NeuN+ neurons was obtained by multiplying the percentage of BrdU+NeuN+-co-labeled cells by the stereological BrdU cell counts. An estimate of the total DCX+ population was calculated by taking the average number of DCX+ cells per 40-µm section and multiplying by 60 (the total number of sections through the rostro-caudal extent of the hippocampus).

For comprehensive analysis of SVZ proliferation, coronal sections through the striatal SVZ were sorted and grouped according to preselected stereotactic coordinates (Bregma +1.20, +0.80, +0.40, and +0.10 mm). Counts of Ki67+ cells were made over the ventral-most 500-µm portion of the lateral ventricles to standardize the size and location of the regions that were quantified.

Statistical analyses were performed using GraphPad Prism (Mac OS X version 5.0a). Data sets were tested for normality (D'Agostino and Pearson omnibus normality test) and equal variances (F-test), and group averages were calculated as the average total number of cells per animal or section. Quantifications were analyzed primarily with unpaired t-tests, with application of Welch's correction, where variances differed significantly. One-way analysis of variance (ANOVA) with Bonferroni or Dunnet multiple comparison post hoc analysis was used when testing for inter- or intragroup homogeneity. The Pearson correlation test was used to establish correlations between running distances and cell counts. Significance levels were set at $\alpha = 0.05$, two-tailed.

RESULTS

Voluntary Running Model

In two independent series of experiments (i.e., Series A and B), 2-month-old male CD1 mice were randomized and housed individually for an additional 6 weeks in either standard cages (n = 5/experiment) or in running wheel-equipped cages (n = 5/experiment). CD1 mice are an outbred strain of mice chosen for these experiments to maximize inter-animal variation and hence the significance of any potential findings. Series A and B CD1 mice were treated identically except for the timing of BrdU administration. Series A mice received BrdU injections 24 h prior to sacrifice to identify a cohort of cells passing through S-phase of the cell cycle at the end of the running paradigm. Series B mice were injected with a single dose of BrdU 18 days prior to sacrifice (i.e., after 23 days of running) to study the survival and fate of a cohort of cells proliferating at an intermediate time point of the running paradigm. As shown in Figure 1A, running mice from Series A (nos. E1–E5) ran an average of 6.5–7.8 km per calendar day, while those from Series B (nos. E6–E10) ran an average of 3.5–11.4 km per calendar day. Over the 6-week period, the cumulative distances run (‘‘running performance’’) were between 146.0 and 468.0 km. Notably, the average daily running distances for six out of the ten runners increased over the duration of the exercise period while three showed no change and only one decreased (Figs. 1B,C and data not shown), suggesting that the mice did not lose interest in performing the activity over this extended period. For immunohistochemical purposes, brains of Series A and B mice and their standard housed controls were sectioned in the coronal plane through the forebrain lateral ventricles and hippocampus, as shown in Figures 1D–F.

FIGURE 1

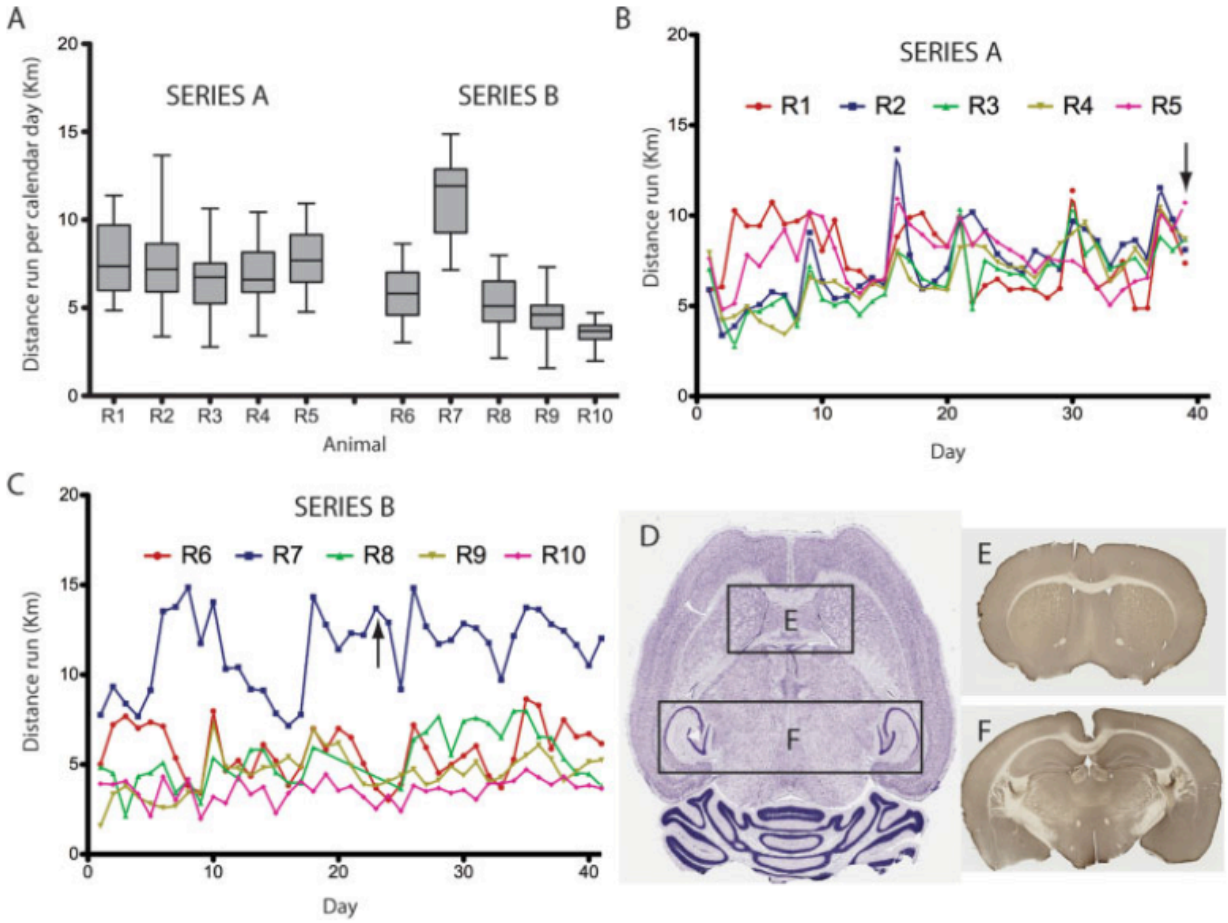


FIGURE 1. Overview of the prolonged voluntary wheel-running paradigm. Two separate series of CD1 mice ran voluntarily for 40–41 days (Series A: R1–R5, Series B: R6–R10). (A) A box and whisker plot (mean + min/max and quartiles) shows that mice from Series A ran an average of 6–8 km/day, while mice from Series B ran an average of 4–12 km/day. (B,C) A day-by-day visual summary shows that animals ran at or near the average for their respective series, with the exception of one outlier (animal no. R7). Arrows denote days on which BrdU was administered to animals in the respective series. (D) The areas of interest for the purposes of this study, outlined in black, include the subventricular zone (SVZ) (E) and the DG of the hippocampus (F). (D) Modified from www.brainmaps.org photo.

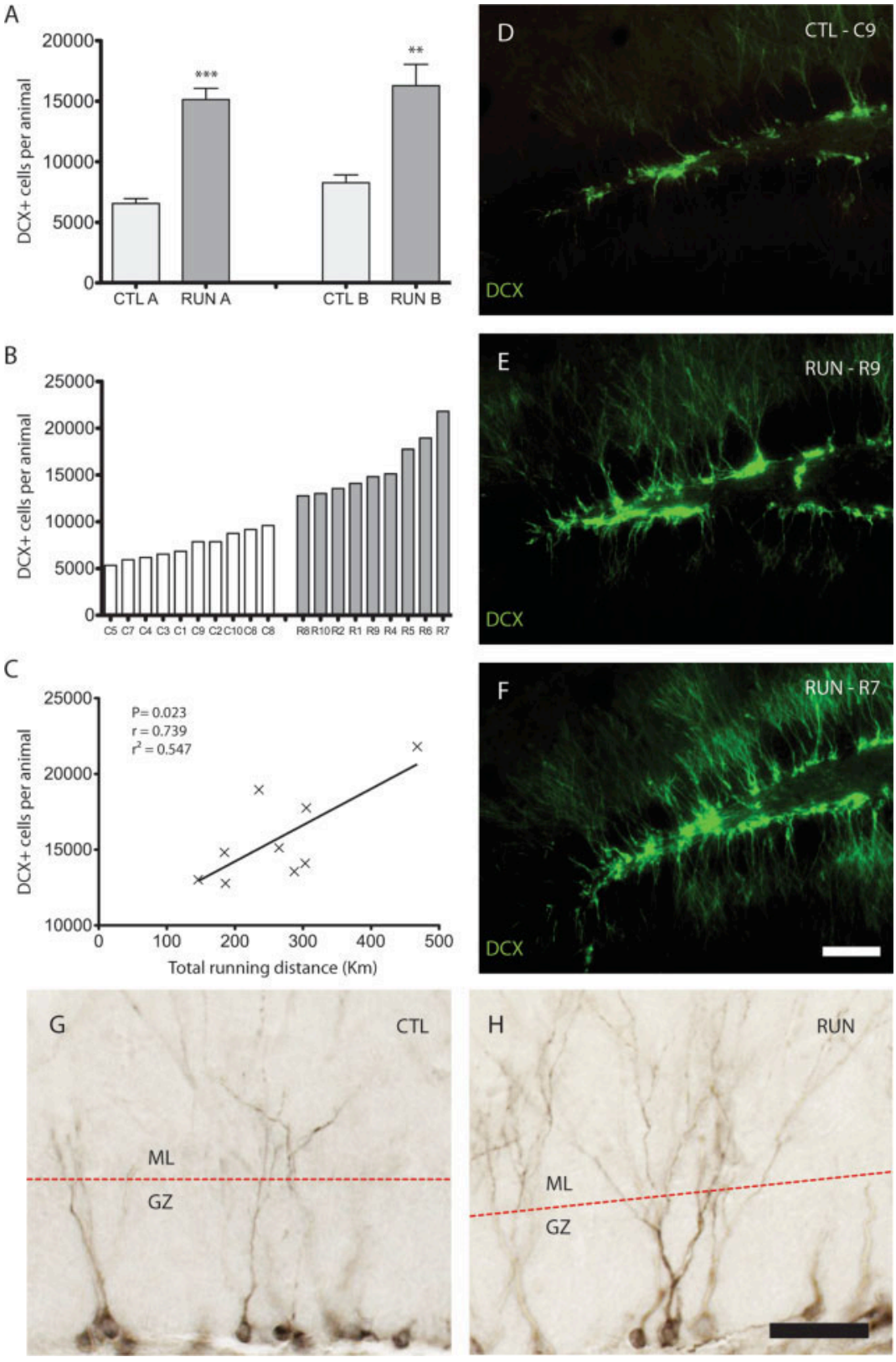
Neurogenesis and Proliferation Remain Strongly Increased in the Hippocampal DG at the End of 6 Weeks of Voluntary Wheel-Running

To evaluate how DG neurogenesis is affected following 6 weeks of voluntary wheel-running, we processed 40-μm sections through the dorsal arm of the hippocampus for fluorescence immunohistochemistry against DCX, a cytoskeletal-associated protein expressed by migratory neuroblasts and newly born neurons. Running mice consistently displayed a strong increase in DCX immunoreactivity within the SGZ of the DG, involving both the number of DCX+ neurons and the apparent density of DCX+ dendrites within the molecular layer (Figs. 2D–H). Stereological quantifications revealed that the number of hippocampal DCX+ neurons/animal increased significantly in both Series A and B (Fig. 2A). The Series A wheel-running group (‘‘RUN’’) averaged 15,135 ± 932.9 standard error of mean (SEM) DCX+ cells, compared to 6,552 ± 412.4 SEM for their control (‘‘CTL’’) group ($P = 0.0001$, unpaired t-test). The Series B running group averaged 16,278 ± 1,772.0 SEM DCX+ cells, compared to 8,268 ± 932.9 SEM for the control group ($P = 0.003$, unpaired t-test). These results were highly statistically significant, and comparison of the data from individual animals surprisingly revealed that even the highest DCX counts from control animals were lower than the lowest counts from the wheel-running group (Fig. 2B). To perform a correlation analysis of running performance versus neurogenesis, we combined the data from the two series as there was no statistical difference between control groups or between wheel-running groups ($P > 0.050$, unpaired t-tests followed by ANOVA with Bonferroni post hoc). Correlation analysis revealed that there was a statistically significant positive correlation between the number of DCX+ neurons per section and the total running performance within the running group ($P = 0.023$, $r = 0.719$, $r^2 = 0.547$) (Fig. 2C). This

correlation is illustrated by comparison of DCX expression in a median control (Fig. 2D), median runner (Fig. 2E), and the highest runner (Fig. 2F). Taken together, these results indicate that hippocampal neurogenesis is highly and reproducibly increased in CD1 mice following this 6-week voluntary wheel-running paradigm.

FIGURE 2. (FOLLOWING PAGE) Hippocampal neurogenesis increases with prolonged voluntary wheel-running. (A) The average number of doublecortin (DCX)-positive cells per animal in control (CTL A, B) and running (RUN A, B) mice from Series A and B. There are higher numbers of DCX+ cells in both Series A and B runners when compared to their control animals (unpaired t-test, one-way ANOVA and Bonferroni post hoc test). (B) Quantifications of DCX+ populations in individual animals from control and wheel-running groups, arranged in ascending order. (C) Correlation analysis of running distance versus total number of DCX+ cells per running animal. The number of DCX+ cells in the running mice was positively correlated with running performance (Pearson correlation test). (D–F) Photomicrographs of DCX fluorescence immunohistochemistry in the hippocampi of the median control ("C9," D), median runner ("R9," E), and the most prolific runner ("R7," F). Note the particularly marked increase in DCX expression in the most prolific runner. (G,H) Visualization of DCX+ cells in the SGZ/GZ using DAB immunohistochemistry. DCX+ cells in runners display greater dendritic complexity in the molecular layer than those of control animals. Dotted line in G and H identifies the border between the granular zone (GZ) and molecular layer (ML). (D–F) Scale bar = 100 μm, (G, H) scale bar = 30 μm; *P ≤ 0.050, **P ≤ 0.010, ***P ≤ 0.001.

FIGURE 2:


A
DCX+ cells per animal
20000
15000
10000
5000
0
***
**
CTL A
RUN A
CTL B
RUN B
B
DCX+ cells per animal
25000
20000
15000
10000
5000
0
C5 C7 C4 C3 C1 C9 C2 C10 C6 C8
R8 R10 R2 R1 R9 R4 R5 R6 R7
C
DCX+ cells per animal
25000
20000
15000
10000
P= 0.023
r = 0.739
r² = 0.547
0
100
200
300
400
500
Total running distance (Km)
D
CTL - C9
DCX
E
RUN - R9
DCX
F
RUN - R7
DCX
G
CTL
ML
GZ
H
RUN
ML
GZ

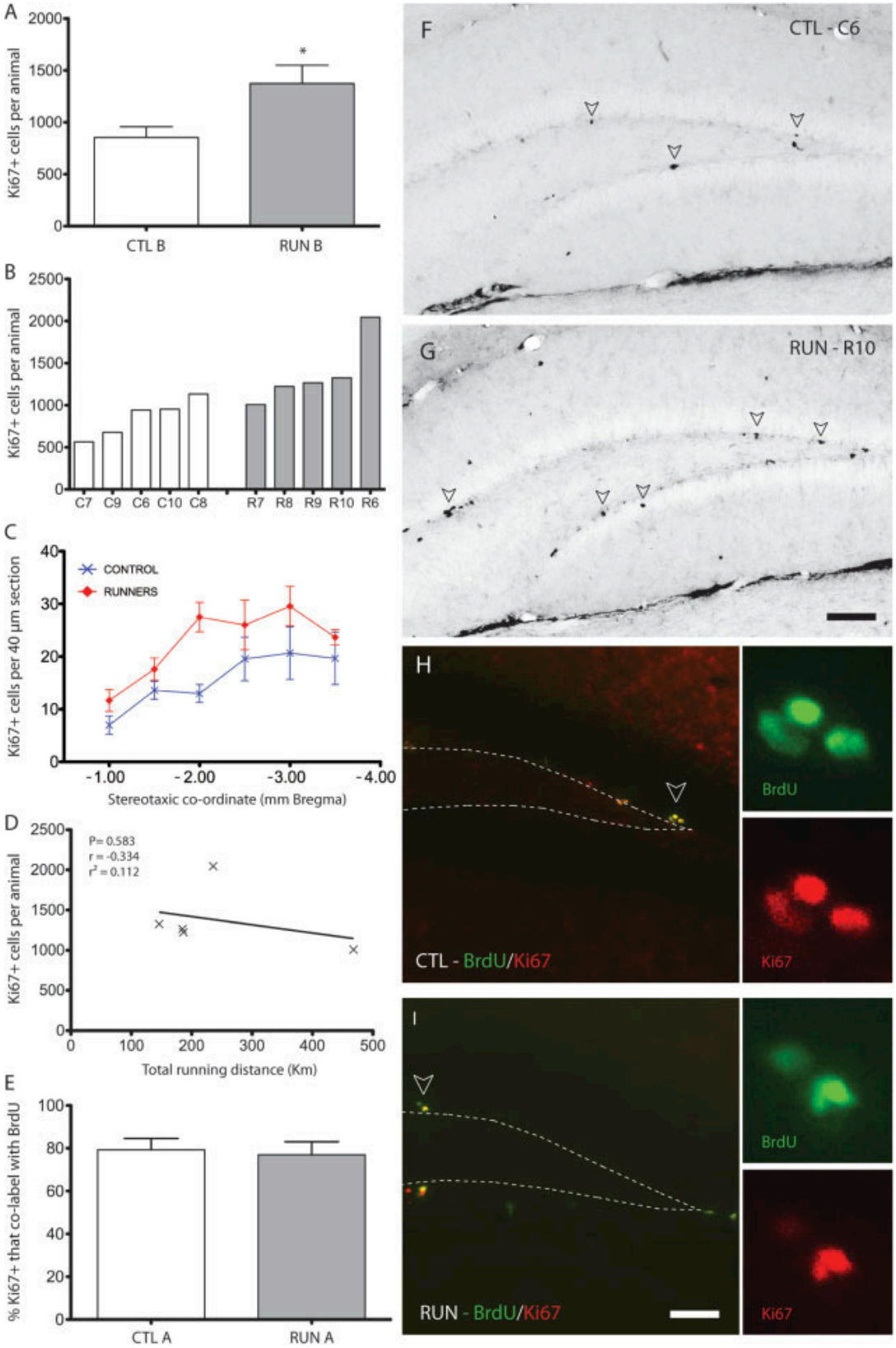
To measure the levels of cell proliferation occurring in these mice following 6 weeks of wheel-running, tissue sections through the hippocampus of Series B mice were processed for DAB immunohistochemistry against Ki67 (Fig. 3), a nuclear protein expressed in mitotically active cells. Quantification of the number of Ki67-expressing cells within the SGZ/GZ indicated that there was an average of 854.4 ± 102.8 SEM cells in control mice versus 1,374 ± 176.4 SEM cells in runners, an increase of 60% ($P = 0.034$, unpaired t-test) (Fig. 3A). Individualized data from exercise and control groups are shown in ascending order in Figure 3B, illustrating the distinct shift to higher proliferation levels in the running mice. Changes in Ki67 expression were predominantly observed within the SGZ and internal granule cell layer of the DG, where hippocampal stem and progenitor cells reside (Kronenberg et al., 2003; Suh et al., 2007). Ki67+ cells were also occasionally found in the hilus or molecular layer, although these were not included in the quantifications for the present purposes. Representative images of Ki67 expression are shown in Figures 3F,G. Comparison of the data along the rostro-caudal stereotactic coordinates illustrates that Ki67 expression was increased over the entire 2.5-mm segment of the hippocampus that was studied (Fig. 3C). However, unlike with DCX, no positive correlation was found between the magnitude of this increase and total running performance ($P = 0.583$, $r = 20.334$, $r^2 = 0.112$) (Fig. 3D). To the contrary, among the runners, the highest runner (no. R7) actually yielded the lowest average Ki67 counts (Fig. 3B).

Since changes in cell cycle duration in mammalian cells are predominantly the result of changes in the length of the G1 phase of the cell cycle (DiSalvo et al., 1995), changes in overall cell cycle length can be detected as corresponding increases or decreases in the proportion of proliferating cells that are in S-phase. To assess the possibility that the increased number of

proliferating cells observed in runners is due to an accelerated cell cycle speed, we measured the proportion of Ki67+ cells undergoing DNA synthesis (i.e., is in S-phase) by performing double-label fluorescence immunohistochemistry for Ki67 and BrdU in the Series A mice that received three BrdU injections 1 day prior to sacrifice. A total of 60–120 Ki67+ cells were quantified from each of three controls and three runners. Consistent with the findings from the Series B mice (Figs. 3A–D), mice from the Series A running group had more Ki67+ proliferating cells than their control group (data not shown). However, quantification revealed that there was no detectable difference in the percentage of Ki67+ cells that were BrdU-labeled (Figs. 3E,H,I). This suggests that there are no sustained changes in cell cycle length associated with the major changes in hippocampal neurogenesis and proliferation measured following 6 weeks of prolonged voluntary wheel-running.

FIGURE 3. (FOLLOWING PAGE) Prolonged voluntary wheel-running increases cell proliferation in the hippocampus. (A) Quantification of the total number of Ki67+ proliferating cells in the DG of Series B mice. Running animals have a significantly higher average number of proliferating cells (unpaired t-test). (B) Quantifications of Ki67+ populations in individual animals from control and wheel-running groups, arranged in ascending order. (C) Rostro-caudal distribution of Ki67+ cells in control and running mice. There is a uniform increase in Ki67+ expression across all stereotactic levels. (D) Correlation analysis of running distance versus total number of Ki67+ cells per running animal. The number of Ki67+ cells in the running mice was not correlated with running performance (Pearson correlation test). (E) Quantification of the percentage of Ki67+ cells that have incorporated BrdU 1 day after BrdU administration. There is no significant difference in BrdU/Ki67 co-labeling percentage between control and running animals (unpaired t-test), suggesting similar cell cycle lengths. (F,G) Photomicrographs of Ki67-expressing cells in the DG of control and running mice (arrowheads). (H,I) Fluorescence immunohistochemical visualization of Ki67 and BrdU colabeling 1 day after BrdU injection. The majority of Ki67+ cells are colabeled with BrdU in control and running mice. Insets show higher magnification images of individual BrdU and Ki67 channels for selected cells (arrowheads). (F, G) Scale bar = 100 μm, (H, I) scale bar = 100 μm; *P ≤ 0.050, **P ≤ 0.010, ***P ≤ 0.001.

FIGURE 3:


A
Ki67+ cells per animal
2000
1500
1000
500
0
*
CTL B
RUN B
B
Ki67+ cells per animal
2500
2000
1500
1000
500
0
C7 C9 C6 C10 C8
R7 R8 R9 R10 R6
C
Ki67+ cells per 40 µm section
40
30
20
10
0
CONTROL
RUNNERS
-1.00
-2.00
-3.00
-4.00
Stereotaxic co-ordinate (mm Bregma)
D
Ki67+ cells per animal
2500
2000
1500
1000
500
0
P= 0.583
r = -0.334
r² = 0.112
0
100
200
300
400
500
Total running distance (Km)
E
% Ki67+ that co-label with BrdU
100
80
60
40
20
0
CTL A
RUN A
F
CTL - C6
G
RUN - R10
H
BrdU
CTL - BrdU/Ki67
Ki67
I
BrdU
RUN - BrdU/Ki67
Ki67

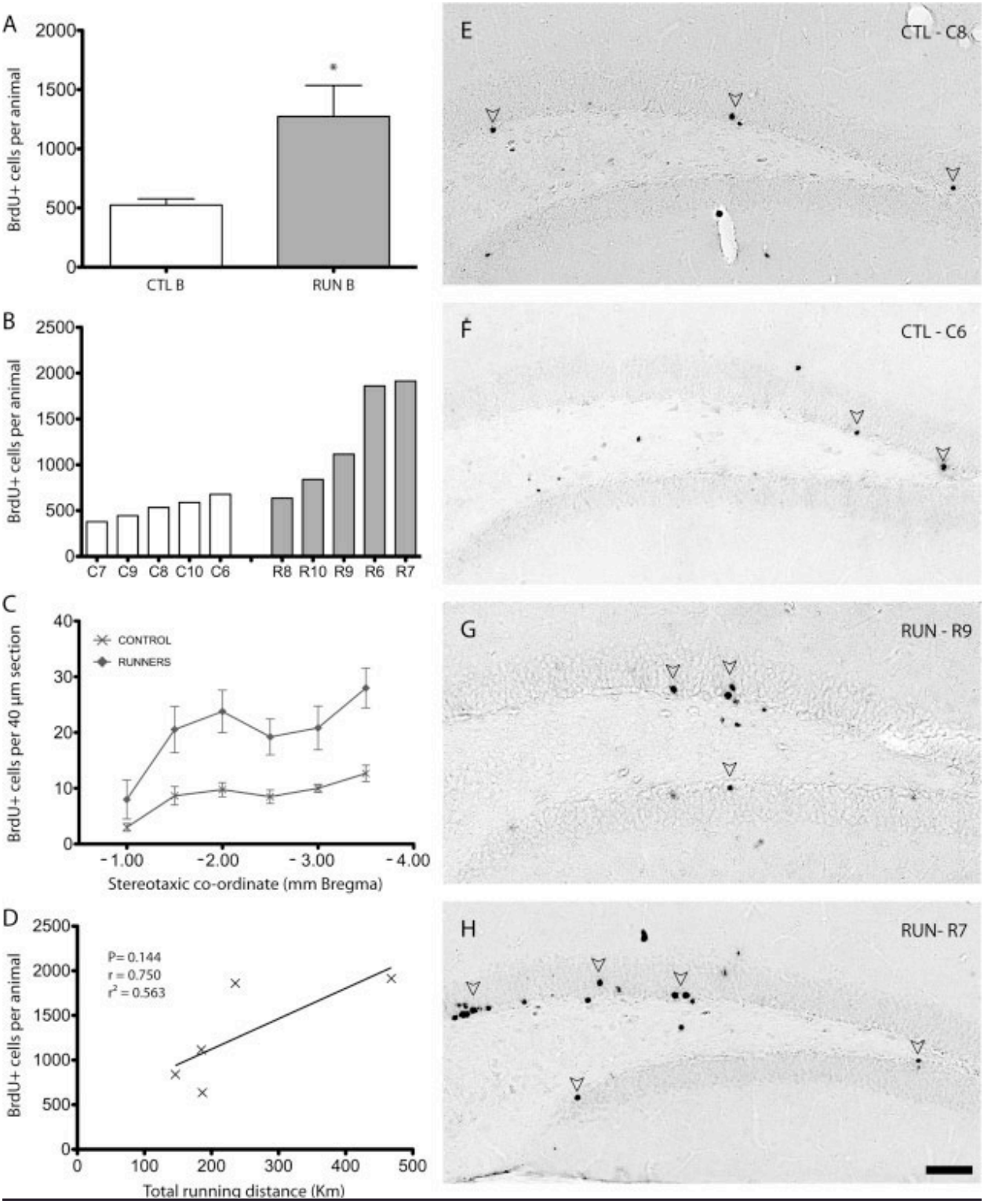
Hippocampal Neural Precursors Dividing During the Latter Stage of the 6-Week Wheel-Running Paradigm Remain Biased Toward Proliferation and Neuronal Differentiation

DCX is reported to be expressed for 2–3 weeks in newborn neurons (Brown et al., 2003b) suggesting that the DCX-expressing cells observed at the end of the 6-week running period (above) were born during the latter half of the wheel-running period. To more conclusively identify the effects of voluntary running on the fate of cells proliferating at the latter stage of the 6-week exercise paradigm, we examined the BrdU-retaining cell population in Series B mice, which had been injected with BrdU after 3 weeks of running and sacrificed 18 days later (Fig. 4). Following sacrifice, BrdU-labeled cells were detected using DAB immunohistochemistry. Quantification revealed that the average number of BrdU+ cells in the hippocampi of each animal increased from 524.4 ± 52.74 SEM in control mice to 1,273 ± 262.0 SEM cells in running mice ($P = 0.049$, unpaired t-test with Welch's correction; $P = 0.010$, F-test) (Fig. 4A). Examination of the individualized animal data showed that the relatively constant number of BrdU-positive cells in control mice increased two to three fold in the wheel-running group (Fig. 4B). This increase was detected along the entire 2.5-mm rostro-caudal region of the dorsal hippocampus (Fig. 4C). Sections from the highest running Series B mice (nos. R7 and R6) also contained the highest number of BrdU+ cells (Fig. 4B). Among the running mice, there was a tendency toward a positive correlation between running performance and BrdU counts ($P = 0.144$, $r = 0.750$, $r^2 = 0.563$). While this did not reach a 5% statistical significance level with only five animals, the relatively low P-value suggests that analysis of a larger sample size could confirm this correlation. Representative photos of the DG of the median and highest BrdU+ animals from both the control and wheel-running groups are shown in Figures 4E–H, and

illustrate the more numerous BrdU+ cells in the runners. In all mice, BrdU labeling was still predominantly found within the DG, SGZ and internal layer of the GZ.

FIGURE 4. (FOLLOWING PAGE) Hippocampal late-stage mitotic cells produce larger numbers of 18-day BrdU-retaining cells in running animals. (A) Quantification of the total number of BrdU-retaining cells in the DG of Series B mice. There is a significant increase in the number of BrdU-retaining cells labeled 18 days prior to sacrifice (unpaired t-test). (B) Quantifications of BrdU-retaining cell populations in individual animals from control and wheel-running groups, arranged in ascending order. (C) Rostro-caudal distribution of BrdU-retaining cells in control and running mice. There is a uniform increase in the number of BrdU-retaining cells across all stereotactic levels. (D) Correlation analysis of running distance versus total number of BrdU-retaining cells per running animal. A trend toward a positive correlation is observed, although statistical significance is not reached (Pearson correlation test). (E–H) Photomicrographs of BrdU-retaining cells in the DG of control (E,F) and running (G,H) mice (arrowheads). Scale bar = 100 µm, *P ≤ 0.050, **P ≤ 0.010, ***P ≤ 0.001.

FIGURE 4:


A
BrdU+ cells per animal
2000
1500
1000
500
0
*
CTL B
RUN B
B
BrdU+ cells per animal
2500
2000
1500
1000
500
0
C7 C9 C8 C10 C6
R8 R10 R9 R6 R7
C
BrdU+ cells per 40 µm section
40
30
20
10
0
CONTROL
RUNNERS
-1.00
-2.00
-3.00
-4.00
Stereotaxic co-ordinate (mm Bregma)
D
BrdU+ cells per animal
2500
2000
1500
1000
500
0
P= 0.144
r = 0.750
r² = 0.563
0
100
200
300
400
500
Total running distance (Km)
E
CTL - C8
F
CTL - C6
G
RUN - R9
H
RUN- R7

To determine whether wheel-running altered the proportion of late-stage proliferating cells that differentiated into mature neurons or that remained in a proliferative state, we double-labeled the BrdU-retaining cells with either NeuN or Ki67, respectively (Fig. 5). In sections double-labeled with BrdU and the mature neuronal marker NeuN, a total of 20–42 cells were analyzed from each control mouse and 48–171 cells from each running mouse. As expected, the larger pool of BrdU-retaining cells in runners produced a greater total number of BrdU+/NeuN+ neurons (655.2 ± 133.6 SEM vs. 163.6 ± 34.73 SEM, P = 0.024, unpaired t-test with Welch's correction; P = 0.050, F-test) (Figs. 5A,B). Among the runners, there was a trend toward a higher number of double-labeled cells in the highest performers (P = 0.099, r = 0.806, r^2 = 0.650) (Fig. 5C), reminiscent of the correlation between DCX expression and running performance (Fig. 2C). Notably, normalization of the number of BrdU+NeuN+ cells to the size of the BrdU+ populations revealed that the proportion of BrdU+ cells that differentiated into NeuN+ neurons rose from 33.35 ± 6.963% SEM in control mice to 51.52 ± 1.707% SEM in running mice (P = 0.035, unpaired t-test), indicating an increased bias toward neuronal differentiation/survival (Fig. 5D). Representative fluorescence images are shown in Figures 5F,G.

To assess the proportion of the 18-day BrdU-retaining cells that remained proliferative, sections were double-labeled for BrdU and Ki67 (Fig. 5). The total number of BrdU+ cells quantified from three sections from each animal varied from 15 to 40 for control mice and from 40 to 75 for running mice. Quantifications of the percentage of BrdU+ cells that were Ki67+ are summarized in Figure 5E and reveal that the percentage of BrdU-retaining cells that remain proliferative increased from 18.58 ± 6.37% SEM in control mice to 41.40 ± 6.52% SEM (P = 0.037, unpaired t-test) in the runners. Thus, there is a higher proportion of hippocampal

precursors that continue to remain proliferative 18 days after BrdU incorporation in the runners. Representative fluorescence images are shown in Figures 5H,I. The effects of prolonged wheel-running on late-stage dividing hippocampal neural precursors are summarized in Figure 6.

FIGURE 5. (FOLLOWING PAGE) Prolonged voluntary wheel-running increases neuronal differentiation and long-term proliferation by late-stage mitotic cells. (A) Quantification of the total number of NeuN+ BrdU-retaining cells in the DG of Series B mice. There is a significant increase in the number of neurons produced by late-stage mitotic cells in running animals when compared with controls (unpaired t-test with Welch's correction). (B) Quantifications of NeuN+ BrdU-retaining cell populations in individual animals from control and wheel-running groups, arranged in ascending order. (C) Correlation analysis of running distance versus total number of NeuN+ BrdU-retaining cells per running animal. A trend toward a positive correlation is observed, although statistical significance is not reached (Pearson correlation test). (D) The percentage of BrdU-retaining cells that colabel with NeuN in control and running mice. The proportion of late-stage mitotic cells that differentiate into neurons increases in running mice (unpaired t-test). (E) The percentage of BrdU-retaining cells that co-label with Ki67 in control and running mice. There is a significantly higher proportion of BrdU retaining cells that co-label for Ki67 18 days after BrdU administration (unpaired t-test). (F,G) Representative fluorescence immunohistochemistry photomicrographs of BrdU and NeuN co-labeling in the DG of control (F) and running (G) mice. For each photomicrograph, the area of interest (arrows) is shown at higher magnification in the upper inset, and the corresponding area with BrdU channel removed is shown in the lower inset. The control animal photomicrograph (F) shows an example of a BrdU-retaining cell that does not co-label with NeuN. The running animal photomicrograph (G) shows an example of a double-labeled cell. (H,I) Fluorescence immunohistochemical visualization of BrdU and Ki67 co-localization 18 days after BrdU injection. For each photomicrograph, higher magnification insets of the individual channels for the area of interest (arrowheads) are shown. Of note is the increased proportion of BrdU-retaining cells that co-localize with Ki67 in running mice. (F, G) Scale bar = 50 µm, (H, I) scale bar = 50 µm; *P ≤ 0.050, **P ≤ 0.010, ***P ≤ 0.001.

FIGURE 5:

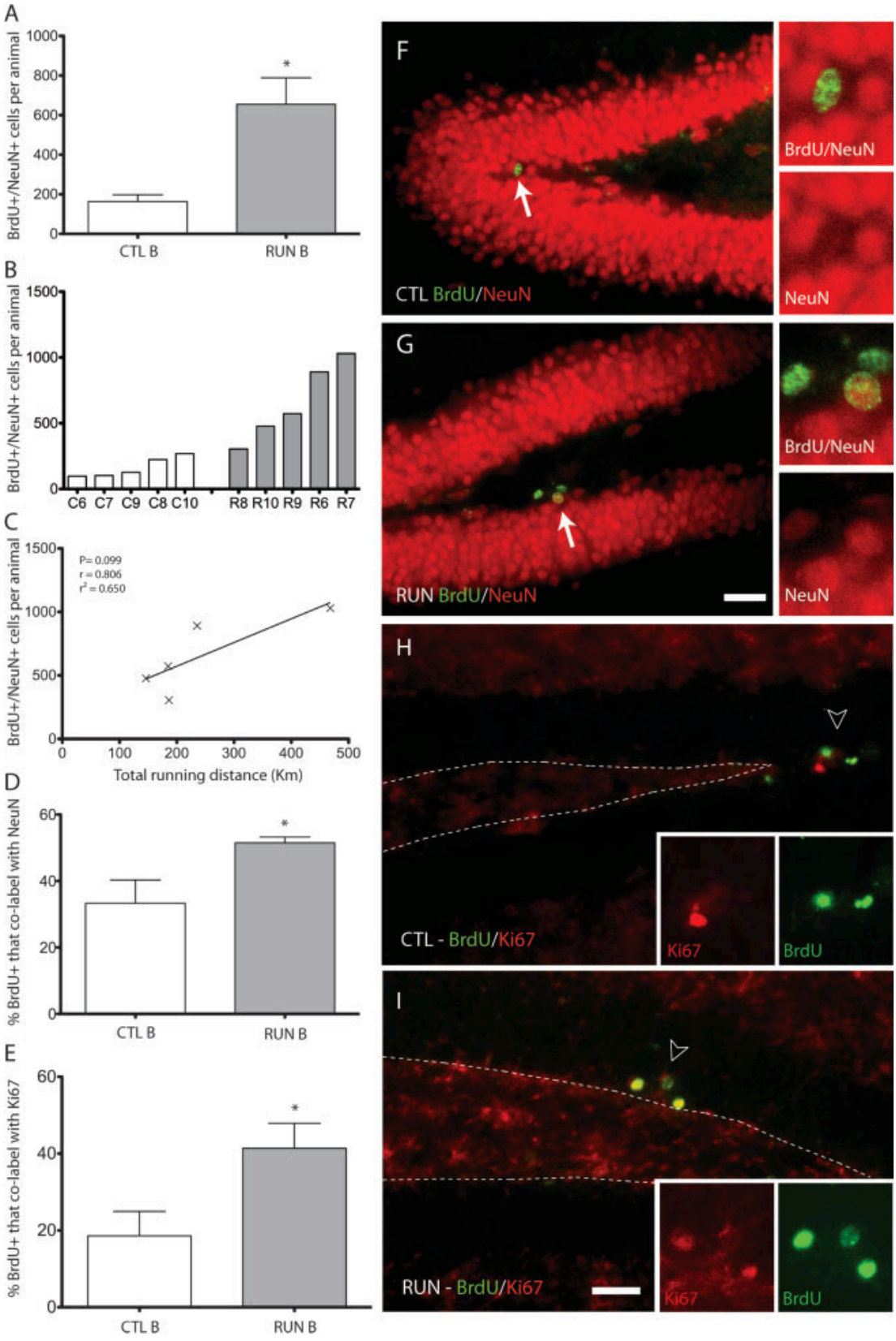

A
BrdU+/NeuN+ cells per animal
1000
800
600
400
200
0
*
CTL B
RUN B
B
BrdU+/NeuN+ cells per animal
1500
1000
500
0
C6 C7 C9 C8 C10
R8 R10 R9 R6 R7
C
BrdU+/NeuN+ cells per animal
1500
1000
500
0
P= 0.099
r = 0.806
r² = 0.650
0 100 200 300 400 500
Total running distance (Km)
D
% BrdU+ that co-label with NeuN
60
40
20
0
*
CTL B
RUN B
E
% BrdU+ that co-label with Ki67
60
40
20
0
*
CTL B
RUN B
F
CTL BrdU/NeuN
BrdU/NeuN
NeuN
G
RUN BrdU/NeuN
BrdU/NeuN
NeuN
H
CTL - BrdU/Ki67
Ki67
BrdU
I
RUN - BrdU/Ki67
Ki67
BrdU

FIGURE 6:

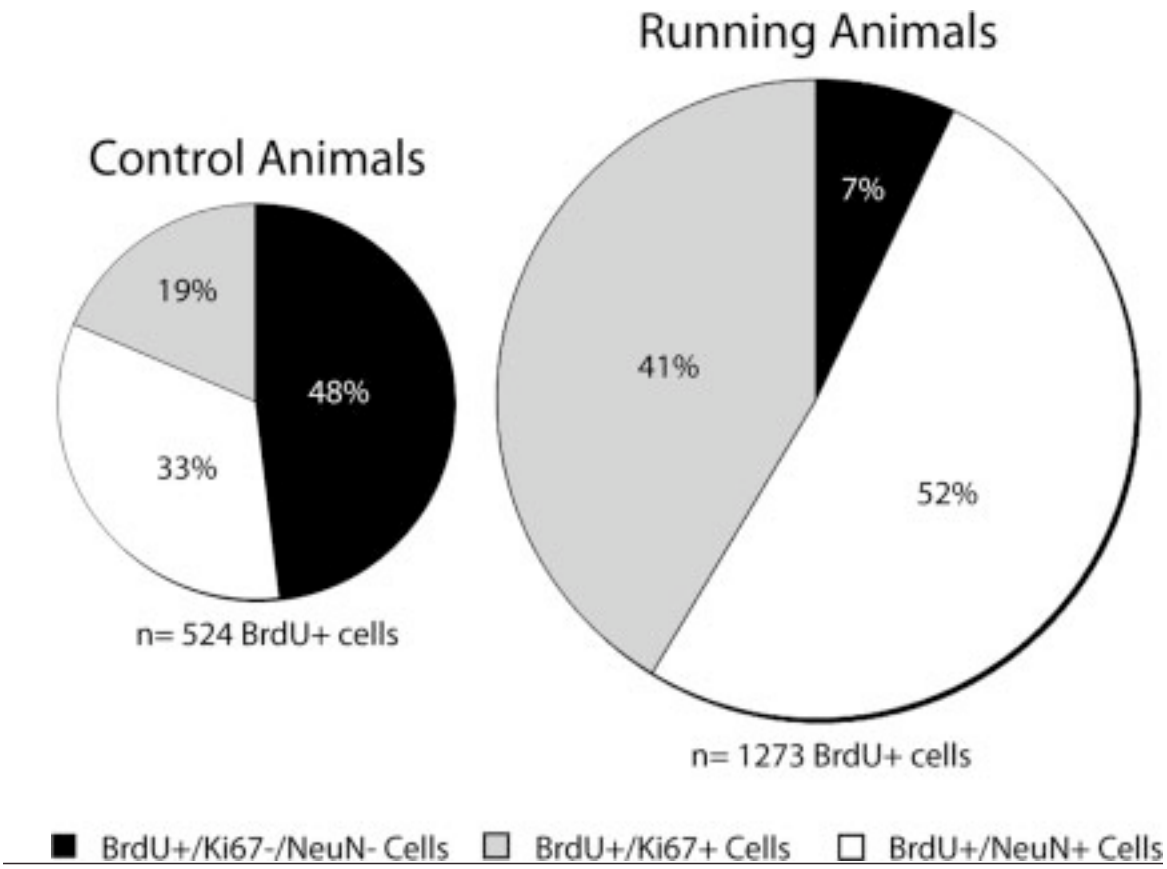


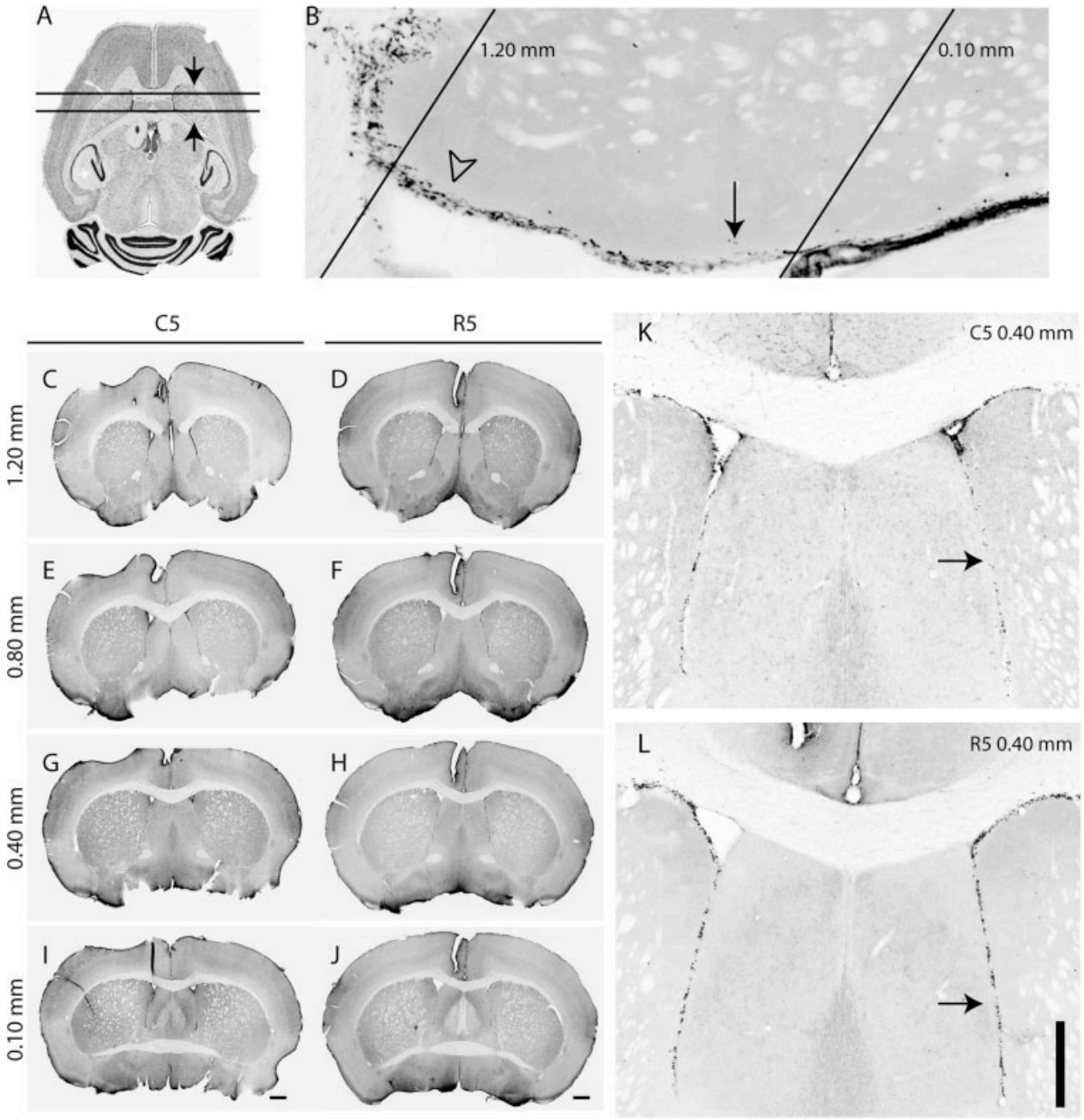
FIGURE 6. Representation of the differentiation of late-stage mitotic cells in control and running animals. The total number of BrdU-retaining cells is represented in this figure as two pie charts, one for control animals and one for running animals. The difference in the size of the pie charts represents the increase in the number of BrdU-retaining cells between control and running animals. Segments of the pie charts represent different identities of BrdU-retaining, late-stage mitotic cells in the DG, categorized as BrdU+/NeuN+ neurons (white), BrdU+/Ki67+ proliferating cells (gray), or unidentified BrdU+ cells (black). Notably, there are increased proportions of late-stage mitotic cells that differentiate into neurons or that remain proliferative in running animals.

Neural Precursors in the Ventral Forebrain SVZ Are Susceptible to the Proliferation Promoting Effects of Prolonged Running

Given the robust effects of prolonged wheel-running on neural precursors within the hippocampus, we extended our analyses of Ki67 immunoreactivity to the forebrain stem cell niche located within the walls of the lateral ventricles. In standard-housed mice, the density of Ki67+ cells gradually decreases along the rostro-caudal axis of the SVZ (Figs. 7A,B), as previously reported by others (Doetsch et al., 1997). In light of this fact, we performed Ki67 immunohistochemistry on coronal sections taken at regular rostro-caudal intervals through the SVZ of control and wheel-running mice (Figs. 7C–J). Inspection of these sections revealed visually apparent differences in Ki67 expression within the ventral extension of the SVZ (Figs. 7K,L).

FIGURE 7. (FOLLOWING PAGE) Prolonged voluntary wheel-running affects Ki67 expression in the SVZ. (A) For analysis of SVZ proliferation, sections were taken between rostral and caudal stereotactic coordinates (Bregma 1.20 to 0.10 mm) (arrows). (B) Within this region, the number of Ki67+ cells in the SVZ normally decreases from rostral (arrowhead) to caudal (arrow), as shown in this horizontal section. (C–J) Forty-micrometer sections matched according to their stereotactic coordinates and processed for Ki67 immunohistochemistry. (K,L) Higher magnification of these coronal sections following Ki67 immunohistochemistry reveals visually apparent differences in the ventral portions of the SVZ at the more caudal stereotactic coordinates examined (arrows). (A) Modified from www.brainmaps.org; scale bars = 500 µm.

FIGURE 7:


A
B
1.20 mm
0.10 mm
C5
R5
C
D
E
F
G
H
I
J
1.20 mm
0.80 mm
0.40 mm
0.10 mm
K
C5 0.40 mm
L
R5 0.40 mm

To quantify this observation, the number of Ki67+ cells was counted in the ventral-most 500 μm of the SVZ at two stereotactic levels, +0.80 and +0.40 mm from Bregma. Comparison of the raw data showed largely overlapping levels of Ki67 expression between control and running mice (Fig. 8A). Nevertheless, at both levels there was an apparent shift toward higher numbers, as further illustrated by binning the data in frequency histograms (Figs. 8B,C). At +0.40 mm Bregma, the difference in number of Ki67+ cells reached statistical significance, as the average number of Ki67+ cells per counting area increased from 29.72 ± 1.74 SEM to 41.54 ± 3.44 SEM ($P = 0.007$, unpaired t-test with Welch's correction; $P = 0.050$, F-test). Side-by-side comparison of Ki67 expression in this ventral SVZ region in control and running animals confirmed that similar numbers of Ki67+ proliferating cells were present at Bregma +0.80 mm (Figs. 8D–I), but that control mice had significantly fewer Ki67+ proliferating cells at Bregma +0.40 mm (Figs. 8J–O). We also noted that this ventral region of the lateral ventricle was often dilated in control but not running mice (i.e., Figs. 8D–F), an observation whose significance has yet to be determined. These results indicate that neural precursors within the ventral SVZ of the forebrain lateral ventricles of young adult mice are susceptible to proliferation-related changes following prolonged wheel-running.

FIGURE 8:

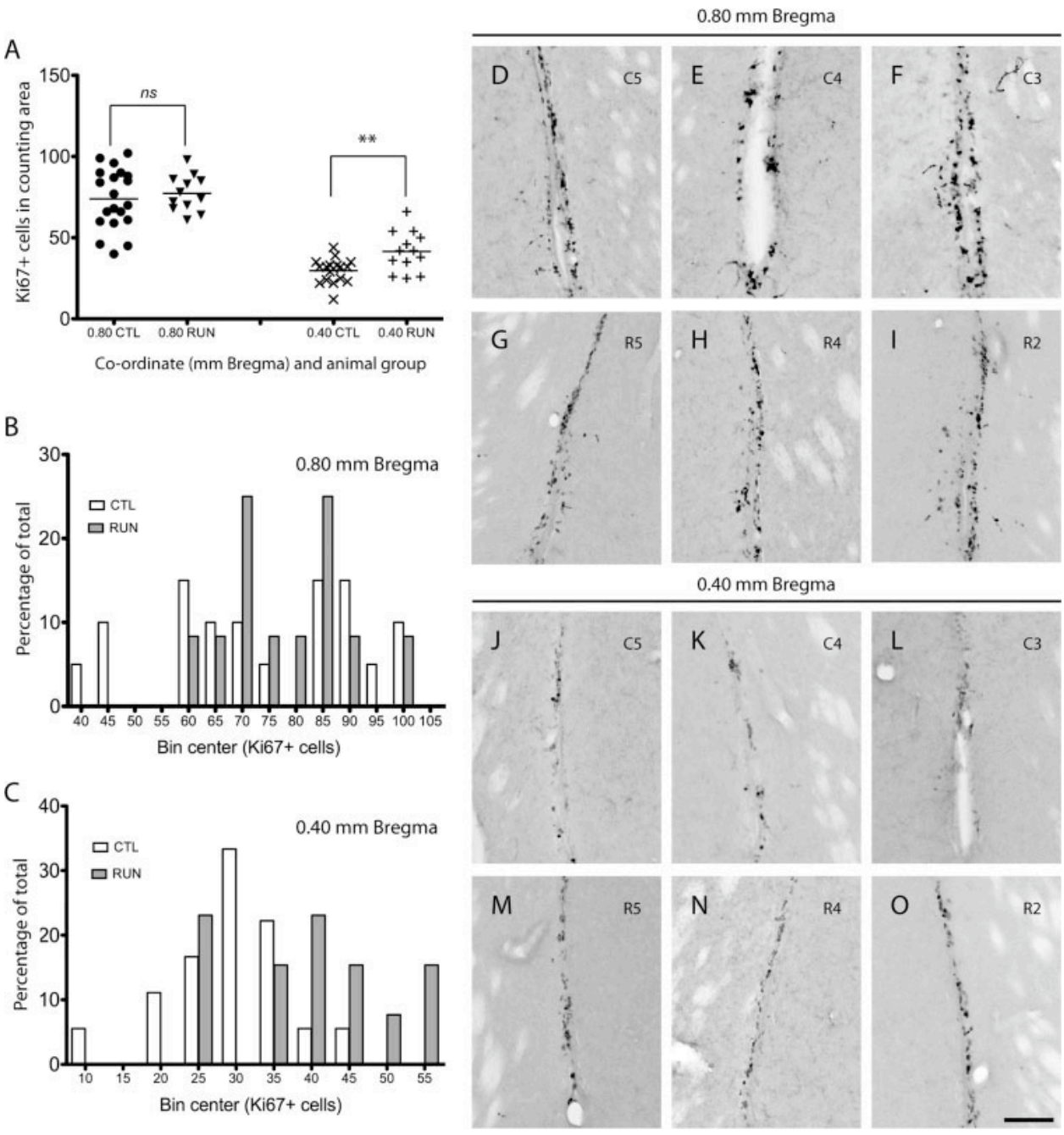

A
Ki67+ cells in counting area
150
100
50
0
ns
**
0.80 CTL
0.80 RUN
0.40 CTL
0.40 RUN
Co-ordinate (mm Bregma) and animal group
B
0.80 mm Bregma
CTL
RUN
Percentage of total
30
20
10
0
40 45 50 55 60 65 70 75 80 85 90 95 100 105
Bin center (Ki67+ cells)
C
0.40 mm Bregma
CTL
RUN
Percentage of total
40
30
20
10
0
10 15 20 25 30 35 40 45 50 55
Bin center (Ki67+ cells)
0.80 mm Bregma
D C5
E C4
F C3
G R5
H R4
I R2
0.40 mm Bregma
J C5
K C4
L C3
M R5
N R4
O R2

FIGURE 8. (PRECEDING PAGE) The number of Ki67+ cells increases in the ventral portions of the caudal SVZ. (A) Scatter plot of the number of Ki67+ cells in the counting area of control (CTL) and running (RUN) animals of Series A mice. A statistically significant increase is observed at 0.40-mm Bregma, which is the more caudal level of the SVZ analyzed. (B,C) Frequency histograms of the data from the 0.80-mm Bregma (B) and 0.40-mm Bregma (C) levels. Running mice have fewer sections with low levels of Ki67+ cells at the 0.80-mm Bregma level and have a clear positive shift in the number of Ki67+ cells at 0.40-mm Bregma. (D–O) Representative photomicrographs of Ki67+ cells in the counting area in control (D–F, J–L) and exercising (G–I, M–O) mice at 0.80-mm Bregma (D–I) and 0.40-mm Bregma (J–O). There is a noticeable increase in Ki67 expression in running mice (M–O) versus control mice (J–L) at the 0.40-mm Bregma level. Scale bar = 100 μm, *P ≤ 0.050, **P ≤ 0.010, ***P ≤ 0.001.

DISCUSSION

We have investigated the consequences of a prolonged voluntary wheel-running regime on neural precursors of the adult mouse hippocampus and forebrain. Our results enable us to draw three key conclusions: (i) Neurogenesis and proliferation remain elevated in the hippocampus following 6 weeks of wheel-running and the number of newly born neurons correlates statistically with the total running performance over this period. (ii) The stimulating effect of wheel-running on hippocampal neural precursors is not transient; rather, it is maintained throughout the latter half of the wheel-running regime. Dividing cells tagged with BrdU 3 weeks into the wheel-running regime continued to produce greater proportions of mature neurons and proliferating cells in runners. (iii) Prolonged voluntary wheel-running can stimulate proliferating CNS precursors outside of the hippocampus. Mice subjected to prolonged wheel-running had greater numbers of proliferating cells within the SVZ stem cell niche of the ventral forebrain.

Characterization of a 6-Week Voluntary Wheel-Running Model in Adult CD1 Mice

CD1 mice are an outbred strain of laboratory mice that are widely used due to their relative genetic heterogeneity, high reproduction rates, docility, and rapid growth. The CD1 mice used in this study proved to be prolific yet variable runners, whose daily running distances ranged from 1.6 to 14.9 km. In spite of this inter-animal variability in running performance, the magnitude of exercise-induced changes in neurogenesis was similar across two experimental series that were separated in time by several months. Moreover, because our animals were housed individually, the inter-animal variability in running distances could be recorded and this permitted us to assess possible correlations between individual running distance and the changes in neurogenesis and proliferation. These correlation analyses revealed that the running performance of mice in the voluntary running group was statistically correlated to the total number of DCX-expressing cells present after 6 weeks ($P = 0.050$), but was not correlated to the number of Ki67-expressing proliferating cells ($P = 0.583$). It has been more difficult to show such correlations when using inbred C57BL/6 mice due to their consistent running performance (Kronenberg et al., 2006).

Six Weeks of Prolonged Voluntary Wheel-Running Increases the Number of Newly Born Neurons and Proliferating Cells in the Hippocampal Dentate Gyrus

An important and unresolved question concerning the underlying mechanisms of running-induced adult neurogenesis is the extent to which increases result from alterations in hippocampal precursors (i.e., proliferation or neuronal differentiation) versus in newly generated neurons (i.e., survival or neuronal maturation). At the end of 6 weeks of wheel-running, there were two to three times as many DCX-expressing cells in the hippocampi of wheel-running mice when compared to control mice. This increased neurogenesis was accompanied by a 60% increase in Ki67+ proliferating precursors in the SGZ/GZ. Correlation analyses of wheel-running distances versus the number of DCX+ cells within the wheel-running group enabled us to conclude that running distances correlated with the number of DCX+ cells (Fig. 2C) but not with the number of proliferating cells (Fig. 3D). This suggests that nonproliferation based processes (i.e., improved cell survival or maturation) are at least partly responsible for changes in DCX expression. It is, however, difficult to directly compare the sizes of the newly born neuron and proliferating cell populations. DCX has a protracted 2- to 3-week-long expression pattern in dividing neuroblasts and newly born post-mitotic DG granule neurons (Brown et al., 2003b). In comparison, the Ki67+ population that was visualized in these experiments identifies only those cells proliferating at the end of the running paradigm. As such, it is possible that the more numerous DCX+ population could potentially derive from higher earlier levels of proliferating cells.

Based on the above considerations, we suggest a voluntary wheel-running model where mechanisms associated with increased stem/progenitor proliferative activity are activated in a generalized fashion in response to voluntary wheel-running, while mechanisms associated with improved neuronal survival/differentiation are activated in a running performance-dependent manner. Supporting the growing relative importance of cell survival mechanisms during prolonged wheel-running, Kronenberg et al. examined C57BL/6 mice following 3, 10, or 32 days of voluntary wheel-running, and found that while the number of DCX+ cells progressively increased over the entire running period, Ki67 levels peaked at 10 days and were nearly back to control levels by 32 days (Kronenberg et al., 2006). These findings support the idea that the size of the proliferative population rises acutely but then declines with prolonged exercise, at which time survival or maturation-promoting mechanisms become predominant. These survival or maturation-promoting mechanisms are most likely mediated by local neurotrophic support, in the form of growth factors such as IGF, BDNF, and VEGF (Fabel et al., 2003a; Gomez-Pinilla et al., 1997; Li et al., 2008; Rossi et al., 2006; Zhu et al., 2006).

Hippocampal Late-Stage Mitotic Cells of the Prolonged Running Paradigm Continue to Yield Increased Proportions of Both Mature Neurons and Proliferative Cells

Hippocampal precursors labeled with BrdU during the initial stages of an experiment will produce larger numbers of mature neurons in running rodents versus nonrunning rodents (van Praag et al., 1999a; van Praag et al., 1999b). What is less clear is whether wheel-running will continue to affect the progeny of mitotic precursors in the context of a prolonged running

paradigm. In the present study, mice from Series B were pulsed with BrdU 3 weeks after wheel-running began to assess the fate of hippocampal precursors that are dividing during the latter half of the 6-week prolonged running paradigm. The total size of the BrdU-retaining population generated by these late-stage mitotic cells increased two to three fold in running mice by the end of the running regime. This increase is expected as the acute stimulating effects of wheel-running on the proliferating cell population (Kronenberg et al., 2006) suggests there were already more BrdU-incorporating cells present at the time of injection. However, analysis of the BrdU retaining population 18 days after injection revealed that the proportion of BrdU-retaining cells that differentiated into mature, postmitotic neurons (NeuN+) increased significantly from 33% in control to 52% in running mice (i.e., 1.5 fold). Similarly, the proportion of BrdU-retaining cells that remained proliferative (Ki67+) increased from 19% in control to 45% in runners (2.3 fold). This indicates that the ongoing generation of both mature neurons and proliferative precursors remains elevated in running animals during the latter half of our prolonged voluntary wheel-running paradigm. Interestingly, in control animals, 48% of the 18-day BrdU-retaining cells was neither proliferating nor mature neurons (Fig. 6). We speculate that this population consists of post-mitotic DCX+ cells that have not yet expressed NeuN, non-proliferating glial progeny, and quiescent stem-like cells (Suh et al., 2007). The size of this population decreased to only 7% of the population following prolonged wheel-running, concomitant with the increase in the NeuN+ and Ki67+ populations.

The Adult Forebrain SVZ Is Susceptible to the Effects of Prolonged Exercise

An issue with wide-ranging implications is whether behavioral modifications such as physical exercise or environmental enrichment are capable of influencing neural precursor activity in regions outside of the hippocampus. Our examination of cell proliferation in the forebrain SVZ revealed increased numbers of Ki67+ cells in the ventral aspect of the caudal SVZ in prolonged wheel-running mice. These data indicate that neural precursors in the SVZ are susceptible, directly or indirectly, to the effects of prolonged voluntary exercise.

Previous studies have yielded contradictory findings concerning the ability of SVZ precursors to respond to physical exercise and environmental enrichment. An earlier study by Brown et al. showed that mice examined after 2 weeks of voluntary running had increased neural precursor proliferation within the hippocampus but not within the lateral ventricle SVZ (Brown et al., 2003a). In contrast, Yasuhara et al. reported that the endogenous levels of proliferation in both the SVZ and hippocampus are diminished following 2 weeks of hindlimb suspension in rats, a model of inactivity, which suggests that baseline levels of SVZ proliferation may be influenced by physical activity (Yasuhara et al., 2007). In another study, Hicks et al. found that neural precursors transplanted into the rat SVZ displayed more proliferation and migration if the rats were housed in an enriched environment (Hicks et al., 2007). Likewise, Komitova et al. demonstrated that, in a cortical infarct model, the stroke-induced decrease in SVZ proliferation and neurogenesis could be prevented by post-stroke environmental enrichment (Komitova et al., 2005a; Komitova et al., 2005b). Direct comparison of our findings with these previous studies is precluded for a variety of methodological reasons, including our focus on the ventral SVZ and

differences in species (rat vs. mouse), exercise periods (2 vs. 6 weeks), and proliferation detection approaches (multi-day BrdU incorporation vs. Ki67 expression). Given the positive correlation between running performance and the size of the increase in hippocampal neurogenesis, it is also possible that there is a minimum running performance threshold that must be crossed before detectable changes can occur within the SVZ.

It is important to note that increased numbers of Ki67+ cells in the SVZ may not necessarily indicate a direct, exercise-induced stimulation of proliferation. In a previous study by Luo et al. (2006), it was demonstrated that the SVZ of CD1 mice decreases in thickness with age, resulting in gradual decreases in proliferation and neurogenesis primarily within the ventral SVZ (Luo et al., 2006). Given the modest running-induced shift in counts of Ki67-expressing cells in the ventro-caudal SVZ over our 6-week paradigm, it is possible that the increase in Ki67 expression represents a prevention of age-related decline in SVZ proliferative activity rather than a direct stimulation. Supporting this, a model of exercise-induced prevention of age-related changes to neural precursors has been proposed for hippocampal neural precursors (Kronenberg et al., 2006). Another formal possibility is that the ventrally located changes in Ki67+ cells that we have measured are the result of an alteration in neuroblast migration patterns. SVZ neuroblasts are highly migratory, undergoing rostrally directed chain migration within the walls of the lateral ventricles, until they merge into the rostral migratory stream toward the olfactory bulb (Doetsch and Alvarez-Buylla, 1996; Doetsch et al., 1997). Differentiating among these possibilities will require further experimentation.

The mechanism by which physical exercise might actually influence SVZ proliferation is also unclear. However, a flurry of recent studies has demonstrated that SVZ stem cells reside

within a vascular niche (Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008) as has previously been reported for proliferative cells in the hippocampus (Palmer et al., 2000). Proximity to vasculature increases the probability that neural precursors can be directly influenced by circulating exercise-induced systemic factors and, in the case of the hippocampus, endorphins appear to represent one of these systemic factors (Koehl et al., 2008).

CONCLUSIONS

Consistent and prolonged periods of exercise have numerous beneficial effects with respect to CNS, cardiovascular and overall health and well-being. Here we established a model of prolonged voluntary wheel-running using CD1 mice. We showed that hippocampal neural precursors continue to display increased levels of neurogenesis after long-term exercise, and that there is a correlation between total long-term exercise performance and the level of hippocampal neurogenesis. Moreover, we demonstrated that the effects of prolonged physical activity on neural precursors are not limited to the hippocampus, but can influence regions such as the SVZ. These findings have important implications for the development of stem cell-based strategies for the treatment of CNS pathologies and degenerative diseases and suggest that appropriate behavioral modifications may be an important component of stem cell-based approaches to CNS repair. Further study of exercise and enrichment-related neurogenesis will enhance our understanding of CNS health and function and will become an increasingly important area of research for our rapidly growing aging population.

ACKNOWLEDGMENTS

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ARTICLE 2: Distinct stages of adult hippocampal neurogenesis are regulated by running and the running environment

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Hippocampus (In Press)

PREFACE

In a number of important studies, both environmental enrichment and wheel running have been shown to increase proliferation and/or neurogenesis in the hippocampus (Brown et al., 2003a; Kempermann et al., 1997; van Praag et al., 1999a; van Praag et al., 1999b). However, because wheel running alone, without any additional enrichment, can influence proliferation and neurogenesis, it has become the "de facto" focus of research into activity-mediated neurogenesis. Because of this, there is a running presumption that exercise or, at the very least, physical activity is solely responsible for running-induced effects on neurogenesis. Conversely, few researchers have considered the relative importance of the environment in which wheel running is performed.

One methodological dividing line in the current body of research into running-mediated neurogenesis pertains to the use of appropriate control animals as a basis of comparison. On the one hand, the majority of researchers seem to favor the ubiquitous and low-cost standard laboratory housing as a control environment. On the other hand, a small proportion of researchers feel that a locked running-wheel cage is the more appropriate control environment. While the use of a standard housing cage is definitely appropriate for some of these studies, there is also just as much justification for the use of a locked running wheel cage as a control environment. Simply, it is possible that the mere presence of a running wheel, which modifies the environment considerably, constitutes some form of environmental enrichment, which could influence neurogenesis independent of any physical activity. Thus, from a conceptual point of

view, the locked running-wheel cage is a better control environment because it minimizes the difference in neurogenesis that may result from simple environmental enrichment.

To date, there is no extant study that directly addresses the question of whether or not the running wheel environment is capable of influencing hippocampal neurogenesis. Thus, we designed an experimental paradigm that would allow us to answer this question. By using both standard housed and locked running-wheel cages in addition to unlocked running wheel cages in a normal running paradigm, this study will be able to show if the running environment is sufficiently "enriching" to have an effect on hippocampal neurogenesis. Besides the obvious methodological implications, this study can also give important insight into how wheel-running itself, independent of environmental enrichment, actually modulates neurogenesis.

AUTHOR CONTRIBUTIONS

Experimental conception: M.R.B., K.F., L.H., S.F-N.

Tissue Processing: M.R.B., K.F., A.A.

Immunohistochemistry experiments: M.R.B, L.H., S.F-N.

Running paradigm setup: M.R.B., A.A.

Manuscript: M.R.B. and K.F.

AUTHOR AND EDITOR AUTHORIZATION IS PRESENTED IN ANNEX I

ABSTRACT

Hippocampal neurogenesis continues into adulthood in mammalian vertebrates, and in experimental rodent models it is powerfully stimulated by exposure to a voluntary running wheel. In the present study, we demonstrate that exposure to a running wheel environment, in the absence of running, is sufficient to regulate specific aspects of hippocampal neurogenesis. Adult mice were provided with standard housing, housing enriched with a running wheel, or housing enriched with a locked wheel (i.e., an environment comparable to that of running animals, without the possibility of engaging in running). We found that mice in the running wheel and locked wheel groups exhibited equivalent increases in proliferation within the neurogenic niche of the dentate gyrus; this included comparable increases in the proliferation of radial glia-like stem cells and the number of proliferating neuroblasts. However, only running animals displayed increased numbers of post-mitotic neuroblasts and mature neurons. These results demonstrate that the running wheel environment itself is sufficient for promoting proliferation of early lineage hippocampal precursors, while running per se enables newly generated neuroblasts to survive and mature into functional hippocampal neurons. Thus, both running-independent and running-dependent stimuli are integral to running wheel-induced hippocampal neurogenesis.

INTRODUCTION

Neurogenesis in the adult mammalian hippocampus occurs within the dentate gyrus (DG) and involves a multi-step neurogenic process whose principal stages have been largely identified (Kempermann et al., 2004a; Seri et al., 2004). According to the current model, adult hippocampal neurogenesis begins with infrequently-dividing, radial glia-like precursor/stem cells located within the subgranular zone (SGZ) of the DG (Fukuda et al., 2003; Seri et al., 2001; Suh et al., 2007). When stimulated to divide, these neural precursors produce rapidly dividing progenitor cells that will differentiate primarily into immature neuroblasts. Under the appropriate conditions, these immature neuroblasts have the potential to mature into fully functional dentate granule neurons (Jessberger and Kempermann, 2003; Song et al., 2002; Toni et al., 2008; Toni et al., 2007; van Praag et al., 2002). While the functional roles of adult hippocampal neurogenesis are still under investigation, newly formed neurons could play a role in spatial pattern separation (Clelland et al., 2009), in spatial learning and spatial memory (Zhang et al., 2008), in improving long-term memory or memory consolidation (Aimone et al., 2009; Bruel-Jungerman et al., 2005; Deng et al., 2010; Deng et al., 2009), and in the associative memory of fear and conditioning (Kitamura et al., 2009).

Remarkably, the production of adult-born hippocampal neurons is highly sensitive to external manipulations, such as environmental enrichment (i.e., an environment that is supplemented with toys, tunnels, running wheels and cognitive stimuli) (Kempermann et al., 1997; Nithianantharajah and Hannan, 2006). With environmental enrichment, there is a robust increase in neural precursor proliferation and neurogenesis that leads to an overall increase in the

number of granule neurons within the DG (Brown et al., 2003a; Kempermann et al., 1997). The neurogenesis-promoting effects of environmental enrichment have been primarily attributed to physical activity, as exposure to a running wheel without additional forms of enrichment is sufficient to induce these effects (van Praag et al., 1999a; van Praag et al., 1999b). Exposure to a voluntary running paradigm has been shown to activate proliferation of early lineage radial glia-like stem cells (Suh et al., 2007), as well as to increase levels of a variety of neural and vascular growth factors within the hippocampus (Cao et al., 2004; Carro et al., 2000; Hunsberger et al., 2007; Neeper et al., 1996).

In the present study, we investigated the possibility that running wheels might promote neurogenesis, in part, through mechanisms that are independent of running. Specifically, we hypothesized that the presence of a running wheel might by itself constitute a form of environmental enrichment. To test this idea, we modified a previously characterized prolonged voluntary wheel-running paradigm in which mice given free access to a running wheel display marked increases in proliferation, production of neuroblasts and mature neurons (Bednarczyk et al., 2009). By adding a third experimental group, with locked running wheels, we demonstrate here that the neurogenic effects of running wheel exposure are comprised of a running-independent component that promotes proliferation of early lineage cells, and a running-dependent component that promotes survival and maturation of post-mitotic neuroblasts and neurons. This work provides important mechanistic insight into how different aspects of environmental enrichment can work together to promote adult hippocampal neurogenesis.

METHODS

Animals and experimental model

All experiments were conducted in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the animal care committee of the Université de Montréal. Fourteen 2-month-old adult male CD1 mice (Charles River, Senneville, Quebec, Canada) were used in the evaluation of the experimental paradigm. Mice were given three intraperitoneal injections of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, Oakville, ON, Canada, 50 mg/kg) at 3-hour intervals and were randomized and separated into one of three different environments the following day: 4 mice in the control or standard cage group (herein referred to as "CTL" mice), 5 mice in the locked wheel cage group ("LOC" mice) and 5 mice in the running wheel or "running" group ("RUN" mice). For the following days 1 to 6, mice in both the LOC and RUN groups were housed with the running wheels locked. At the start of the trial period, day 7, running wheels in the RUN group cages were unlocked. Running cages were outfitted with odometers and a computerized recording system to measure and record the daily and total running distance. All animals were provided with food and water *ad libitum* and nesting material, with no additional environmental enrichment. Mice were sacrificed on day 40, i.e., 33 days following the start of the trial period (Figure 1a).

Tissue Preparation

Mice received a lethal dose of chloral hydrate (7%) and were then perfused trans-cardially with 25 ml of phosphate-buffered saline (PBS) followed by 40 ml of 4%

paraformaldehyde. The brains were removed and post-fixed in 4% paraformaldehyde for 2 h, and then kept in PBS at 4°C until sectioning. The entire brain of each animal was cut into 40 µm coronal sections using a vibrating microtome (Leica VT1000S, Leica Microsystems, Richmond Hill, ON, Canada) and the tissue sections were stored at -20°C in an anti-freeze solution (glycerol:ethylene glycol:PBS 1X, 3:3:4).

Immunohistochemistry

The primary antibodies used in these experiments were mouse anti-human Ki67 (1:200, BD Biosciences, Mississauga, ON, Canada), goat anti-human Doublecortin (DCX; 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-mouse Neuronal Nuclei (NeuN; 1:200, Chemicon, Billerica, MA, USA), rabbit anti-cow Glial Fibrillary Acidic Protein (GFAP; 1:1000, Dako, Glostrup, Denmark), rat anti-BrdU (1:200, AbD Serotec, Oxford, UK), rabbit anti-human Calretinin (1:2500, Swant, Bellinzona, Switzerland), rabbit anti-Iba1 (1:500, Wako, Osaka, Japan) and mouse anti-cow S100β (1:1000, Sigma-Aldrich, Oakville, ON, Canada). For immunohistochemical labeling of Ki67, NeuN, GFAP and DCX, free-floating 40 µm sections were washed in PBS (pH 7.4) and then blocked for 2h in 10% normal donkey serum (NDS)/0.1% Triton-X/PBS (for fluorescence-based immunohistochemistry) or 4% bovine serine albumin (BSA)/0.1% Triton-X/PBS (for diaminobenzidine (DAB)-based immunohistochemistry). Sections were then incubated at room temperature overnight in primary antibodies diluted in either 5% NDS or 2% BSA in PBS. For BrdU immunohistochemistry, sections were washed in PBS, rinsed in double-distilled water to remove excess buffer, and then treated with 2N HCl for 40 min at 37°C to denature the DNA. The HCl was subsequently washed out in PBS and the

sections then blocked for 2h in 4% BSA/0.1% Triton-X/PBS. Sections were incubated overnight at room temperature in the rat anti-BrdU antibody (alone or in combination with other antibodies) diluted in 2% BSA/PBS.

For DAB-based detection of primary antibodies, sections were washed in PBS and then incubated in the appropriate biotinylated secondary antibody (Jackson Immuno Research, West Grove, PA, USA) for 1h and 30 min at room temperature, diluted in 2% BSA/PBS. Following washes, the signal was amplified using the avidin–biotin–peroxidase system (VectaStain ABC Kit, Vector Laboratories, Burlington, ON, Canada) for 1h and 30 min, and then detected using a DAB containing solution (0.5 mg/ml DAB, 0.015% H_2O_2, 0.040% $NiCl_2$ in PBS) for 1–2 min. Sections were mounted onto glass slides, dried overnight, and were then dehydrated in a graded series of alcohol baths and coverslipped with Permount (Fischer Scientific, Ottawa, ON, Canada). A subset of sections from each condition was counterstained with 0.02% Cresyl Violet for 25 minutes, rinsed, dehydrated in a series of alcohol baths and then coverslipped with Permount.

For fluorescence detection of primary antibodies, sections were rinsed and incubated in appropriate secondary antibodies conjugated to either CY3 (1:200, Jackson ImmunoResearch, West Grove, PA, USA) or Alexa 488, 555 or 647 (1:1,000, Molecular Probes, Invitrogen, Burlington, ON, Canada) diluted in 0.05% Triton-X/PBS for 40 min at room temperature. Sections were then washed and incubated with Hoechst 33342 nuclear counterstain (0.2 μM, Sigma-Aldrich, Oakville, ON, Canada) for 2 minutes. After a final wash, sections were mounted on glass slides and coverslipped with Mowiol anti-fade solution [13% (w/v) polyvinyl alcohol and 2% (w/v) DABCO in 2:1 Tris–HCl (pH 8.5):Glycerol].

Quantification and Statistical Analyses

When using DAB immunohistochemistry, unless otherwise noted, quantifications were performed on every sixth section through the entire hippocampus (10 sections total). Tissue sections processed using DAB were coded and were then digitized using an automated system (NDP Scan, Hamamatsu Photonics K.K., Bridgewater, NJ, USA) and a 40X objective and/or examined using differential interference contrast (DIC) microscopy with a 40X objective (Olympus IX81 microscope, Olympus, Markham, Ontario, Canada). Counts were performed manually by a blinded individual using the digitized images and/or in real-time via DIC microscopy. Slide coding was only broken after all quantifications had been completed for any given marker. Cell counts were converted into the approximate total number of cells by multiplying by six. All counts are expressed as the sum total of cells/brain counted from both hippocampi of each animal, except where otherwise indicated.

For fluorescence labeling, cells were quantified from three to four sections at comparable stereotactic levels, selected from the rostral, medial and caudal hippocampus. Fluorescence signals were imaged in z-stacks at 1 µm intervals using a motorized Olympus IX81 microscope and manually counted from the digital images. To assess co-localization of markers, nuclear (ho/ki67/BrdU/NeuN) and cytoplasmic (DCX, GFAP) labels were initially visualized by epi-fluorescence as described. Co-localization was then confirmed using a Leica TCS-SP confocal microscope (Leica Microsystems, Richmond Hill, ON, Canada) and a 100x objective. Nuclear markers were considered to be co-localized if they overlapped at all z-stack levels. Nuclear and cytoplasmic markers were considered to be co-expressed if the nuclear marker was enveloped by the cytoplasmic marker in all three dimensions. The total number of co-labeled cells (i.e., Ki67+/

DCX+, BrdU+/NeuN+) was determined by multiplying the percentage of co-labeled cells by the total number of DAB-labeled Ki67+ or BrdU+ cells. Quantification of positive cells was limited to cells within the SGZ/GZ of the DG.

All data are expressed as cell counts ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism (Mac OS X version 5.0a). Data sets were tested for normality (D'Agostino and Pearson omnibus normality test) and equal variances (F-test), and group averages were calculated as the average total number of cells in both hippocampi per animal. Quantifications were analyzed primarily with one-way ANOVA and Tukey's post-hoc test. Kruskal-Wallis one-way ANOVA with Dunn's post hoc analysis was used when variances differed significantly between groups. Significance levels were set at $\alpha=0.05$, two-tailed.

RESULTS

Characterization of standard housing, locked wheel and running wheel paradigms: animal behavior and running performance

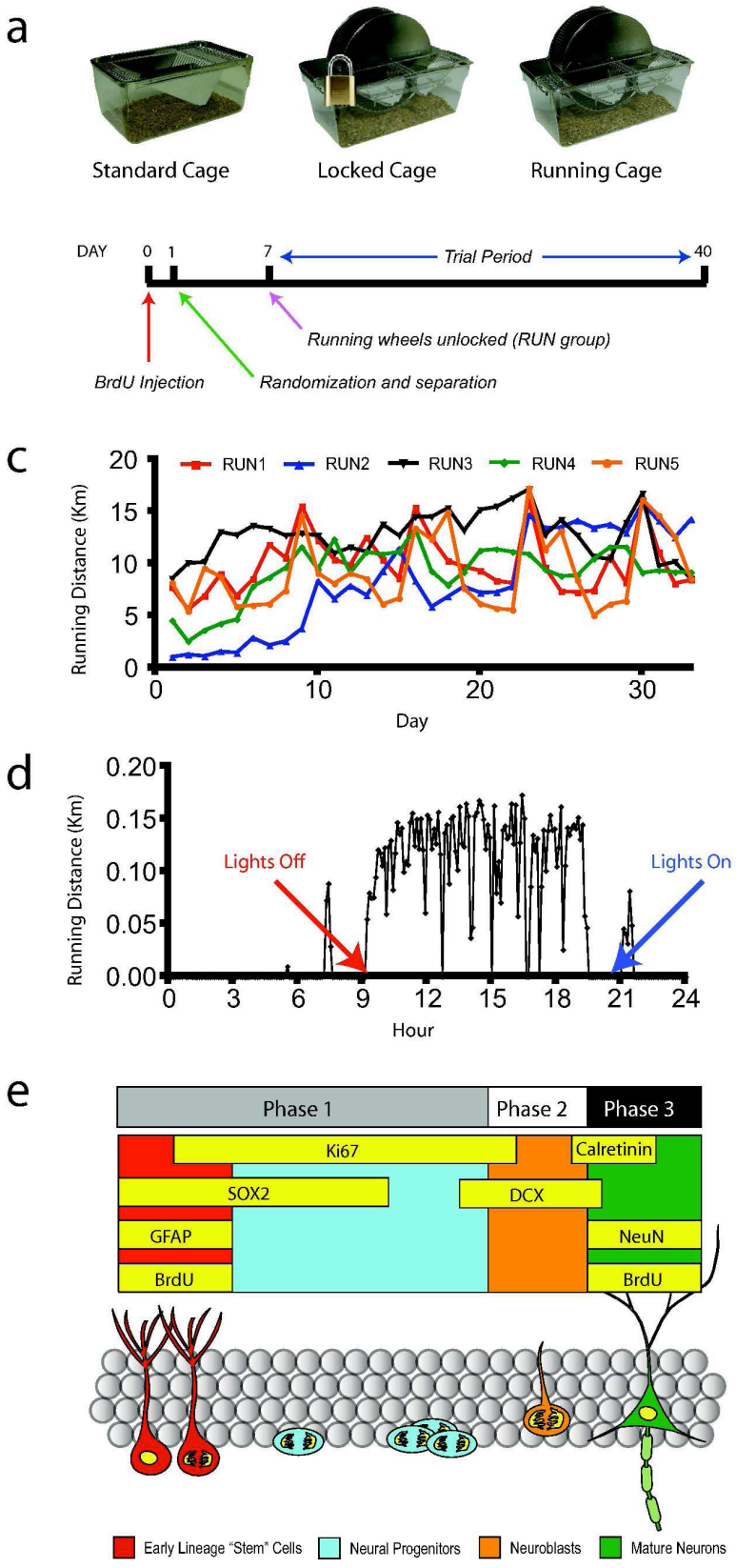
Previous rodent studies have shown that enrichment of living space with toys, tunnels, running wheels and other objects of potential interest can promote neurogenesis within the DG (Brown et al., 2003a; Kempermann et al., 1997). We have previously characterized changes within the DG neurogenic lineage following 6 week exposure to a voluntary running wheel (Bednarczyk et al., 2009). In order to determine whether the running wheel environment contributes to these changes independently of running, we modified our previous experimental protocol. In addition to control mice in standard housing ("CTL" mice) and running mice housed with unlocked running wheels ("RUN" mice), we added a third group housed with locked wheels ("LOC" mice) (Figure 1a). All mice were housed without any additional forms of environmental enrichment, only being provided with food, water and bedding material. LOC mice had the same living environment as RUN mice, but could not engage in any sustained physical activity (i.e., running).

In order to compare the effects of running and locked wheel housing conditions on survival of newly generated cells in the hippocampal DG, we labeled proliferating cells with BrdU 40 days before the time of sacrifice. Following BrdU injections, mice in the LOC and RUN groups were housed in an identical locked wheel environment for a period of six days before unlocking cages in the RUN group, in order to minimize environment-mediated effects on proliferation that could interfere with assessment of cell survival.

While behavioral analysis was not the primary focus of this study, we observed that mice in both the CTL and LOC groups regularly engaged in limited forms of physical activity even in the absence of functional running wheels (Video 1, Video 2, Supplementary Information; see also Dubreucq et al., 2010). At the time of sacrifice, the weights of LOC mice (41.36±1.433g) and CTL mice (42.37±2.275g) were not significantly different from each other, while the weights of RUN mice (35.80±0.5132g) were significantly decreased compared to both these groups ($p<0.050$, one-way ANOVA with Tukey's post-hoc test). RUN animals ran voluntarily for extended periods, averaging 8.25-12.79 km run per night, for a total of 272.4-421.9 km over the 33 days of the experiment (Figure 1b, and Video 3, Supplementary Information). Running mice were generally active for the entire twelve hours of the lights-out, or nighttime period, as is expected for nocturnal animals (Figure 1c).

For the purposes of the present study, we sub-divided the process of hippocampal neurogenesis into three broad and partially overlapping phases (Figure 1d). The Proliferation phase (Phase 1) is defined by the expression of the proliferation marker Ki67 and encompasses the steps from activated radial glia-like precursors to the initial generation of proliferative neuroblasts. The Neuroblast phase (Phase 2) is defined by the expression of Doublecortin (DCX), which is first expressed by neuroblasts during their initial proliferative stage and continues to be expressed for approximately 2-3 weeks into their post-mitotic period. The Survival/Maturation phase (Phase 3) is defined here by the long-term retention of BrdU, and represents the surviving fraction of dividing cells that had been labeled prior to the start of the experimental paradigm. BrdU+ cells that co-label with NeuN represent newly generated mature neurons. Cells expressing Calretinin are newly born post-mitotic neurons at the Phase 2-3 transition.

FIGURE 1:


a
Standard Cage
Locked Cage
Running Cage
DAY
0
1
7
Trial Period
40
Running wheels unlocked (RUN group)
BrdU Injection
Randomization and separation
c
RUN1
RUN2
RUN3
RUN4
RUN5
Running Distance (Km)
Day
d
Running Distance (Km)
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Hour
e
Phase 1
Phase 2
Phase 3
Ki67
Calretinin
SOX2
DCX
GFAP
NeuN
BrdU
BrdU
Early Lineage "Stem" Cells
Neural Progenitors
Neuroblasts
Mature Neurons

FIGURE 1. (PRECEDING PAGE) ***Experimental model and running performance.*** **(a) Mice were housed in a standard cage, a locked running wheel cage or an unlocked running wheel cage for the duration of the trial period. 2 month old CD1 mice were given three injections of BrdU and were then randomized and separated into their respective environments the following day. Running wheels were locked in both the running and locked wheel groups for the following 6 days. At day 7, running group running wheels were unlocked and all animals were allowed to live in the respective environment for a period of 33 days. (b) Mice in the running group averaged 8.25 to 12.79 km per day for the entire 33 day trial period. (c) Hourly running distances for a representative mouse showing that most of the running activity was performed during lights-off, or nighttime hours. (d) A schematic of the neurogenic lineage cells and their antigenic markers in the adult DG. We have divided this lineage into three phases: Phase 1, cell proliferation, Phase 2, production of neuroblasts and Phase 3, maturation and survival of newly-formed neurons.** ***(BrdU= bromodeoxyuridine, DCX= doublecortin, GFAP= glial fibrillary acidic protein, NeuN= neuronal nuclei)***

Phase 1: Proliferation is increased in both running and locked wheel mice

To determine the effects of housing conditions on cells in the Proliferative phase of hippocampal neurogenesis, we analyzed the expression of Ki67, a nuclear protein that is expressed by actively proliferating cells in all phases of the cell cycle (Scholzen and Gerdes, 2000). Immunohistochemistry revealed that mice in both the LOC (Figure 2b) and RUN (Figure 2c) groups displayed visually apparent increases in the number of Ki67+ cells when compared to CTL mice (Figure 2a). Quantification of the number of Ki67+ cells in the DG identified a two-fold increase in the number of proliferating cells in both these groups (Figure 2e). The number of cells increased from an average of 376.5±96.8 cells in CTL mice to an average of 856.8±73.6 cells in LOC mice and 808.8±155.3 cells in RUN mice (Figure 2e). This increase only reached statistical significance in the locked wheel group ($P<0.050$, one-way ANOVA, with Tukey's post-hoc test vs. control), possibly due to the greater inter-animal variability in the RUN mice (Figure 2d). The dorsal and ventral regions of the DG displayed equivalent increases in Ki67+ cells in the LOC and RUN mice (Figure 2f).

FIGURE 2:

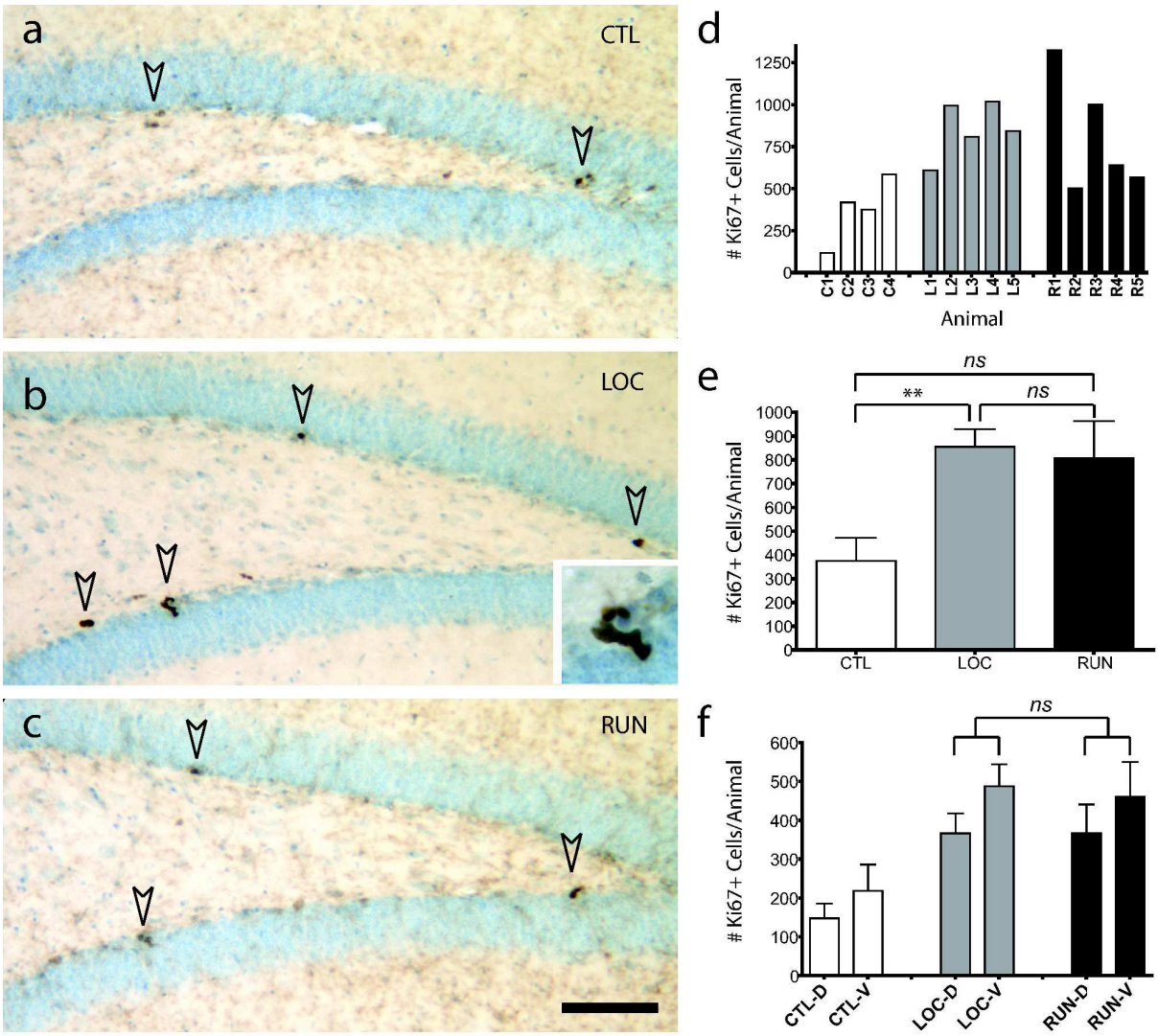


FIGURE 2. ***Cellular proliferation is increased in locked wheel mice.*** **(a,b,c) Photomicrographs of Ki67 expressing cells in the Cresyl-Violet counterstained dentate gyrus (DG) of control ("CTL", a), locked wheel ("LOC", b) and running ("RUN", c) animals. Inset in (b) shows a cluster of Ki67+ cells. (d) Individualized cell proliferation data from CTL (C1-C4; white), LOC (L1-L5; grey) and RUN (R1-R5; black) animals. (e) Quantification of Ki67+ cells reveals a significant, two-fold increase in the total number of Ki67+ cells in LOC mice when compared to CTL mice. (f) Separation of Ki67 quantifications into dorsal (CTL, LOC, RUN-D) and ventral (CTL, LOC, RUN-V) components shows that increases in proliferation were uniform in both dorsal and ventral portions of the DG in both locked and running animals.** ***(Scale bar: 125 µm (a,b,c); ns = P>0.050, * = P≤0.050, ** = P≤0.010,*** = P≤0.001, One-way ANOVA with Tukey post-hoc test)***

Phase 2: The total number of neuroblasts is increased in running mice but not in locked wheel mice

The production of DG neuroblasts increases markedly with prolonged voluntary wheel running (Bednarczyk et al., 2009). We assayed the number of neuroblasts produced under our three housing conditions by quantifying the number of cells immunoreactive for DCX, a microtubule associated protein that is expressed by newly born and migrating neuroblasts (Couillard-Despres et al., 2005). Photomicrographs of DCX immunohistochemistry in the DG revealed a striking increase in DCX expression in RUN mice (Figure 3c) compared to CTL mice (Figure 3a), and that unlike for Ki67, this increase did not occur in LOC mice (Figure 3b). Individualized data is shown in Figure 3d. Quantifications confirmed that there was a two-fold increase in the number of DCX+ cells in running mice (9762±838.1 cells) and no increase in locked wheel mice (4171±515.4 cells) when compared with standard-housed mice (4125±435.8 cells) (Figure 3e). The effects of running on the expression of DCX were highly statistically significant ($P<0.001$, one-way ANOVA, with Tukey's post-hoc test vs. control), and occurred approximately equally in the dorsal and ventral regions of the DG (Figure 3f).

FIGURE 3

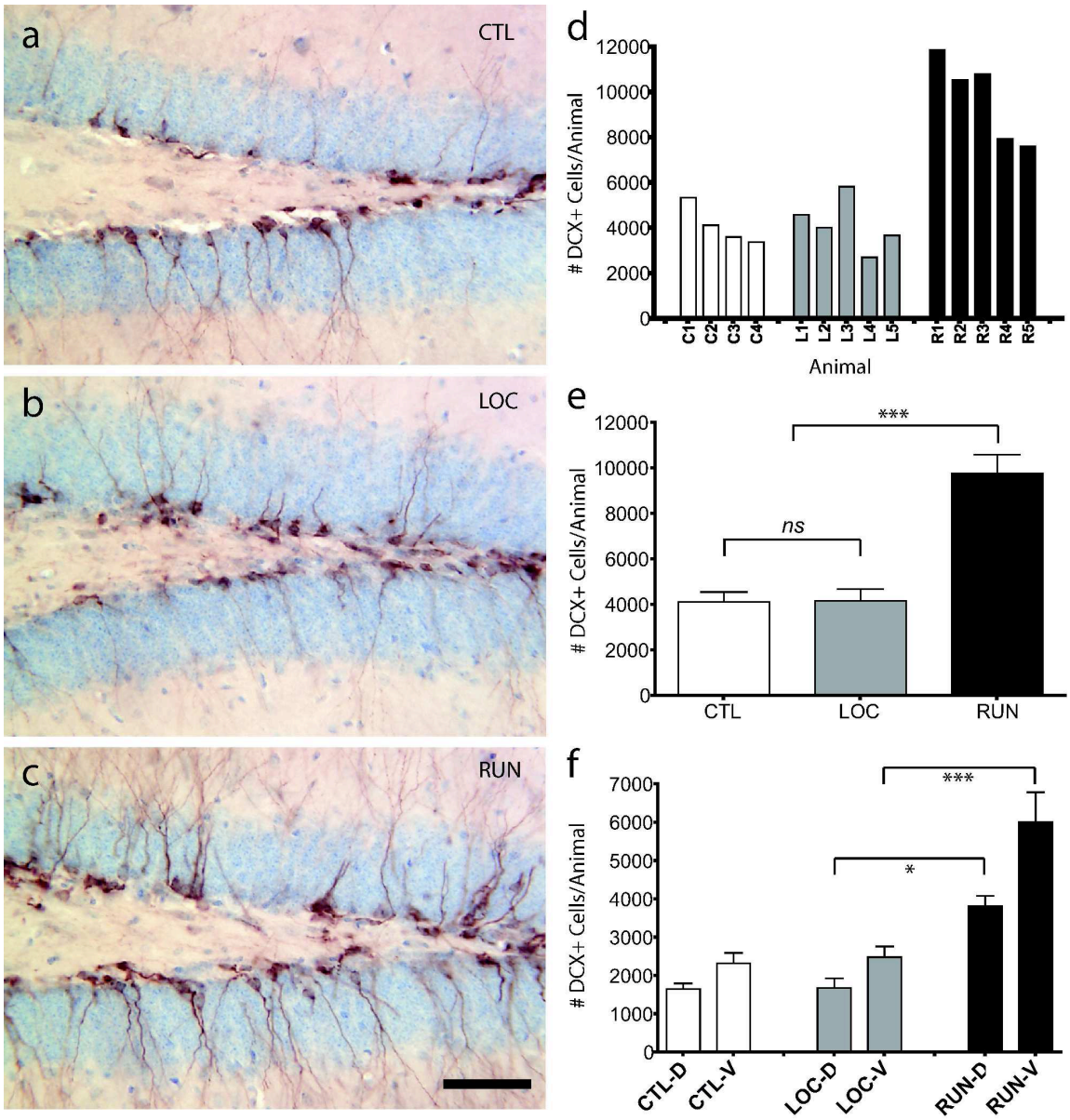


FIGURE 3. ***The number of neuroblasts is increased in running but not locked wheel mice.*** **(a,b,c) Photomicrographs of Doublecortin (DCX) expressing cells in control ("CTL", a), locked wheel ("LOC", b) and running ("RUN", c) animals. (d) Comparison of the total number of DCX+ cells from individual animals in CTL (C1-C4; white), LOC (L1-L5; grey) and RUN (R1-R5; black) groups. (e) Quantification of DCX+ cells shows a significant, two-fold increase in the average number of DCX+ cells in RUN animals, when compared to both LOC and CTL animals. (f) Separation of DCX quantifications into dorsal (CTL, LOC, RUN-D) and ventral (CTL, LOC, RUN-V) components shows that DCX expression increased proportionately in dorsal and ventral components in running animals.** ***(Scale bar: 50 µm (a,b,c); ns = P>0.050, * = P≤0.050, ** = P≤0.010,*** = P≤0.001, One-way ANOVA with Tukey post-hoc test)***

Phase 3: The number of newly born neurons is increased in running mice but not in locked wheel mice

In order to compare the effects of RUN and LOC housing conditions on survival of newly generated cells, we first quantified changes in the number of BrdU-retaining cells in the DG. Photomicrographs of BrdU-immunoreactive cells in the DG illustrate a marked increase in the number of BrdU+ cells in RUN mice (Figure 4c) when compared to both CTL (Figure 4a) and LOC (Figure 4b) mice. Quantifications revealed that there was a two-fold increase in the number of surviving, BrdU-labeled cells in RUN mice (1020.0±101.5 cells) and no significant increase in LOC mice (500.4±65.8 cells) when compared to CTL mice (474.0±70.7 SEM cells) (Figure 4e) (running vs. standard-housed mice, $P<0.010$, one-way ANOVA, with Tukey's post-hoc test). Both the dorsal and ventral regions of the DG exhibited increased numbers of BrdU+ cells (Figure 4f).

To determine the percentage of BrdU-retaining cells that had differentiated into neurons, we co-labeled for BrdU and NeuN (a nuclear marker of mature neurons) (Figure 4g). A total of 15-53 BrdU+ cells were counted per animal (3-4 sections/animal). Quantification revealed that a similar proportion of the BrdU-retaining cells had differentiated into neurons in the CTL (70.28±5.32%), LOC (68.31±2.14%) and RUN (67.94±3.32%) groups (Figure 4h). Given the running-induced increase in the size of the BrdU-retaining population, this translated into a significant running-induced increase in the total number of BrdU+/NeuN+ newly generated neurons in RUN mice (691.2±78.4 cells) versus CTL mice (340.9±70.5 cells) and LOC mice (342.9±47.8 cells) (RUN vs. CTL mice, $P<0.050$, one-way ANOVA, with Tukey's post-hoc test; Figure 4h). Immunofluorescence labeling for BrdU with the differentiated astrocytic marker S100β or the microglial marker Iba1 did not show any co-labelling with the BrdU-retaining cells within the DG (data not shown).

FIGURE 4:

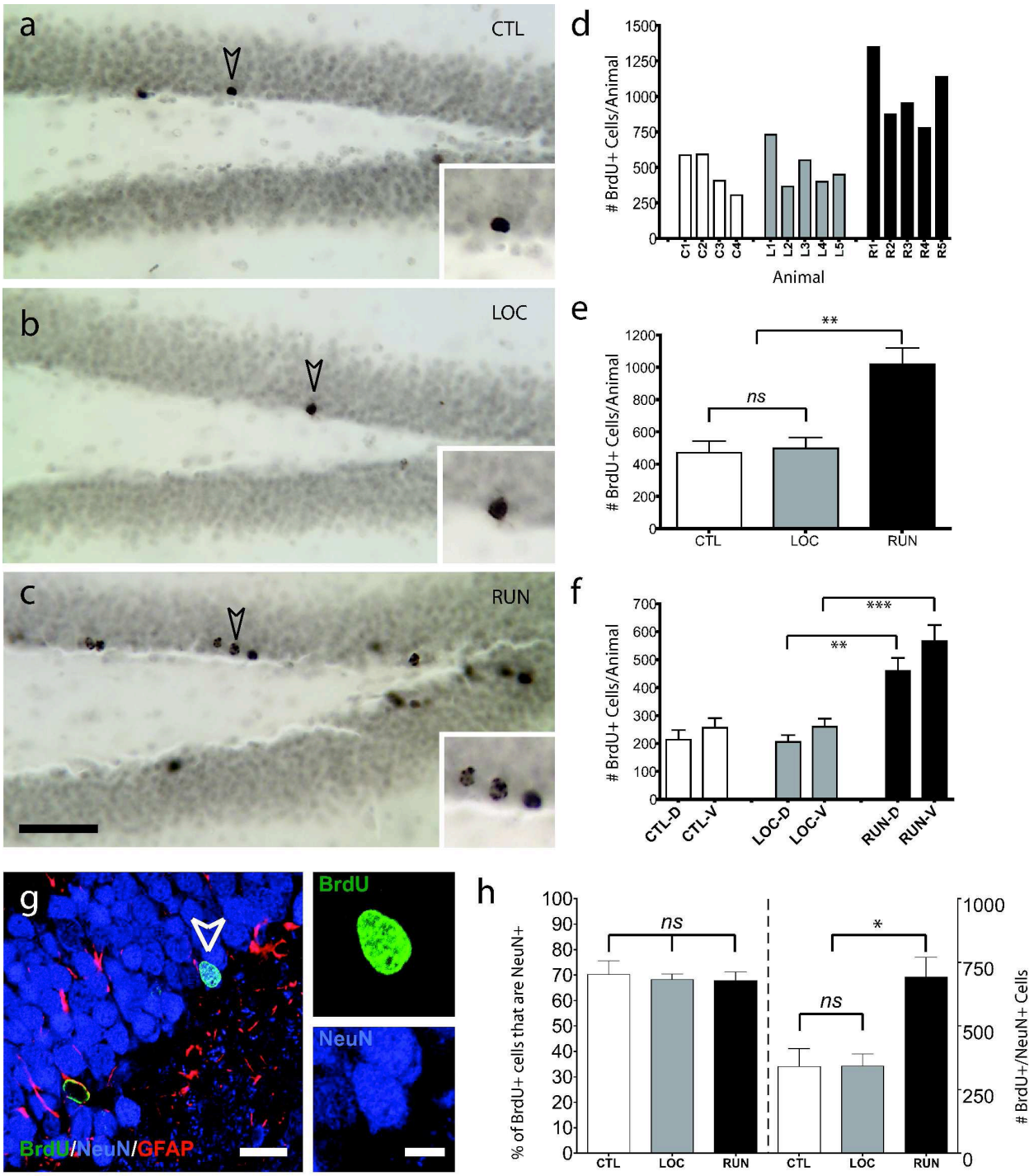

a
CTL
b
LOC
c
RUN
d
BrdU+ Cells/Animal
Animal
C1 C2 C3 C4 L1 L2 L3 L4 L5 R1 R2 R3 R4 R5
e
BrdU+ Cells/Animal
ns
**
CTL
LOC
RUN
f
BrdU+ Cells/Animal
***
**
CTL-D
CTL-V
LOC-D
LOC-V
RUN-D
RUN-V
g
BrdU
NeuN
BrdU/NeuN/GFAP
h
% of BrdU+ cells that are NeuN+
# BrdU+/NeuN+ Cells
ns
*
ns
CTL
LOC
RUN
CTL
LOC
RUN

FIGURE 4. (PRECEDING PAGE) ***The number of 40-day, BrdU-retaining cells and BrdU+/NeuN+ cells is increased in running but not locked wheel mice*****. (a,b,c) Photomicrographs of BrdU-retaining cells in the DG of control ("CTL", a), locked wheel ("LOC", b) and running ("RUN", c) animals. Note that BrdU signal was slightly overdeveloped to show the granule layer. Insets show select cells (arrowheads) in greater detail. (d) Comparison of the total number of BrdU+ cells from individual animals in CTL (C1-C4; white), LOC (L1-L5; grey) and RUN (R1-R5; black) groups. (e) Quantification of BrdU+ cells reveals a significant, two-fold increase in the average number of BrdU+ cells in RUN animals when compared to both LOC wheel and CON animals. (f) Separation of BrdU quantifications into dorsal (CTL, LOC, RUN-D) and ventral (CTL, LOC, RUN-V) components shows that BrdU expression increased proportionately in dorsal and ventral components in running animals. (g) Triple-labelling fluorescence immunohistochemistry for BrdU, NeuN and GFAP. A representative photomicrograph of a newly-formed mature neuron in the granule cell layer of the DG. Higher magnification insets of the cell (arrowhead) show clear co-labeling of NeuN with BrdU. (h) There are no significant differences in the percentage of BrdU+ cells that are also NeuN+ in any of the three experimental groups (left). Because of the marked increase in the total number of BrdU+ cells in running animals ("RUN"), there are significantly more BrdU+/ NeuN+ newly-formed mature neurons in this group when compared to both locked wheel ("LOC") and control ("CTL") animals (right).** ***(SGZ = subgranular zone. Scale bar: 50 µm (a,b,c), 25 µm (g), 10 µm ("g" insets); ns = P>0.050, * = P≤0.050, ** = P≤0.010,*** = P≤0.001, One-way ANOVA with Tukey post-hoc test)***

To confirm that newly born post-mitotic neurons were increased only in the RUN group, as well as to ensure that our findings were not confounded by a proliferation-induced dilution of BrdU, we assessed expression of the calcium binding protein calretinin (Figure 5). Calretinin is transiently expressed in post-mitotic neurons at the Phase 2-3 transition (Figure 1e) (Brandt et al., 2003). Calretinin-immunoreactive neurons were found within the SGZ/GZ and were more numerous in RUN mice than in LOC or CTL mice (Figure 5a-c). Quantification revealed that the number of calretinin+ neurons was 2462±360 cells in CTL mice and 1901±222 cells in LOC mice, and increased to 9196±493 cells in RUN mice ($P<0.001$, one-way ANOVA, with Tukey's post-hoc test) (Figure 5d,e). This increase occurred to a similar extent in both the dorsal and the ventral regions of the hippocampus (Figure 5f).

Together, these results demonstrate that only running mice displayed an increase in overall cell survival and the generation of mature neurons.

FIGURE 5:

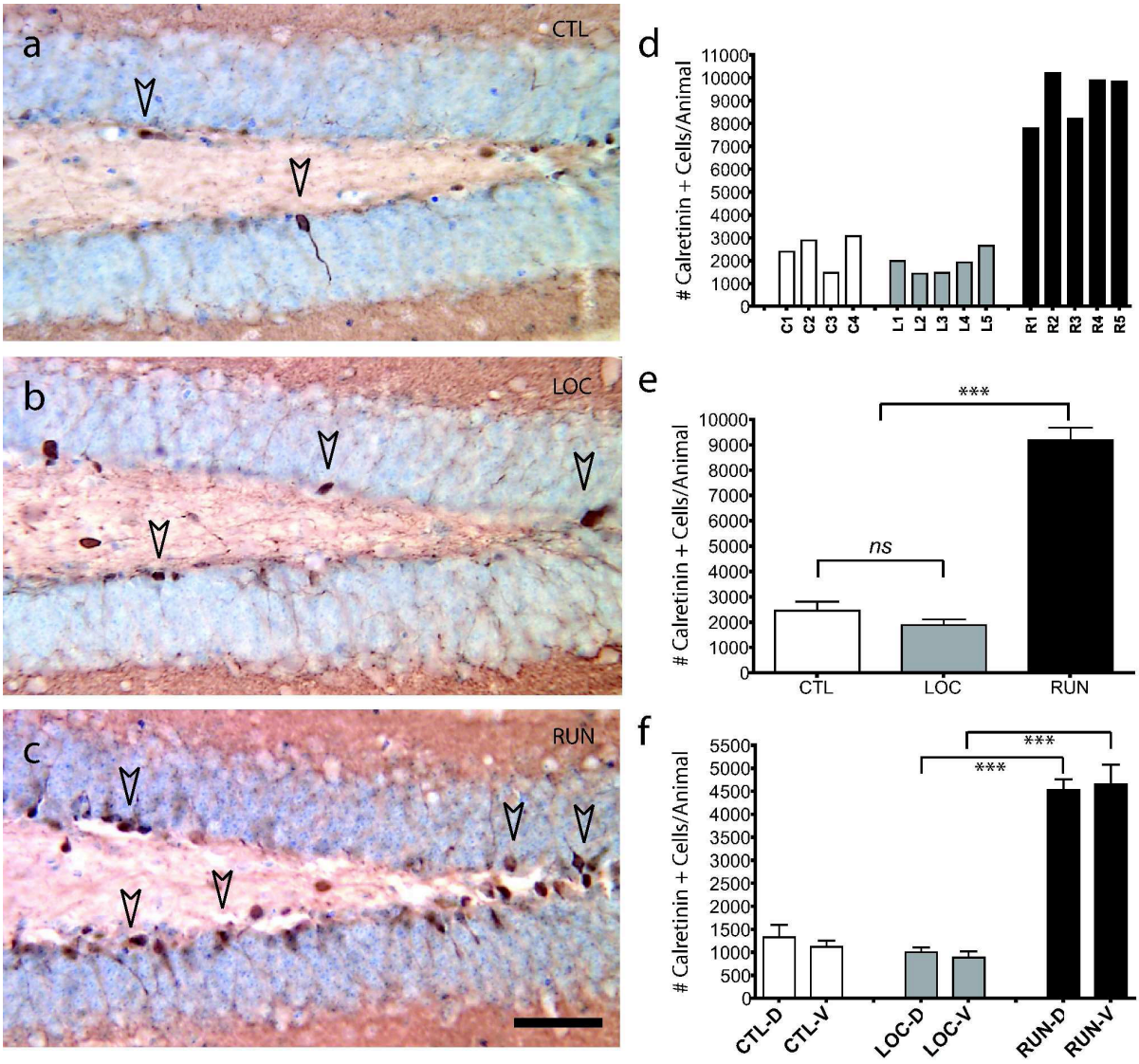


FIGURE 5. ***The number of new, immature neurons is increased in running, but not locked wheel mice.*** **(a,b,c) Photomicrographs of Calretinin expressing cells in control ("CTL", a), locked wheel ("LOC", b) and running ("RUN", c) animals. (d) Comparison of the total number of Calretinin+ cells from individual animals in CTL (C1-C4; white), LOC (L1-L5; grey) and RUN (R1-R5; black) groups. (e) Quantification of Calretinin+ cells shows a significant, four-fold increase in the average number of Calretinin+ cells in RUN animals, when compared to both LOC and CTL animals. (f) Separation of Calretinin quantifications into dorsal (CTL, LOC, RUN-D) and ventral (CTL, LOC, RUN-V) components shows that Calretinin expression increased proportionately in dorsal and ventral components in running animals.** ***(Scale bar: 50 µm (a,b,c); ns = P>0.050, * = P≤0.050, ** = P≤0.010,*** = P≤0.001, One-way ANOVA with Tukey post-hoc test)***

Running wheel exposure is sufficient to activate early lineage GFAP-expressing cells.

Dividing GFAP-expressing cells located within the SGZ are neurogenic precursors/stem cells (Fukuda et al., 2003; Seri et al., 2001; Suh et al., 2007). To determine whether the size of this infrequently dividing GFAP+ population of cells increased in response to locked wheel or running wheel housing conditions, we quantified the percentage of 40 day BrdU-retaining SGZ cells that co-expressed GFAP (Figure 6a). A total of 15-53 BrdU+ cells were counted per animal (3-4 sections/animal). Comparison of the CTL, LOC and RUN groups showed there was no significant difference detected in either the percentage of BrdU+ cells that co-expressed GFAP or the total number of BrdU+/GFAP+ cells/brain within the SGZ (Figure 6b), suggesting that running wheel exposure did not expand the size of the population of GFAP+ precursor/stem cells.

Previous work suggests that physical activity recruits GFAP-expressing neurogenic progenitors into the cell cycle within the SGZ (Suh et al., 2007). To investigate whether SGZ GFAP-expressing cells are equally activated in our locked wheel and running wheel housing conditions, we performed immunofluorescence co-labeling for Ki67 and GFAP (Figure 6c). A total of 30-109 Ki67+ cells were counted from each animal in all three experimental groups (3-4 sections/animal) and the relative percentage of Ki67+ cells that were also GFAP+ was determined from these quantifications. While no Ki67+ cells that were GFAP+ were found in CTL mice, 3.85±1.34% of Ki67+ cells co-expressed GFAP in LOC mice and 7.32±2.40% of Ki67+ cells co-expressed GFAP in RUN mice (running vs. standard-housed conditions, $P<0.050$, one-way Kruskal-Wallis ANOVA, with Dunn's post-hoc). There was no significant difference between LOC and RUN groups ($P>0.050$, one-way Kruskal-Wallis ANOVA, with Dunn's post-

hoc test Figure 6d). Conversion of these percentages into the total number of Ki67+/GFAP+ cells using the Ki67 quantitative data obtained earlier showed that the average number of Ki67+/ GFAP+ cells increased from 0.00 in CTL mice to 33.13±13.76 cells in LOC mice and 61.90±24.43 cells in RUN mice. Again, there was a significant difference in the number of cells between CTL and RUN mice ($P<0.050$, one-way Kruskal-Wallis ANOVA, with Dunn's post-hoc test), and no significant difference between LOC and RUN mice ($P>0.050$, one-way Kruskal-Wallis ANOVA, with Dunn's post-hoc test; Figure 6d).

Together, these results indicate that while none of our experimental groups underwent an expansion of their GFAP-expressing stem cell pool, quiescent GFAP-expressing precursors were activated to proliferate in similar numbers following exposure to either the locked wheel or running wheel.

FIGURE 6:

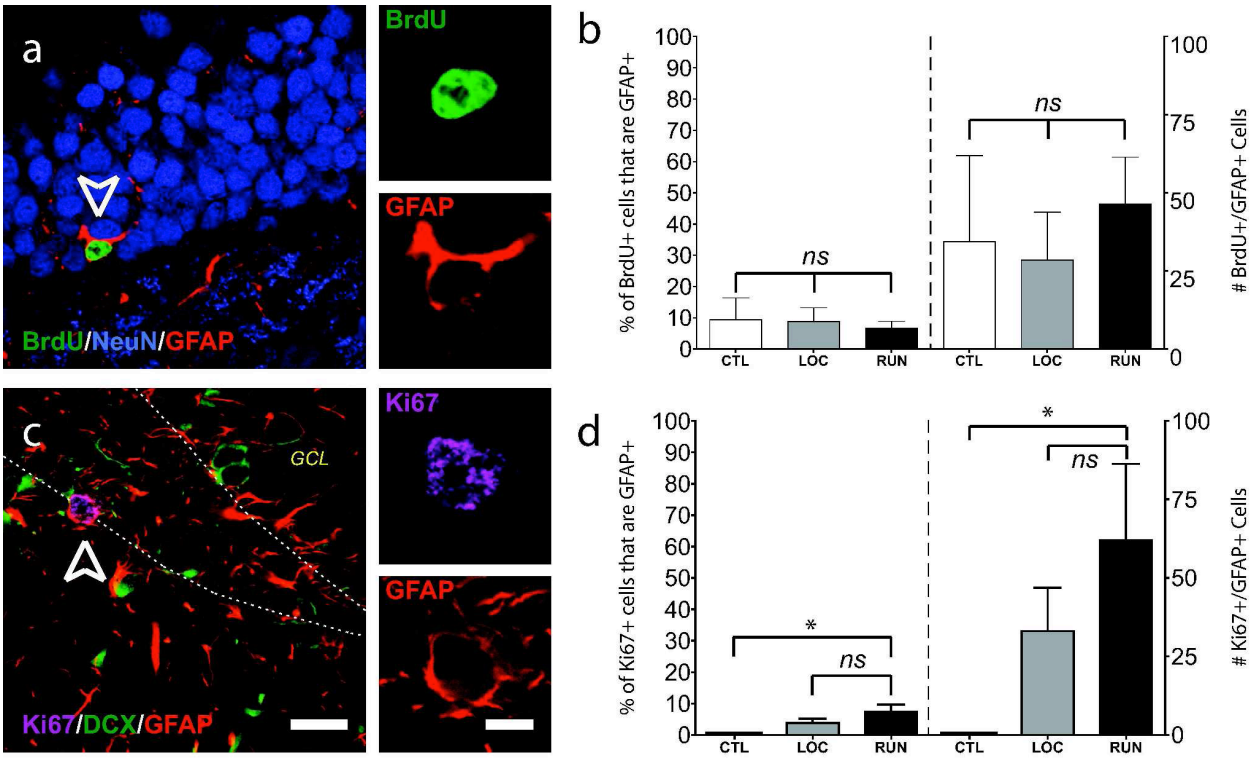


FIGURE 6. ***Number and proliferation of early GFAP+ precursor cells.*** **(a) A representative photomicrograph of a label-retaining GFAP+ cell in the subgranular zone (SGZ) of the DG. Higher magnification insets of the cell (arrowhead) show clear co-labeling of GFAP with BrdU. (b) Quantification of both the relative percentage of BrdU+ cells that are also GFAP + and the average number of BrdU+/GFAP+ cells per animal shows that there are no significant differences between any of the three experimental groups. (c) A representative photomicrograph of a proliferating GFAP+ in the SGZ of the DG. Higher magnification insets of the cell (arrowhead) shows a clear co-labeling of GFAP with Ki67. (d) Quantification of the relative percentage of Ki67+ cells that are also GFAP+ (left) reveals a significant increase between control ("CTL") and running ("RUN") mice. Quantification of the average number of Ki67+/GFAP+ cells per animal (right) shows a significant increase between CTL and RUN mice.** ***(GCL = granule cell layer, SGZ = subgranular zone. Scale bar: 25 µm (a,c), 10 µm (insets); ns = P>0.050, * = P≤0.050, ** = P≤0.010,*** = P≤0.001, One-way ANOVA with Tukey post-hoc test or one-way Kruskal-Wallis ANOVA with Dunn's post-hoc test.)***

The Phase 1-2 transition: Locked wheel and running wheel mice generate equivalent numbers of proliferative neuroblasts

The above results demonstrate that mice exposed to locked wheels and running wheels display similar levels of Phase 1 proliferating cells, while only running mice have elevated total numbers of Phase 2 neuroblasts and Phase 3 surviving cells/mature neurons. To determine whether LOC mice fail to generate neuroblasts or whether they generate neuroblasts that fail to persist, we examined the proliferative neuroblast stage (Ki67+/DCX+) at the transition from Phase 1 to Phase 2 of the neurogenic pathway. Ki67-expressing and DCX-expressing cells were identified using immunofluorescence, and a total of 30-109 Ki67+ cells were analyzed for DCX co-expression in each animal (3-4 sections/animal) from all three experimental groups (Figure 7a). Quantification revealed that the percentage of Ki67+ cells that co-expressed DCX increased from 6.73±2.67% in CTL mice to 15.55±1.35% in LOC mice and 16.49±2.72% in RUN mice (CTL versus RUN mice, $P<0.050$, one-way ANOVA, with Tukey's post-hoc). There was no significant difference between LOC and RUN animals (Figure 7b). Conversion of these percentages into the total number of cells/brain confirmed that the number of Ki67+/DCX+ cells in CTL mice (30.68±12.00 cells) increased by a similar amount in LOC mice (131.90±13.50 cells) and RUN mice (134.70±30.31 cells) ($P<0.050$, one-way ANOVA, with Tukey's post-hoc test; Figure 7b). Conversely, the number of post-mitotic neuroblasts was 4096±428 in the CTL group, 4042±516 in the LOC group and 9654±832 in the RUN group. Thus, mice exposed to either a locked wheel environment or a running wheel exhibit a similar increase in cell proliferation and initially generate equivalent numbers of proliferating neuroblasts, but these neuroblasts do not persist post-mitotically in the absence of wheel running.

FIGURE 7:

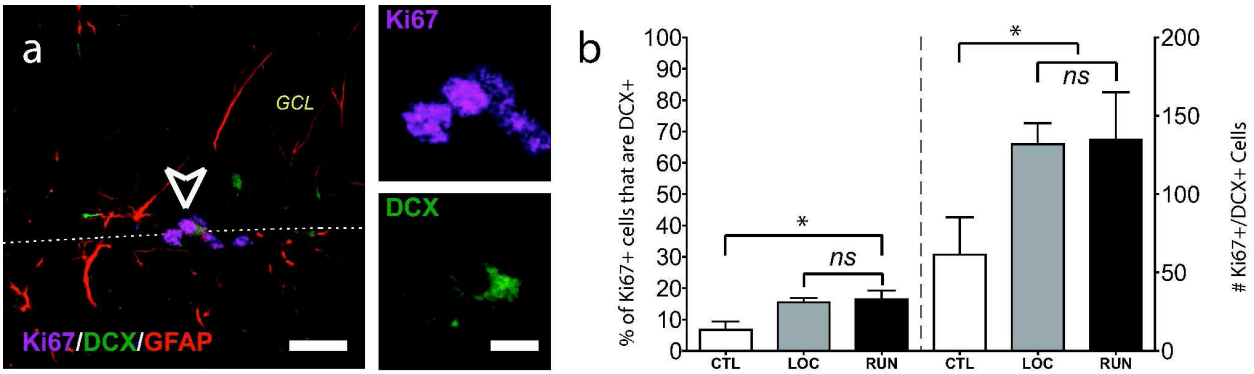


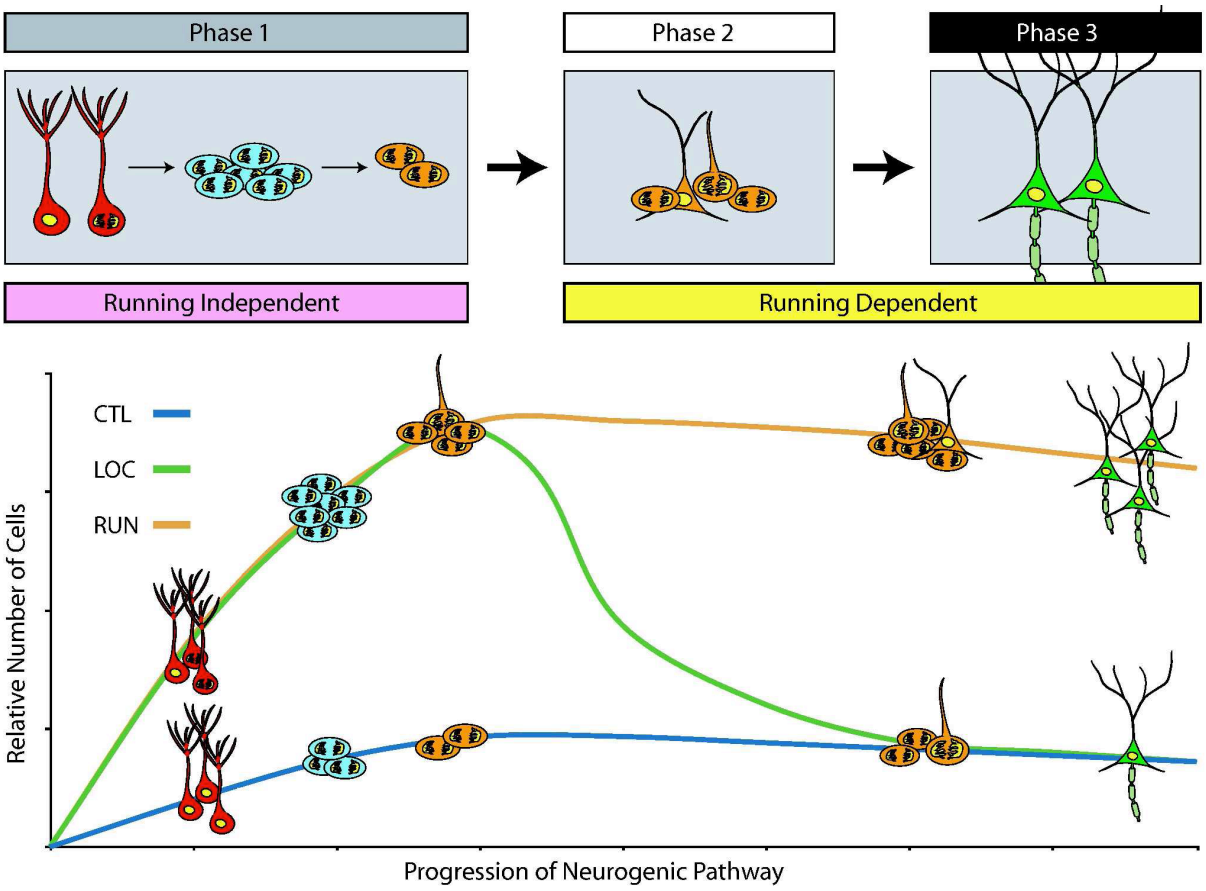
FIGURE 7. ***Locked wheel and running mice produce equal numbers of proliferating neuroblasts.*** **(a) A representative photomicrograph of proliferating neuroblasts in the SGZ of the DG. Higher magnification insets show a cluster of Ki67+ cells (arrowhead) that co-label with DCX. (b) Quantification of the relative percentage of Ki67+ cells that are also DCX+ (left) and the average number of Ki67+/DCX+ cells per animal (right) shows that running ("RUN") and locked wheel ("LOC") mice have identical numbers of proliferating neuroblasts.** ***(GCL = granule cell layer, SGZ = subgranular zone. Scale bar: 25 µm (a), 10 µm (insets); ns = P>0.050, * = P≤0.050, ** = P≤0.010, *** = P≤0.001, One-way ANOVA with Tukey post-hoc test)***

DISCUSSION

Adult hippocampal neurogenesis can be potently stimulated in rodents by increasing the complexity of the living environment, i.e., environmental enrichment. In the present study, we investigated how one specific form of environmental enrichment, exposure to a running wheel, promotes neurogenesis. Remarkably, we found that proliferation of neural precursors (Phase 1) was increased within the hippocampal neurogenic niche to the same degree in mice exposed to either locked or unlocked running wheels. This proliferating cell population contained both the same number of dividing GFAP-expressing stem-like cells and the same number of dividing DCX-expressing proliferative neuroblasts. On the other hand, the total number of post-mitotic DCX-expressing neuroblasts (Phase 2) and the Calretinin+ or NeuN+ neurons they generated (Phase 3) were increased only in mice housed with unlocked running wheels. These results suggest a model where running wheels are a source of multiple, functionally complementary, neurogenic stimuli: 1) running-independent stimuli that activate the early proliferative stages of neurogenesis and 2) running-dependent stimuli that promote survival and maturation of post-mitotic neuroblasts into mature neurons (summarized in Figure 8).

FIGURE 8. (FOLLOWING PAGE) ***Model of the influence of running-independent and -dependent stimuli on hippocampal neurogenesis*****. (upper panel) In this summary figure, a representation of the three broad phases of hippocampal neurogenesis is shown in comparison to where running-dependent and running-independent stimuli exert their primary effects. Running-independent stimuli promote increases in proliferation (Phase 1), while running-dependent stimuli enhance the survival and maturation of newly-formed neuroblasts (Phase 2) and mature neurons (Phase 3). (lower panel) A representation of the relative changes in the number of cells at each stage of the neurogenic process in CTL, LOC and RUN mice. During the first phase, locked wheel (LOC) and running (RUN) mice display identical increases in proliferation of early stem cell-like precursors, neural progenitors and mitotic neuroblasts. As the process continues, however, only running mice retain a larger total number of post-mitotic neuroblasts and fully mature neurons.**

FIGURE 8:



The running wheel environment stimulates proliferation in the hippocampal dentate gyrus in the absence of wheel-running

Comparison of Phase 1 proliferating cells in mice housed with locked and unlocked running wheels suggests that increased cell proliferation observed following running wheel exposure occurs entirely independently of running. Cell proliferation in the DG of locked wheel mice was comparable to that of running mice. In order to determine the identity of these proliferating cells, we co-labeled Ki67+ cells in the DG with markers for early progenitor/stem cells and for neuroblasts. We found that locked wheel and running mice were indistinguishable in

terms of the percentage and number of Ki67+/GFAP+ stem cells or Ki67+/DCX+ proliferative neuroblasts. Intriguingly, some of our findings are corroborated by a recent study that used locked wheel cages as a control environment (Fuss et al., 2009); that study also found no difference in Ki67 expression between running and locked wheel mice, however, the authors could draw no conclusions on whether the locked wheel itself increased proliferation as their study did not include a standard-housed control group.

We hypothesize that several potential mechanisms may mediate the proliferation induced by the locked wheel environment. In comparison to standard cages, the locked wheel cages represent a source of multiple forms of cognitive stimulation. The running wheel surface and rungs provide numerous tactile and visual stimuli that are not present in the standard housing environment. Moreover, because of the arrangement of the running wheel in the cage, mice are also provided with "hidden" spaces around, beneath and in the running wheel, in which they were frequently found to nest and burrow. The walls and hidden spaces created by the running wheel could result in modification of spatial maps in the hippocampus and related cortical structures (Derdikman et al., 2009; Lu and Bilkey, 2009), potentially necessitating changes in DG proliferation and neurogenesis. From a behavioral or psychological perspective, more places for hiding and burrowing may also have effects on cell proliferation by helping reduce stress and anxiety (Czeh et al., 2001; Malberg and Duman, 2003).

While formally possible, the effects of the locked wheel are unlikely to be due to exercise-related mechanisms. Mice in both the standard housing and locked wheel conditions engaged in apparently similar types of low intensity climbing and hanging behaviors and had no difference in weight, while the running mice ran up to 13 km per night and weighed

approximately 17% less at the time of sacrifice. Furthermore, the effects of running on cell proliferation were not greater than the effects of a locked wheel environment, as might have been expected if the effects of these two environments on proliferation were additive or at least partially dissociable. Moreover, in previous analyses of running mice, we found that the intensity of exercise correlated with production of DCX+ neuroblasts but not the number of Ki67+ proliferating cells (Bednarczyk et al., 2009). A recent study by Dubreucq and colleagues (Dubreucq et al., 2010b) has provided additional evidence of exercise-independent influences of the running wheel environment.

What is the fate of the proliferating Ki67+/DCX+ neuroblasts that are produced in locked wheel mice? While locked wheel mice produced the same number of mitotic neuroblasts as running mice, only running mice had larger numbers of post-mitotic neuroblasts and mature neurons. It is clear that Ki67+/DCX+ cells do not persist within the SGZ/GZ of the DG, as the number of BrdU-retaining cells, i.e., surviving cells, did not increase in locked wheel mice (Figure 4). It is also considered unlikely that these Ki67+/DCX+ cells differentiated into non-neuronal cells that migrated out of the granule cell layer; however, in this respect, it should be noted that multi-potential DCX-expressing cells have recently been reported to exist within the adult hippocampus (Walker et al., 2007). The most likely explanation is that, in the absence of running-induced factors (described below), these proliferating neuroblasts fail to be recruited or fail to survive the transition into post-mitotic neuroblasts (Biebl et al., 2000; Young et al., 1999). Due to the small size of this proliferating neuroblast population (<150 cells/brain; Figure 7), it is likely to be difficult to identify a difference in the number of pyknotic or apoptotic cells between the locked wheel and running groups. However, it would be interesting to determine whether

treatment with cell death-inhibiting factors would increase the number of post-mitotic neuroblasts in locked wheel mice.

Running-dependent mechanisms affect post-mitotic stages of the hippocampal neurogenic pathway

In our study, the specific effects of running on neurogenesis were first identifiable at the post-mitotic neuroblast stage (Phase 2). Our results showed that while the number of mitotic neuroblasts was increased equally in locked wheel and running wheel mice, the number of post-mitotic neuroblasts and newly generated neurons was only increased in running mice. Running wheel animals had increased total numbers of DCX+ cells, Calretinin+ cells, BrdU+ cells and BrdU+/NeuN+ cells relative to both control and locked running wheel animals.

A variety of interrelated mechanisms may mediate the effects of running or exercise on neurogenesis. Running mice have been consistently shown to have elevated levels of local and systemic growth factors and secreted hormones, including BDNF (Adlard and Cotman, 2004; Duman et al., 2008; Fuss et al., 2009), VEGF (Cao et al., 2004; Fabel et al., 2003a), IGF (Carro et al., 2000; Llorens-Martin et al., 2008; Trejo et al., 2001), FGF-2 (Gomez-Pinilla et al., 1997), corticosterone (Fediuc et al., 2006) and endorphins (Koehl et al., 2008). Running also induces structural changes, such as increased vascularisation, which could increase local oxygenation, metabolism and delivery of secreted factors (Palmer et al., 2000; Van der Borght et al., 2009).

It is also important to note that running could have psychological consequences that impact on hippocampal neurogenesis. A number of studies suggest that exercise has anti-depressive effects in rodents (Brene et al., 2007; Duman et al., 2008), and that treatment with

anti-depressant drugs is correlated with increased hippocampal neurogenesis (Boldrini et al., 2009; Czeh et al., 2001; Malberg and Duman, 2003). Interestingly, Takahashi et al. recently showed that rodent intracranial self-stimulation, an autonomous method of directly stimulating the pleasure centers of the brain, increased proliferation, survival and neuronal integration in the DG (Takahashi et al., 2009). If running is indeed a pleasurable activity for mice, and one that potentially contributes to survival in the wild, then running-induced neurogenesis may reinforce these behaviors and the contextual memories associated with them (Kempermann, 2002; Kempermann, 2008). Moreover, as discussed previously, because the hippocampus plays a role in spatial mapping, nocturnal wheel-running (Figure 1d) may increase hippocampal neurogenesis through unknown mechanisms in order to accommodate a larger perceived spatial environment. Interestingly, a previous study (Keith et al., 2008) found that training rats in spatial navigation tasks could increase the pool of DCX+ neuroblasts.

From a functional or mechanistic perspective, it should be noted that environmental enrichment of laboratory raised mice may actually be a closer approximation of the normal mouse environment, while standard housing might be more accurately considered as a model of sensory deprivation or inactivity (Cummins et al., 1977). In a recent study by Hauser et al. (2009), it was found that wild mice did not exhibit running induced hippocampal neurogenesis (Hauser et al., 2009). While it is possible that this could be due to differences in strain, it also raises a broader question about environmental "enrichment". For laboratory raised mice, an environment equipped with a running wheel and/or toys is enrichment because these animals are raised in a sterile environment lacking predators or the need for survival behaviors. For wild mice, the same "enriched" environment is greatly deprived in terms of external stimuli and may

not provide the same neurogenic stimuli. More generally, if the effects of wheel-running were entirely due to exercise, then different forms of exercise, performed at the same level of intensity, should yield comparable neurogenic increases. Unfortunately, only a few studies have directly or indirectly tested this idea (Liu et al., 2009; van Praag et al., 1999b). Additional studies are clearly required to understand how environmental enrichment and running enhance hippocampal neurogenesis.

CONCLUSIONS

In this study, we demonstrate that the neurogenic effects of running wheels are mediated by running-dependent and running-independent mechanisms that are dissociable and functionally complementary. Our findings lead us to conclude that the hippocampal neurogenic pathway is activated by running–independent processes that culminate in the accumulation of proliferating neuroblasts, and that running-dependent processes are subsequently required for the generation of post-mitotic neuroblasts and for their maturation into newly born neurons. A greater understanding of the biochemical and physiological events underlying these processes is an important future objective. Collectively, this work provides insight into how multiple aspects of environmental enrichment can collaborate to regulate adult hippocampal neurogenesis.

ACKNOWLEDGEMENTS

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GENERAL DISCUSSION

From the two studies presented in this thesis, one can draw two principal conclusions. First, animals that are given access to a running wheel for a prolonged period display increased hippocampal neurogenesis and both hippocampal and SVZ proliferation. Thus, prolonged running paradigms can directly influence the neurogenic process in the SVZ. Second, animals that are housed in a running environment, without any sustained physical activity, display increases in hippocampal proliferation, but not in hippocampal neurogenesis. Thus, there are both running-dependent and running -independent stimuli that affect the hippocampal neurogenic process in different ways.

Because of the implications of these conclusions, it is important to discuss and contextualize these findings. General points of discussion were addressed in the presented articles. Here, I will discuss some of the more specific controversies inherent to these studies and will present a general postulate about the mechanisms and reasons for running-induced increases in hippocampal neurogenesis.

1. PERSPECTIVES ON AND THE IMPLICATIONS OF OUR STUDIES

Wheel running and SVZ-OB neurogenesis

On the basis of a study by Brown and colleagues, there is a general consensus that physical activity does not influence forebrain and olfactory bulb neurogenesis (Brown et al.,

2003a). However, this study, while formidable in its own right, has several limitations in that it does not completely address the question of the influence of physical activity on forebrain neurogenesis.

To briefly outline the methodology of the Brown et al. study, mice were randomized and housed in groups in one of three experimental environments (Control, Enriched Environment, Running Wheel). They received 12 daily intraperitoneal injections of BrdU beginning on the day when they were separated into their experimental groups. To assay proliferation, one cohort of animals was sacrificed 1 day after the last injection (day 13). The remaining animals were allowed to continue living for a period of 43 days in order to assess cell survival and olfactory bulb neurogenesis.

As a first point of contention, the period of voluntary wheel running that was used to assess proliferation in this study was of only two weeks. While this serves as an adequate reference for a baseline level of physical activity, it does not approximate more robust regimes of physical activity or the type of sustained activity that a wild animal would engage in in its day-to-day living. Thus, while a two-week running regime might not have any acute effects with respect to SVZ neurogenesis, it is possible that a longer running period could indeed produce some sort of stimulation. The second cohort of animals had run for a period of 6 weeks, but could not be assessed for proliferation via BrdU labeling. After a delay of 21 days from the time that these animals received their BrdU injections, most of the BrdU-retaining cells would have migrated out of the SVZ to the olfactory bulb. Cells retaining a signal in the SVZ proper, therefore, would represent surviving and immobile stem cells (or the like) rather than proliferating precursors.

As a second point of contention, the manner in which BrdU was administered to measure proliferation does not necessarily give a good estimation of SVZ proliferation. Because BrdU was injected over the course of 12 days, on the day of sacrifice, many of the same labeled cells would have long since stopped proliferating. Moreover, a sizable portion of cells labeled during the first few days of physical activity would have already begun to migrate out of the SVZ, thus reducing the overall number of BrdU-labeled cells in the SVZ proper.

There is, however, comparatively little that can be criticized in the finding that the number of newly-formed olfactory bulb neurons was not increased by physical activity or environmental enrichment. These results were determined by assessing the total number of BrdU-labeled cells in the olfactory bulb 43 days after injection and by assessing the percentage of these cells that were co-labeled for NeuN, a marker of mature neurons. However, in this instance, there were still some lacunae which need to be addressed. With the long-term BrdU labeling protocol, it cannot be excluded that the number of new olfactory neurons simply represented surviving cells, rather than an absolute number. As previously discussed, there is a large surplus of neuroblasts entering the olfactory bulbs via the rostral migratory stream. The vast majority of these neurons will die, instead of being integrated into the olfactory bulb networks. Thus, in the study of Brown et al. (2003a) even if fewer surviving neurons were detected, this could be attributable to other factors, such as a lack of olfactory novelty or enrichment rather than physical activity. Moreover, because BrdU-labeling took place during the first 12 days of physical activity, it is possible that proliferation in the SVZ was not as high during these first few days in running mice and that, as a result, there were fewer proliferating cells to label. Consequently, there could still be higher rates of olfactory bulb neurogenesis in

running animals, although in this case, it would be impossible to detect with BrdU co-localization.

A final point to consider with respect to the conclusions drawn by the Brown et al. study (2003a) is that animals were housed in groups in their respective environments. For one, with this arrangement, it is impossible to assess the individual activity of any particular animal. It becomes impossible, therefore, to correlate or link the level of physical activity with the level of proliferation or neurogenesis. More importantly, however, the social hierarchy that results from cohabitation can directly influence some aspects of hippocampal neurogenesis and the olfactory signals therein could influence olfactory bulb neurogenesis. Thus, these external stimuli could mask any changes that are occurring because of physical activity.

Because of these controversies, we set up an experiment to more thoroughly assess the affects of physical activity on SVZ proliferation and olfactory bulb neurogenesis. In our study, animals were housed individually and were allowed to run for a protracted period of 6 weeks. We then assessed SVZ proliferation by using Ki67, which is a robust marker of proliferation at the time of sacrifice. In doing so, we were able to show that, while modest, there were quantifiable changes in proliferation in the SVZ following this prolonged running period.

While the fact that proliferation in the SVZ can be stimulated by wheel-running is interesting in itself, this finding also provides some insight into the influence of exercise in the CNS. Basically, the increase in SVZ proliferation is small when compared to the large increase in hippocampal proliferation (and neurogenesis). Given that the SVZ is highly vascularized, located in close proximity to the lateral ventricles and is relatively devoid of innervation, it would seem that “exercise” (or, the structural changes and secreted factors associated with it) alone can

influence proliferation in this structure. On the other hand, it is unlikely that exercise alone accounts for increases in proliferation and neurogenesis in the hippocampus, as, for example, simply activating the excitatory perforant path is sufficient to increase proliferation and neurogenesis in this region (Cameron et al., 1995). Therefore, the hippocampus is likely subject to additional or parallel stimulation from cognitive stimuli, while the SVZ, by comparison, would not be heavily influenced by these. If we consider these facts on a purely theoretical basis and "subtract" the influence of cognitive stimuli from both the SVZ and hippocampus, we are left with an entirely exercise-dependent component. Using the relatively small increase in proliferation in the SVZ as a guideline, it appears that exercise by itself may have little to do with the large changes that are seen in the hippocampus following a wheel-running paradigm.

The small effect of exercise in a 6-week running period (both in the SVZ and, hypothetically, in the hippocampus) is not particularly surprising, considering the time that it takes for either cognitive or exercise-dependent stimuli to exert their effects in CNS. The effects of cognitive stimuli are, for all intents and purposes, instantaneous. The instant that a stimulus is presented to an animal, there is a broad activation in a number of CNS regions, and, if the stimulus is a "hippocampal-dependent" one, hippocampal activity is concomitantly increased. Thus, without much delay, it should be possible to directly or indirectly stimulate neural precursor activity in the hippocampus. Conversely, purely exercise-dependent changes, such as changes in vasculature or metabolism, may take much longer to manifest in CNS structures and the germinal regions of the SVZ and hippocampus.

Given that it takes between 2-4 weeks for a neural precursor to produce a mature and integrated neuron in both the SVZ-OB and hippocampal system, a 6-week running paradigm

might simply not be long enough to really show changes in neurogenesis that occur as the result of exercise. At the time of sacrifice (after this 6-week period), it is possible that some exercise-related changes in vasculature or metabolism have taken place. Still, for the majority of new cells that have long since exited the cell cycle, it would be impossible to demonstrate any exercise-dependent effect, as they would no longer be "subject" to direct regulation by secreted factors or the like. Thus, if one were to truly evaluate the effects of exercise on neural precursor activity, it would be wiser to design running paradigms that span over many months, i.e., over a significant portion of the life of a rodent. If exercise indeed promotes systemic and local changes in metabolism and vasculature, it would then be easier to visualize their neurogenesis-promoting effects, as large numbers of cells would have been born and would have matured during this time in neurogenic structures.

Wheel-running and hippocampal neurogenesis: What is the influence of the running environment?

With respect to the second study presented in this thesis, it is important to differentiate between exercise-dependent and exercise-independent effects on neurogenesis. With all of the possible cognitive stimuli inherent to wheel running and the wheel running environment, it seems reasonable to conclude that cognitive stimuli alone can, at the very least, influence some aspects of hippocampal neurogenesis. Indeed, according to our study, animals that are housed in a locked-cage environment produce more proliferating precursors and neuroblasts than their control counterparts, to a level comparable to that of running animals. Therefore, without any

significant amount of exercise, cellular proliferation is increased in the hippocampus. It is important to note, however, that there are numerous behavioral differences between the three experimental groups (CTL, LOC, RUN) and that there were confounding or contentious elements in our study.

One could reasonably argue that animals with an increased living space, such as that provided by a locked cage, could be "exercising" more by virtue of the fact that they have more space to explore. Indeed, one study (Koteja et al., 1999) found that animals housed in locked cages spend as much as one-third of their active (night) time hanging from or exploring the running wheel. However, lid hanging, where an animal hangs from the bars of the cage cover, is a common phenomenon in all types of animal housing (Steele et al., 2007), including standard control cages. Hanging from a locked wheel could just be replacing, rather than supplanting the normal lid-hanging behavior. Moreover, wheel-running itself can be broken down into time spent running and time spent hanging from said running wheel. It has been reported that mice running on a free wheel spend as much as one-third of that time hanging onto or from it (Koteja et al., 1999), rather than actively "exercising." Furthermore, active use of a running wheel does not supplant normal behaviors. Rather, it takes away from the time in which an animal spends moving about the cage, such that the use of the running wheel is a behavior which eats into the time an animal would otherwise spend foraging, lid-hanging, etc.. All in all, the most robust measure of whether or not an animal has engaged in sustained exercise comes from the gross weight of the animal after the experimental period. In our study and in other reports, animals that are housed with a freely-rotating running wheel will consistently record a lower body weight

than their locked-wheel or standard-housed counterparts (Burghardt et al., 2006; Koteja et al., 1999; Swallow et al., 1999).

The question of whether a locked wheel, or the stimulus provided by the running environment, can affect CNS function has not yet been explored in great detail. However, one recent study hints at the fact that there are measurable differences in the complexity of the standard housed, locked and unlocked running wheel environments that can manifest as differences in animal behavior (Dubreucq et al., 2010b). For example, mice housed in unlocked and locked running-wheel cages show similar levels of contextual fear and less anxiety (via a light/dark-box test) when compared to control animals. Moreover, mice with access to a locked running wheel showed less behavioral despair (via a forced swim test), although the precise conclusion of this type of test is somewhat controversial. It could be, then, that subtle differences in the environment can influence an animal's behavior and could potentially affect hippocampal proliferation or neurogenesis.

While the cognitive influence of a locked-wheel environment is certainly evident, it is also highly likely that wheel running is a cognitive stimulus. It is the informed opinion of this author that exercise, *per se*, could have little or nothing to do with increases in neurogenesis in the hippocampus and that, in fact, it is just masking the influence of more subtle stimuli, such as spatial memory dynamics, navigation, novelty, reward mechanisms and mood. Unfortunately, it is difficult to experimentally isolate "exercise" from any cognitive stimuli, such as wheel running, as they are all invariably connected to one another. It is imperative, therefore, that future studies address this fundamental question.

With respect to the question of "exercise" vs. "cognitive stimuli," the types of controversies and questions presented here have wide-ranging implications for the field of human health. For example, based on current research, it would seem "reasonable" to recommend a regime of physical activity to persons suffering from depression, Alzheimer's disease, etc. where hippocampal neurogenesis is presumed to be severely compromised. While there is certainly no harm in engaging in exercise, it is entirely possible that, in humans, physical activity does nothing by itself to rescue or promote neurogenesis. One must remember that rodents and humans differ greatly in terms of behavior and "life priorities." To the modern, more-or-less sedentary human, physical activity might only be of secondary or tertiary importance, rather than a function that supports "living." This is not to say that it could not influence neurogenesis, just that perhaps its effects would be minimal in comparison to those produced when a rodent engages in sustained physical activity. Within this realm, it is entirely possible that, for a human, simply engaging in hippocampus-dependent cognitive tasks would suffice to stimulate neurogenesis in that structure. As such, from a therapeutic point of view, it is of crucial importance to study the effects of cognitive tasks and training on cellular plasticity, as it could prove beneficial for the treatment of certain diseases where neurogenesis is severely compromised.

2. WHEEL-RUNNING AS A COGNITIVE STIMULUS AND ITS INFLUENCE ON NEUROGENESIS

As outlined previously, it is highly likely that wheel-running is itself a cognitive stimulus. Therefore, I hypothesize that exercise is only of secondary or tertiary importance with respect to the mechanisms underlying increases in hippocampal neurogenesis following voluntary wheel running. To date, there has been some speculation about this hypothesis (Kempermann, 2002; Kempermann, 2006; Kempermann, 2008). Here, I will present some candidate mechanisms that may be responsible for these changes in neurogenesis as well as some novel insight into why neurogenesis might increase in response to wheel-running.

Contextual memories and running: Is the brain rewarding a "good" behavior?

Fundamentally, physical activity is primordial to the survival and success of a given organism. While physical activity has mainly been relegated to a recreational activity for many humans, the overwhelming majority of animals depend on it for their day-to-day survival. For example, a wild mouse must travel over relatively long distances and forage continuously if it is to find adequate sustenance. Similarly, the mouse must be capable of avoiding predation by finding suitable places to burrow and hide, and by fleeing at the sight of danger. Thus, engaging in physical activity can be correlated with an animal's survival and is, therefore, "rewarding" in and of itself.

The mesolimbic structures of the brain are central to reward and reinforcement in mammals. The most studied and most well-known of the reward pathways is the dopaminergic ventral tegmental area (VTA) to nucleus accumbens pathway. Because the relationship between these structures and the hippocampus has not been thoroughly studied, the hippocampus has not traditionally been considered in influencing reward and reinforcement. Nonetheless, some recent studies have elucidated several important pathways between the hippocampus and the reward centers of the mesolimbic system (Cooper et al., 2006; Floresco et al., 2001; Floresco and Grace, 2003; Floresco et al., 2003; Legault and Wise, 2001; Lisman and Grace, 2005; Samson et al., 1990; Thierry et al., 2000). For example, the subiculum, which interfaces with the entorhinal cortex and the CA1 pyramidal cells, serves as a bridge between the hippocampus and some reward structures, such as the nucleus accumbens and prefrontal cortex (Cooper et al., 2006). From this, and given the role of the hippocampus in memory consolidation, it is reasonable to believe that the contextual memories of particular rewards (i.e., "was this behavior good or bad for me; what type of behavior is better for me?") may be processed, in part, by the hippocampus. Consequently, there is a growing body of evidence to suggest that the hippocampus may be involved in remembering/analyzing the rewards and risks of miscellaneous behaviors (Kumaran et al., 2009; Okatan; Okatan, 2009; Rolls and Xiang, 2005; Tracy et al., 2001; Vanni-Mercier et al., 2009) and may, therefore, be an important contributor to reward mechanisms.

With respect to adult neurogenesis, a recent study demonstrated that intracranial self-stimulation (ICSS), via an electrode implanted into the VTA, induced potent increases in hippocampal neurogenesis (Takahashi et al., 2009). The authors use physical activity, or wheel running, as a basis of conceptual comparison because the running-wheel behavior seems to be

"rewarding" for rodents and could affect mesolimbic reward structures (Belke and Hancock, 2003; Iversen, 1993; Pierce et al., 1986; Ralph et al., 2002; Vargas-Perez et al., 2003; Wilson and Marsden, 1995). Indeed, without any additional impetus from experimenters, mice will run voluntarily for great distances on a running wheel, as was the case in both of the studies presented in this thesis (Bednarczyk et al., 2009). Hypothetically, in the case of both wheel-running and ICSS, the animal "learns" that these behaviors are "rewarding" and, potentially, the hippocampus accommodates these new memories via increased neurogenesis. On an evolutionary level, it seems perfectly reasonable that the brain would "reward" such behaviors as running, as they are essential for the survival of the animal. An increase in hippocampal neurogenesis resulting from wheel running, then, could serve as one mechanism for ensuring that the animal continues to perform such a behavior.

Mechanisms of reward and reward-induced changes in neurogenesis

On a mechanistic level, it is important to consider the role of certain secreted factors, such as the endorphins, in mitigating or, potentially, producing the rewards attributable to certain behaviors. It has already been demonstrated that physical activity increases CNS levels of endogenous opiates, like beta-endorphins (Boecker et al., 2008), and that this directly influences hippocampal neurogenesis, as outlined previously (Koehl et al., 2008). However, endorphins are also secreted in response to other "rewarding" activities, such as excitement, substance use/abuse and sexual activity and form part of the general mechanism for producing positive reinforcement (Le Merrer et al., 2009). In accordance with what was previously discussed, beta-endorphins and

other opioids might influence the hippocampus in reward and addiction by influencing the contextual memories of certain pleasurable activities (Dong et al., 2006; Drake et al., 2007; Ito et al., 2008; Le Merrer et al., 2009; Sharifzadeh et al., 2006; Stanley et al., 1988; Tracy et al., 2001).

It is also interesting to note that the endogenous cannabinoid system influences and serves in the reward mechanisms of the CNS (Solinas et al., 2008; Solinas et al., 2006; Zangen et al., 2006). Moreover, endocannabinoids directly influence neurogenesis and other CNS phenomena and are relevant when considering the impact of physical activity on the brain. Indeed, secretion of anandamide, an endogenous cannabinoid, is increased in reward situations (Caille et al., 2007; Kirkham et al., 2002) and in physically active humans (Sparling et al., 2003), and could contribute to the psychotropic effects that are described as the "runner's high" (Dietrich and McDaniel, 2004; Fuss and Gass, 2010). More importantly, a recent study has shown that when the CB1 receptor is knocked out ($CB1^{-/-}$) in running mice, thus mitigating the effects of endocannabinoids, total voluntary running distances decrease significantly when compared to $CB^{+/+}$ animals, with a resulting decrease in the total number of neuroblasts, although the latter is not directly attributable to the running distances (Dubreucq et al., 2010a). To rationalize this view, it seems that decreased endocannabinoid efficacy results in decreased volition or motivation and a consequent decrease in running performance. Supporting this finding, acute treatment with the CB1 receptor inverse agonist Rimonabant (SR141716) results in decreased daily running distances in rodents (Keeney et al., 2008). It seems plausible that neurogenesis contributes to this system of reward, in that, independent of external stimuli, cannabinoids mediate proliferation and neurogenesis in the hippocampus and can be anxiolytic

(Aguado et al., 2005; Jiang et al., 2005; Jin et al., 2004). Indeed, as is the case with beta-endorphins, one study suggests that endocannabinoids are absolutely required to produce running-induced increases in neurogenesis (Hill et al., 2010), although this finding is still somewhat controversial (Dubreucq et al., 2010a; Dubreucq et al., 2010b; Fuss and Gass, 2010). Thus, endocannabinoids may be another or complementary mechanism by which running and hippocampal neurogenesis are influenced by rewarding or pleasurable activities (Fuss and Gass, 2010).

Endorphins and endocannabinoids are some examples of how endogenous compounds that are secreted in response to pleasurable or rewarding activities can influence behavior, hippocampal function and, to some degree, neurogenesis. Cognizant of the actions of these compounds, and of the reward mechanisms of the brain, one could hypothesize that hippocampal neurogenesis and function could be influenced directly by the reward of pleasurable activity itself, rather than by complex metabolic or systemic changes. Expanding on this hypothesis, it would be interesting to see if other "rewarding" activities, such as sexual behavior, could also mediate hippocampal neurogenesis.

The influence of mood and well-being on neurogenesis

Surprisingly, mood and feelings of well-being seem to directly influence hippocampal function and neurogenesis and/or result from changes in neurogenesis. There is ample evidence to support that neurogenesis is perturbed in depressed animals and that stimulation of neurogenesis could be instrumental in successful treatment of depression via

pharmacotherapeutics (Duman et al., 2001; Lucassen et al., 2010a; Lucassen et al., 2010b; Sahay et al., 2007; Sahay and Hen, 2007). Conversely, wheel-running and environmental enrichment have been shown to have anti-depressive and anxiolytic effects and qualities, which coincide with increases in neurogenesis. Thus, a link between neurogenesis and mood (specifically depression) has been forged, although the causal relationship between these phenomena is still contested. Hypothetically, it is possible that mice and other rodents find the running activity pleasurable, which is likely due to reward mechanisms and the concomitant secretion and action of factors such as beta-endorphins and endocannabinoids, as well as the influence of acetylcholinergic, noradrenergic and serotonergic innervation.

Novelty as a mediator of neurogenesis

As outlined previously, the hippocampus has several distinct functions within the CNS. While traditional neuroanatomical models tend to compartmentalize and localize cognitive functions to specific structures, recent research has demonstrated that this is not always the case in the CNS. The hippocampus and mesial temporal lobe, for example, do not just assure the "generation" and consolidation of declarative memories (Kumaran and Maguire, 2009). Rather, they host a wide variety of cognitive functions, ranging from short-term or working memory (Hannula et al., 2006; Hartley et al., 2007; Olson et al., 2006a; Olson et al., 2006b) to implicit memory (Greene, 2007), imagination (Hassabis et al., 2007) and perception (Lee et al., 2005a; Lee et al., 2005b). Underlying all these phenomena, however, is the ability to detect novelty,

which may turn out to be the most important characteristic or function of the hippocampus (VanElzakker et al., 2008).

Novelty directly influences how cognitive processes are "computed" within the hippocampus. As such, one can think of any stimulus as having three basic types of novelty inherent to it: 1) stimulus novelty ("Have I seen this stimulus before?") 2) associative novelty ("Is this stimulus presented in the same configuration in which I had previously seen it?") and 3) contextual novelty ("Have I seen this stimulus in this context before?") (Brown and Aggleton, 2001; Kumaran and Maguire, 2007a; Kumaran and Maguire, 2007b; Nyberg, 2005; Ranganath and Rainer, 2003). These different types of novelty, then, will affect how contextual memories and experience are processed not only within the hippocampus, but within other CNS structures as well.

Since neurogenesis has been implicated in hippocampal memory functions (Aimone et al., 2009; Deng et al., 2010; Deng et al., 2009), it would seem entirely reasonable to assume that it can be affected by or can affect the processing of environmental novelty. Surprisingly, there are few extant studies that directly or indirectly addresses this question in vivo. In a 1999 study, Lemaire and colleagues showed that a behavioral trait in rodents that increases reactivity to novelty is inversely correlated (although not causally linked) to levels of hippocampal neurogenesis (Lemaire et al., 1999). A more recent study by Vayrac and colleagues demonstrated that with olfactory enrichment, there is a strong increase in olfactory bulb neurogenesis and that this depends on the novelty of the experimental stimuli, rather than on the presence of said stimuli (Veyrac et al., 2009). Unfortunately, Veyrac and colleagues did not look at how hippocampal neurogenesis is affected by olfactory novelty.

From these two studies, it is hard to draw a strong conclusion about how novelty might affect hippocampal neurogenesis. However, one can still hypothesize that the complexity and novelty of the running environment/behavior might directly influence hippocampal neurogenesis. This rational comes from how the running animal might perceive his/her running environment. For example, mice that have access to a free running wheel might perceive the running activity as being continuously novel, in that the experience of running is completely "new" to them at every opportunity (i.e., each new running period could affect the associative and contextual novelty of the activity). Conversely, mice in a locked wheel/control environment might, at first, react to the stimulus novelty but, because the environment is more or less static, will not perceive any sustained contextual or associative novelty.

Some evidence for this hypothesis comes indirectly from a study by Hauser and colleagues that examined the effects of wheel-running on neurogenesis in wild-caught mice (Hauser et al., 2009). Surprisingly, this study showed that these mice, when exposed to voluntary running in the laboratory setting, did not produce the same increases in hippocampal proliferation and neurogenesis that are seen in laboratory-raised animals, despite running comparatively enormous distances. As discussed in the second article, it is possible that this difference could result from characteristics inherent to the strain of animals (CD1, C57 vs. Wood Mice).

A more elegant explanation, however, is that the types of stimuli that are provided by laboratory voluntary wheel-running cannot compare (in terms of complexity and novelty) to those provided by the natural world. The vast majority of mice used in the experimental setting of laboratory research are obtained from sterile in-house colonies or via large-scale suppliers,

such as Charles River. In those settings, animals live a relatively quiet and peaceful life with food and water being provided to them *ad libitum*. Moreover, they live in an environment free of natural predators and, to some extent, free of any significant complexity. Conversely, wild mice grow up and live in an environment full of natural predators and which they must constantly explore in order to find sustenance. It stands to reason, therefore, that when wild mice are introduced to the laboratory setting, they are suffering from a significant lack of external stimuli as compared to their normal living environment. When laboratory-raised animals are presented with a running wheel or enriched environment, there is a marked increase in all types of novelty from their perspective. Conversely, wild-caught animals might only perceive a transient form of novelty that comes from their transition to this new setting, instead of a sustained stimulus. Surprisingly, this perspective on "environmental un-enrichment" has been considered for quite some time by researchers in this discipline (Cummins et al., 1977). In this line of thinking, one can hypothesize that the stimulating actions of wheel running are relatively insignificant to the wild-caught mouse and that, consequently, this activity provides no additional novelty which could increase neurogenesis in these animals.

Spatial memory, navigation and its influence on neurogenesis

While reward, memory formation and novelty are all important contributors to hippocampal function and neurogenesis, it is also necessary to consider how the spatial memory and navigation functions of the hippocampus are affected by physical activity. Basically, any modification of the dimensions or layout of the environment, as is the case with wheel-running

and/or compartmentalization, is sure to influence how the hippocampus and related structures process this new information.

Importantly, many pyramidal cells in the CA1 and CA3 regions of the Cornu Ammonis, as well as granule cells in the dentate gyrus, serve as place cells, i.e., cells that fire in a specific location within a given space (Moser et al., 2008; O'Keefe and Dostrovsky, 1971). Moreover, the entorhinal cortex contains grid cells, whose behavior is similar to place cells, except that they may fire in several different locations within a given environment (in a triangular, grid-like pattern), providing a "meta-representation" of said environment (Fyhn et al., 2004; Hafting et al., 2005; Jacobs et al.; Moser et al., 2008). The activity of these two types of cells is related, as the entorhinal cortex ennervates and receives input from the dentate granule cells and CA pyramidal cells respectively. As such, place and grid cells, along with head direction cells (Sargolini et al., 2006) and spatial view cells (Georges-Francois et al., 1999), contribute to representing the spatial layout of a particular environment in the normal function of the hippocampus. The behavior of these diverse cells is well characterized in a static environment, but it is only recently that researchers have studied how the firing of these cells changes when the environment itself is modified. In a definitive study, Moser and colleagues demonstrated that if an environment is compartmentalized, the firing pattern of grid cells is completely re-organized (Derdikman et al., 2009). Furthermore, this same group had previously demonstrated that place cells re-organize completely (i.e., fire in a different place) when grid cell firing is changed (Fyhn et al., 2007). Thus, it would appear that compartmentalization or re-organization of an environment induces changes in the behavior of cells within the entorhinal cortex, pyramidal cell layer and dentate gyrus granule cell layer.

Consequently, when an animal is exposed to a locked or unlocked running wheel cage, there is a significant increase in spatial complexity when compared to the standard housing cage. The running wheel effectively compartmentalizes the environment and adds a third, vertical dimension that can be explored by the animal, thus (transiently) altering grid and place cell firing patterns. More importantly, if an animal is actively engaged in wheel running, then, from its perspective, the running wheel provides an infinitely long, linear track, that may implicate or result in reorganization of spatial maps in the hippocampus. To date, there are a large number of studies that look at grid/place cell firing in freely-moving rodents, usually in a large, open environment, and these results alone support a dynamic spatial representation system in the hippocampus. It would be interesting, however, to see if wheel running or similar activities result in changes in the firing and patterning of grid or place cells.

It is important to note that a running wheel "tricking" a rodent into thinking that the spatial environment has changed is a perfectly plausible hypothesis. As it stands, grid cell firing does not depend on environmental stimuli or any regularity in said environment. Rather, the meta-representation that grid cells produce is a synthetic a priori, i.e., is innate or is limited to the "mind." Therefore, it derives from integration of many different intrinsic signals (vision, movement, etc.), which depend heavily on perception. As such, if the perception of the environment has changed, irrespective of any "actual" changes, then it is perfectly plausible that the grid/place cell firing/patterning follows suit. Some evidence for this hypothesis comes from a recent study that, for the first time, described grid-cell activity in the human brain (Doeller et al., 2010). In order to look for grid cells, this group used fMRI in combination with a virtual environment (that mimics running and foraging), which was presented to the subjects as they

were being imaged. Surprisingly, this study found conclusive evidence for grid cell-like activation in the entorhinal cortex, the peculiarities of which go beyond the scope of this study. Still, by just perceiving movement, rather than actually engaging in it, there was a significant activation of putative grid cell-like structures, which (indirectly) supports that perception can influence hippocampal activity.

As a result of hypothetically increased hippocampal activity resulting from changes in the layout of the environment, and because of a potential need to accommodate new spatial memories or to increase processing of said information, it is possible that new neurons must be produced and integrated to maintain proper hippocampal function. However, this is still largely speculative and has yet to be tested.

CONCLUSIONS

In summary, the studies that I have undertaken have allowed me to draw two principal conclusions about the regulation of adult neurogenesis by external stimuli. First, neurogenesis in both the forebrain and hippocampus is susceptible to regulation by a prolonged period of physical activity. Second, voluntary wheel running is a complex behavior and its effects can be divided into running-dependent and running-independent components, both of which influence different aspects of adult neurogenesis in the hippocampus.

From this work, I have gained important insight into the potential regulators of neurogenesis and how they may contribute to the function of the hippocampus and SVZ in the healthy and pathologic CNS. As such, this work has provided insight not only into the influence or regulation of neurogenesis in the hippocampus and SVZ, but to the large-scale functions of these structures in both the healthy and the pathologic CNS.

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