The notion that new neurons are added to the adult brain has been the subject of controversy ever since the mid-1980s, when Fernando Nottebohm's lab reported that adult neurogenesis occurs in the canary brain and is functionally linked to seasonal acquisition of new song. Early debates focused on whether or not this phenomenon was restricted to birds, but then came incontrovertible evidence of adult neurogenesis in rodents, specifically in the dentate gyrus of the hippocampus and the olfactory bulbs. Now, almost 30 years later, the debate is centered on the extent to which these findings in birds and rodents generalize to non-human primates and humans, and if they do, whether there are temporal and/or spatial (brain region) limits to postnatal neurogenesis in monkeys and humans? Nottebohm himself has stayed out of the fray, maintaining that the important question is how species solve problems that are relevant to them in nature. On the other hand, Pasko Rakic has taken the viewpoint that adult neurogenesis, particularly in the hippocampus and cortex, would in principle be evolutionarily disadvantageous to humans, because as a species, it would not be a good idea to sacrifice longevity of memory in the service of plasticity. Rakic was a long-time and staunch disbeliever of any type of adult neurogenesis in non-human primates and humans, but made small concessions every now and then, like the time when he himself found adult-born neurons in the hippocampus of macaque monkeys. Rakic has been particularly critical of work by Elizabeth Gould in rodents and non-human primates, and work by Fred Gage in humans has also figured prominently into the debate. A 2001 article from The New Yorker accompanies this question; it provides a human context for the debate as it stood some 10 years ago. Please address the following questions in this ongoing controversy.

1. What is the evidence for a gradual decrease in the prevalence of *adult* neurogenesis as one moves up the phylogenetic tree from birds to rodents, to monkeys, to humans?

2. To the extent that you find evidence for *postnatal* neurogenesis in rodents, non-human primates, and humans, does postnatal neurogenesis appear to be restricted to particular brain regions or to particular stages of development? In other words, evaluate the evidence for temporal or spatial constraints on postnatal neurogenesis in rodents, non-human primates, and humans.

3. To what extent do methodological considerations influence the debate? Do you think there are legitimate reasons for differences in interpretation of results based on methodology alone? If so, is it a matter of old vs new methods for identifying adult-born neurons, or is one method sufficiently flawed so as not to be trusted?

Controversy Question

Student 5

Pass, with considerable range of opinions on writing style and organization of the answer.

PASS PLUS: Excellent essay – well-written and informative. Explictly contrasts neurogenesis across species rather than just giving evidence. Temporal constraints was glossed over when comparing species. Could have given more of an opinion about Part III and what we can conclude about neurogenesis given the methods at our disposal.

Introduction: Adult Neurogenesis

The initial reports of adult neurogenesis overthrew a roughly one-hundred year old phenomenon stating that neuronal renewal was virtually impossible beyond birth. When it was first reported that neurons were unable to undergo post-natal mitosis, it seemed rather unfortunate that the cell type most critical for normal human functioning was the one type incapable of regeneration after trauma. Yet when both Joseph Altman and Michael Kaplan first discovered adult neurogenesis in the rodent hippocampus, their work was greeted with pure skepticism as it seemed nearly impossible that their scientific predecessors had not uncovered this process earlier. It was not until 40 years later when Frank Nottebohm clearly identified continuously high quantities of neurogenesis in adult canaries and songbirds that the field was re-visited. When combined with the technological advances in biology and immunohistochemistry, evidence for adult neurogenesis was then continually uncovered in species that spanned the length of the phylogenetic tree, including mice, rats, primates, and later humans. Surprisingly, the underlying purpose or benefit for adult neurogenesis, particularly in higher order species, is still rather unknown, and some even postulate that a gradual decline in adult neurogenesis in a given species is evolutionarily advantageous. Regardless, the potential function that adult neurogenesis plays in behavior, learning, and memory as well as its enormous therapeutic implications, render this process one of the most significant discoveries for the future of human health.

Comment [S1]: Really well written introduction!

I) Adult Neurogenesis across Species: Birds

Frank Nottebohm and his colleagues initially searched for evidence of adult neurogenesis in canaries in the 1980s, despite the fact that initial data for adult neurogenesis in rodents had been largely dismissed [1]. In the avian brain, neural progenitors are housed within the ventricular zone (VZ) of the lateral ventricles, and successful recruitment and migration of these neural precursors occurs throughout most of the year into much of the avian forebrain [2, 3]. These avian forebrain regions that continuously recruit new neurons are functionally homologous to the mammalian hippocampus, striatum, and, somewhat, to the mammalian cortex [2]. Additionally there exists substantial, year-long neuronal recruitment to distinct nuclei of the vocal control system in the avian forebrain, most notably to the hyperstriatum ventralis pars caudalis (HVC) [2]. The HVC projects to the robust nucleus of the archistriatum (RA) for proper song production in males, hence the male HVC and RA are much larger than those of the female, and females do not sing unless treated with male gonadal hormones [4]. The male HVC experiences dramatic changes in size due to both the seasonal fluctuations in circulating testosterone, such that in non-breeding seasons when testosterone is low, the HVC is reduced to almost half of its normal neuronal number [4]. Surprisingly, within 12 months the HVC replaces over half of the lost neurons, corresponding to the onset of the breeding season and an increase in circulating testosterone [4]. The female HVC also demonstrates a continuous, year-long recruitment of new neurons, though they differentiate into interneurons within the HVC that neither project to the RA nor promote song production. Goldman et al., 1983 clearly demonstrated that new neurons in the female canary HVC were morphologically neurons, incorporated the ^{3}H thymidine label, produced action potentials, and were activated in response to acoustic stimuli [1, 4]. Later studies corroborated the results from this original study and found that roughly 20,000 new and functional neurons were

added to several, widespread regions throughout the forebrain, in addition to some new recruitment to parts of the avian midbrain, medulla, and cerebellum [4].

This large quantity and expanse of neurogenesis in the avian forebrain suggests that most regions of the avian brain are capable of supporting both neuronal migration as well as later differentiation, and that neurons are able to survive within the forebrain long enough to function in existing circuits. A major reason for the vast quantities and consistency of neural precursor migration to the avian forebrain is the presence of a supportive system for migration and a supportive environment within the forebrain parenchyma that permits the survival of these new neurons. Radial guide cells extend from the VZ proliferative "hot spots" throughout much of the avian forebrain, thus providing a truly supportive scaffold upon which neural precursor cells can travel to distant sites [2, 3]. Additionally, gonadal hormones are crucial for both neuronal recruitment and survival, as estrogen promotes the recruitment and survival of new HVC neurons and testosterone promotes the survival of new RA-projecting HVC neurons [2, 3]. Additionally brain derived neurotrophic factor (BDNF) circulates within the HVC to support the survival and growth of newly recruited neurons [2]. The supportive roles of estrogen and testosterone explain both 1) the seasonal fluctuations in the size of the male HVC that correlates to the concentration of circulating testosterone, and 2) the presence of HVC neurogenesis in females that yields only HVC interneurons incapable of producing song [2, 4]. Furthermore, the expanse of the migratory network and the level of trophic support within the forebrain parenchyma distinguish the avian forebrain from other mammalian species and may explain why adult neurogenesis declines along the phylogenetic tree.

Adult Neurogenesis across Species: Rodents

Unlike the vast adult neurogenesis throughout much of the avian forebrain, adult neurogenesis in rodents is drastically reduced to the olfactory bulb and the dentate gyrus of the hippocampus [5]. Neural progenitor cells from the subventricular zone (SVZ) of the lateral ventricles migrate along the rodent rostral migratory stream (RMS), a structure similar to the radial guide cell system of the avian forebrain but devoid of the supporting radial-glial guide cell scaffold that widely projects to the avian forebrain [3, 5, 6]. Within the hippocampus, the subgranular zone (SGZ) of the dentate gyrus acts as a source of neural progenitor cells that can eventually differentiate into mature granule cells and add to the total population of the dentate gyrus [5, 7]. Initial evidence of hippocampal neurogenesis in rodents appeared roughly 50 years ago through the work of Altman and Kaplan who, like Nottebohm, used the ^{3}H thymidine label and the morphological characteristics of neurons to identify adult neurogenesis [8, 9]. Later evidence in support of neural progenitors within the SVZ came from *in vitro* studies showing that explants from mouse SVZ were able to differentiate into both neurons and glia [10]. Additionally, a study by Lois and Alvarez-Buylla in 1994 discovered that rodents indeed possess a migratory path to the olfactory bulb composed of a dense network of astrocytes that guides migrating neuroblasts rostrally to successfully differentiate into olfactory interneurons [11].

There are notable differences between avian and rodent adult neurogenesis, namely the rarity of neurogenesis in non-neurogenic regions of the rodent forebrain and the decline in neurogenesis in aged rodents. Notably, these distinctions indicate a substantial decline in the prevalence of adult neurogenesis from one species to the next. Neurogenesis in the adult rodent brain is regionally constrained such that neurogenesis in non-neurogenic regions is both rare and often transient. Though Kaplan reported evidence of neurogenesis within the rodent visual cortex [12], and Dayer al., 2005 reported GABAergic neurogenesis within the rodent neocortex [13], the extent of the

Deleted: et

new neuron recruitment appeared substantially lower than the quantity of daily neural recruitment within the avian forebrain. Furthermore, a study by Chen et al., 2004 is currently one of the only studies that has detected mouse neocortical neurogenesis, a finding that has yet to be replicated [14]. Select studies have reported neurogenesis in the hypothalamus of mice [15] and rats [16], as well as in sexually dimorphic nuclei of the rat hypothalamus [17]. During adolescence, gonadal hormone activation led to a significant increase in BrdU labeling within the female anteroventral periventricular nucleus and the sexually dimorphic nucleus of the preoptic area in males, suggesting that adolescent neurogenesis is both hormone dependent and crucial for organizing circuits that control adult sexual behavior [17]. Despite the occasional evidence for neurogenesis in these classically non-neurogenic regions, the process of new neuronal recruitment in such regions either occurs only at one distinct developmental time point or is primarily incomplete [5]. Incomplete neurogenesis holds that a vast majority of newly formed neurons in these regions are incapable of surviving long enough to have any functional relevance in existing circuits [5]. Furthermore, the origin and migratory path of these forebrain and midbrain neurons is not always clear, and differs significantly from VZ progenitors and the radial-glial guide cells that clearly allow for the migration of avian neural precursors to a vast majority of the telencephalon.

The second important difference between rodent and avian adult neurogenesis is the gradual decline in the process overtime in any given rodent, even in the major neurogenic region of the hippocampus. The frequency of hippocampal neurogenesis is not uniform throughout the adult rodent life, such that in comparison to 6 month old rats, 12 to 27 month old rats have a significant reduction in the number of both new and migrating neurons within the granule cell layer of the dentate gyrus [18]. This is in stark contrast to avian neurogenesis within both the forebrain and hippocampus that demonstrate a continuous recruitment of new neurons throughout much of adulthood [2,

4, 19]. Specifically, the HVC of the canary forebrain recruits between 0.1 and 0.74% new neurons per day, and the hippocampus recruits between 0.15 and 0.37% new neurons per day [2]. Therefore, the decline in rodent neurogenesis overtime and the vast regional constraints on rodent neurogenesis indicate that the continuity and the expanse of this process become continually restricted in more complex species. This may suggest that despite its profound implications to the overall function of the brain, adult neurogenesis may not be crucial for the optimal survival of higher order species.

Adult Neurogenesis across Species: Primates and Humans

The eventual acceptance of adult neurogenesis in rodents led investigators in primate neurobiology to search for similar process in non-human primates, as any indication of neurogenesis in any region of the primate brain would hold enormous therapeutic potential. In 1985, Pasko Rakic initially reported that neurogenesis was completely absent throughout the entire primate brain, for injections of the ^{3}H-thymidine label failed to stain any neuron in the neocortex, the hippocampus, the olfactory bulb, the basal ganglia, or any other brain region analyzed [20]. It was postulated that the lack of neurogenesis in such a complex, highly developed species was a purposeful adaptation, such that the continual environmental interactions in primates and humans necessitated a set of stable, persistent synapses for much of the adult life [20]. Yet almost 15 years later, Gould et al., 1998 found convincing evidence for neurogenesis within the dentate gyrus of young, middle-aged and older (23 years) Old World Monkeys [21]. BrdU labeled neurons also co-localized with the distinct marker for immature neurons, TOAD-64, indicating that labeled cells were indeed neurons [21]. Around the same time, despite their initial claims that neurogenesis does not occur within primates, Kornack and Rakic 1999 finally observed and validated neurogenesis in the dentate gyrus of adult macaque monkeys [7]. Taken together, these studies pointed to the presence of *in*

Deleted: leading

vivo neural stem cells within the subgranular zone of the dentate gyrus and their later differentiation into hippocampal granule cells, a finding that was highly significant in light of previous reports indicating that primate neurogenesis was neither necessary nor feasible beyond birth.

Neurogenesis within the hippocampus and olfactory bulb of the primate did not come as much of a surprise considering the vast amount of support for these two processes in lower order rodents. Similar to adult neurogenesis in rodents, few investigators were able to provide support for adult neurogenesis in any other region of the primate brain. However, Gould et al., 1999 attempted to report evidence of new, BrdU-labeled neurons within the neocortex that had traveled to their final position along a migratory stream projecting from the SVZ to their final cortical destination [22]. Not surprisingly, this study faced harsh criticism, disbelief, and mostly skepticism, with critics primarily questioning her interpretations and the methods used in the study [23-25]. The study faced such criticism, that the investigators completed a second study to evaluate the survival of these new neurons that they had reportedly identified within the primate neocortex [26]. They continued to find evidence for both hippocampal as well as neocortical neurogenesis, though it was clear that several of the BrdU-labeled cells in the cortex were unable to survive past approximately 9 weeks [26]. Moreover, Bernier et al., 2001 reported adult neurogenesis within the primate amygdala and piriform cortex as well as the presence of a temporal migratory stream along which the precursor cells from the SVZ had possibly migrated to the cortex [27]. Yet they too noted that several migrating neurons along this supposed temporal stream were unable to survive long enough to reach their cortical destination [27]. In response to both claims, Kornack and Rakic again searched for evidence of cortical neurogenesis, yet their results showed only the growth of new glial cells within the cortex and no evidence for neurogenesis [25]. To this day, there have been very few additional reports of successful

neurogenesis within the cortex of primates, suggesting that any migrating neural precursors from the SVZ are likely to die within weeks of settling in the cortical parenchyma.

Similar to the restrictions of the rodent brain in comparison to the avian brain, the limitations placed on forebrain and midbrain neurogenesis in primates appears to be primarily caused by a decline in basic anatomical support for the process as noted by the grossly underdeveloped RMS in primates. The migratory stream available to primates is limited in its regional and forebrain extension, much like the decreased complexity of the RMS in rodents compared to birds [5]. In the primate RMS neuroblasts rely on neurophilic interactions between one another rather than radial-glial cell guided or even astrocyte guided migration, and extension of the RMS is strictly limited to the olfactory bulb [28]. Additionally, relative to the rodent brain, the migration rate of primate neuroblasts destined for the olfactory bulb is much slower, requiring at least 75 days for full migration after normalizing for differences in brain size between rodents and primates [28]. Thus it appears as though the lost complexity of the primate migratory stream is again a limiting factor in the prevalence of primate neurogenesis in any area other than the olfactory bulb and the hippocampus. This feature places a large restriction on the potential for neural precursors in the SVZ to successfully migrate to any other region of the forebrain. Additionally, the forebrain is essentially considered to be a non-neurogenic region likely void of proper trophic factors to support neural differentiation and survival [5]. Despite reports of neocortical neurogenesis, the ability for new neurons to survive and integrate into functional circuits in any area other than the hippocampus is drastically reduced in comparison to that seen in avian species.

Additionally, there exist important restrictions on hippocampal neurogenesis within the primate, particularly in comparison to rodents. While granule cells that are newly generated in the rodent hippocampus continually add to the pool of hippocampal

neurons in the adult rodent, for example between 30 and 365 days of life, this is likely not the case in primates [28, 29]. Hippocampal neurogenesis in the adult rodent increases in response to several environmental stimuli including exercise [30], sexual experience [31], and learning [32], all of which lead to an overall increase the total number of functional granule cells. However, the primate brain appears to show no significant accumulation of new hippocampal neuron addition, suggesting that hippocampal neurogenesis correlates to an equal rate of neuronal apoptosis [7]. Thus, hippocampal neurogenesis within rodents may facilitate new learning and synaptic plasticity in the face of constant environmental changes or necessary changes in social behavior throughout the adult life. Considering that both primates and humans extensively interact with the environment, it is possible that the gradual decline in neurogenesis in favor of long-lasting synapses and circuits is necessary for long term memory storage and the overall complexity of the primate and human brain [7].

Comment [S2]: Great integration with functional implications of neurogenesis.

Humans show virtually an identical pattern of neurogenesis as primates, with neural progenitor cells housed only in the hippocampus and the SVZ lining the ventricular system. The first reports of human neural progenitor cells were rather astounding, as they truly verified that neurogenesis was indeed possible in the human brain. Eriksson et al., 1998 evaluated the brains of cancer patients that had been previously treated with BrdU, a treatment that had been used to follow the proliferative profile of tumor cells [33]. The study found a substantial quantity of BrdU labeled cells positive for neural markers within the dentate gyrus of these individuals, providing official proof that neural precursors can survive in the human brain [33]. Knoth et al., 2010 analyzed hippocampal tissue samples from individuals ranging from childhood to almost 90 years of age, specifically searching for neural precursors and neural proliferation [34]. Surprisingly they found that several cells co-localized with both immature neuronal and cell proliferation markers at almost all ages, though they noted a significant decline the

number of labeled cells beyond roughly 40 years of age [34]. Additionally, Sinai et al., 2004 confirmed the presence of SVZ neural progenitors in postmortem adult human tissue as well as a unique band of astrocytes extending from the SVZ [35]. However they found no evidence in support of neural precursor migration along this band of astrocytes, suggesting that neural precursors in humans are even more restricted to distinct locations within the brain parenchyma than even non-human primates [35]. Additionally, a separate study used an alternate method of detecting proliferating neural cells, the phenomenon of carbon-14 labeling, and found that neurons in the neocortex of humans were the exact age of the individual [36]. This suggested that, like primates, neocortical neurogenesis in humans is largely restricted to embryological development prior to birth. No other studies have successfully reported human neurogenesis or the presence of neural precursors in any other region outside of the hippocampus or the SVZ. Nonetheless, the presence of individual neural precursors in humans warrants great attention, and highlights the necessity to identify an optimal environment that would allow neurons to differentiate and thrive in several regions throughout the human brain.

Avian and mammal adult neurogenesis differ in both the frequency of neural turn over as well as the constraints placed on neural progenitors as to where they can survive and differentiate. More importantly, the regional constraints appear to be more limiting to the expanse of adult neurogenesis in more complex species. The fact that neural precursors of the SVZ and hippocampus can survive well into adulthood of rodents, primates, and humans, indicates that age does not appear to restrict the potential for neurogenesis, but rather their capacity to survive in various regions of the brain is the more significant limiting factor. Despite reports of neurogenesis within the rodent neocortex or hypothalamus as well as the primate neocortex, such accounts are

Comment [S3]: Good summary

both rare and often conflicting between groups, a finding that is in direct contrast to the widespread and continual neurogenesis throughout the avian forebrain.

II) Temporal vs. Regional Constraints in Adult Neurogenesis

Despite the fact that the total amount of mammalian neurogenesis declines in prevalence both across species and within a given species, adult neurogenesis becomes more regionally constrained when the mammalian brain becomes more complex. Lower order vertebrates such as fish and the aforementioned songbirds experience high quantities of neurogenesis both throughout adulthood and within several higher order forebrain structures. In comparison to birds, mammals as low as rodents have a drastic reduction in the brain regions available for supporting complete neurogenesis. While both avian species and rodents possess a similar pool of neural precursors within the SVZ of the lateral ventricles, their migratory ability and their capability to survive in various forebrain regions is dramatically different. Though both primates and humans still possess distinct pools of neural progenitors that are capable of proliferating well into adulthood, there are even fewer reports of successful integration of such precursors into other brain regions. Additionally, regional constraints on mammalian neurogenesis appears to be the more significant factor in higher order species due to fact that labeling of proliferating and immature neuronal precursors has identified active neural progenitor cells in elderly rodents [18], primates [21], and humans [33, 34]. The fact that any neural precursor exists in the mammalian brain several years after birth indicates that mammalian brains are capable of harvesting and supporting neural stem cells, but that their survival and differentiation requires a distinct environment that appears to be uncharacteristic of higher order brain regions.

Comment [S4]: Needs to answer about whether temporal restrictions differ among species.

It is noteworthy that certain non-neurogenic regions of the mammalian brain, particularly in humans, can suddenly support nascent neurogenesis after trauma due to the change in circulating factors associated with an injured brain region. In a report by Jin et al., 2006, brain biopsy tissue samples from stroke patients were stained for Ki67, a marker of cell proliferation, as well as DCX and βIII tubulin, two markers expressed in new neurons [37]. Particularly within the penumbra, or the surviving cortical tissue surrounding the ischemic core, the study found that several cells were positive for both Ki67 and either DCX or βIII tubulin [37]. As neurogenesis has been shown to be enhanced in such degenerative diseases as Huntington's and Alzheimer's, the authors noted that it is likely that a similar process was taking place in these stroke patients, such that pre-existing neural precursors were stimulated to begin dividing in order to replace lost neurons after the insult [37]. They noted that the source of the precursors could either be the SVZ where progenitors may have migrated along capillary beds, or even local precursors within the cortex [37]. In a similar study, Liu et al., 2008 evaluated tissue samples from epileptic patients undergoing resection of both the hippocampus and the anterior temporal cortex [38]. By staining for DCX in control and epileptic tissue samples, the authors sought to identify if immature, new neurons were more prevalent in epileptic tissue [38]. They found a notable increase in DCX positive cells in both the granule cell layer of the hippocampus and distinct temporal cortical regions of epileptic patients in comparison to control tissue [38]. The authors noted that *in vitro* cultures of epileptic human tissue housed neural progenitor cells, suggesting that the trauma induced by seizure is able to essentially induce proliferation of quiescent progenitor cells within brain regions that are usually non-neurogenic [38].

In both cases, trauma to the brain induced proliferation of neural precursors near the site of the lesion, likely as a last resort attempt to restore function before the level of trauma became fully detrimental. Yet this phenomenon corroborates the idea that adult

neurogenesis in higher order mammals is possible late into adult life but is constrained to distinct regions throughout the brain that appear to have evolved as optimal stem cell niches. Constitutive neurogenesis within the hippocampus is now a well-known, accepted fact in all mammals, as is the presence of neural progenitor cells throughout the SVZ capable of migrating to the olfactory bulb. Though the overall prevalence of neurogenesis appears to decline gradually in aging mammals, it continues to be an ongoing albeit occasional process, and neural progenitors within the hippocampus or SVZ do not appear to suddenly die off as the brain ages. Rather, the major decline in adult neurogenesis that is seen from birds to rodents to monkeys and to humans is due to the major restrictions placed on neural precursors as to the brain environments in which they are capable of thriving. The discovery of adult neurogenesis in humans was an immense milestone in the field of neurological and neurodegenerative disorders, though the therapeutic potential of individual neural precursors cannot be achieved until investigators uncover a method that allows such precursors to survive and function in more widespread regions of the human brain.

III) Methods for Detecting Adult Neurogenesis

When the first reports of adult neurogenesis surfaced, most individuals within the scientific community were hostile to even evaluate the methods used by such scientists as Michael Kaplan and Joseph Altman who had proposed such a phenomenon. Even decades later when investigations into this process became more widespread, non-believers were more skeptical of the idea rather than the accuracy of the methods used. Yet when both BrdU labeling and immunohistochemistry were incorporated into the study of adult neurogenesis, claims of neurogenesis in higher order primates and in non-neurogenic brain regions called for a re-evaluation of the evidence [23]. Much of the criticism of certain groups concerned the specific methods and BrdU protocols used as

well as the individual interpretations of the results. The methodological critiques were both justified and legitimate, considering that the idea of adult neurogenesis in mammals has such profound implications to human health. Though the current use of BrdU is highly efficient and validated, it requires that investigators undergo rigorous controls and consider several alternate interpretations in order for their results to be accepted by the scientific community.

Old versus New Thymidine Analogs

The first reports of adult neurogenesis in both mammals and birds initially used the analog ^{3}H-thymidine that was efficiently incorporated into dividing cells in the DNA synthesis phase of mitosis, thus indicating a cell's "birthday" [4, 9, 39]. Cells exposed to the ^{3}H-thymidine were later exposed to silver grains that would bind the thymidine analog and that would allow for cell visualization via autoradiography [4, 9, 23, 40]. Investigators delineated stringent criteria for cell labeling, such that a mitotically-active cell was one whose overlying silver grains were at least 50% of the labeling in cells that showed maximal labeling [23]. Frank Nottebohm reported that avian neurons were considered labeled if they possessed 3 silver grains per nucleus, while Michael Kaplan reported that a labeled rodent neuron was one with 5 grains over the nucleus in the olfactory bulb or 19 grains over the nucleus of a granule cell from the dentate gyrus [4, 8]. This initial method in detecting cell proliferation was highly efficient and validated, and it was advantageous in that it allowed for an objective, quantitative analysis of proliferating cells.

Though the potential for quantification of cell division was beneficial for early neurobiologists using this method, major issues arose when attempting to identify cell type without the vast array of neural or glial markers available today. Early reports of adult neurogenesis determined cell type using both ultrathin sections stained with either

Nissl or Cresyl violet or electron microscopy to search for morphological clues that would distinguish a neuron from a glial cell [4, 8]. In general, cells were considered to be neurons when they possessed round, compact nuclei with one or two nucleoli when visualized with standard histology, and if electron microscopy (EM) revealed the presence of synaptic terminals with multiple types of vesicles, smooth membrane contours, and long processes containing microtubules [1, 4, 8, 12, 41]. The use of the 3H thymidine label and the detailed characterization of neuronal morphology initially identified the presence of adult neurogenesis in both avian and mammalian speices, a process that had long been considered impossible. Clearly, the phenomenon was initially dismissed, and not until almost 40 years later did innovations in biological techniques allow for the idea to be more highly reconsidered.

The idea of neurogenesis, particularly in the mammalian hippocampus, was reconsidered in the early 1990s during which both neural and glial-specific antibodies were readily available and when researchers first incorporated the use of bromodeoxyuridine (BrdU) to tag neuronal cell division in adult mammals. The use of BrdU to assess the proliferative nature of growing tumors in cancer patients prompted investigators to apply the same marker to label proliferating and migrating neurons within the mature CNS [40]. Similar to ^{3}H thymidine, BrdU could be incorporated into the cell specifically during the S phase of mitosis after which the label was identified, though using immunohistochemistry [40]. Additionally, BrdU held major advantages over that of ^{3}H thymidine in that visualizing the BrdU label took only 1 to 3 days rather than the 1 to 3 months required for the autoradiography associated with ^{3}H thymidine, and specialized facilities were not required to visualize the BrdU label [39]. Additionally, new antibodies that were specific to surface or cytoskeletal markers of neurons or glial cells allowed neurobiologists to distinguish one cell type from another without relying on subjective morphological criteria or the demanding techniques of EM.

To this day, BrdU has arguably been the most significant contribution to the field of adult neurogenesis, and it is the most prominent method used to detect the process. The transition from the ^{3}H thymidine analog to the BrdU label was an immense move in the field that made the study of adult neurogenesis both feasible and accessible to virtually any neurobiologist. Unfortunately, the BrdU method is not without inherent flaws rendering it continually susceptible to much scrutiny throughout the scientific community. The rather subjective nature of immunohistochemistry required to detect the BrdU label is the major factor in the BrdU protocol that may cause investigators to evaluate data rather differently. Due to its current prevalence within neurobiology, there are several standard controls and considerations that investigators cannot ignore in order for their studies to be both accepted and validated by their peers.

Considerations in BrdU Staining: Route and Dose of BrdU

Though the use of BrdU dramatically advanced the field of adult neurogenesis, there exist several variations in individual BrdU protocols that can significantly impact how results are interpreted. One of the major differences identified across protocols is the concentration of BrdU used, as well as the route of its injection [42, 43]. It has been proposed that a dose of 50mg/kg BrdU is sufficient to label any and all dividing neurons, though doses ranging from 50 to 500mg/kg have also been used in order to optimize staining and to account for smaller neurons that might not be labeled with protocols using the lower doses [42, 44]. Though lower doses of BrdU may ultimately underestimate the quantity of proliferating cells, administration of higher doses renders the experimenters more likely to stain cells undergoing DNA turnover or even apoptosis [23, 42, 43]. Cells preparing for apoptosis or in the process of degeneration lose Cdk inhibition, leading them to re-enter the cell cycle prior to death [45, 46]. In such cases, BrdU will stain cells undergoing either process leading to erroneous and false positive

results. Additionally, as with any radioactive or exogenous agent applied to cells, BrdU is considered to be a mutagen, meaning its successful incorporation into nuclear material and the general health of the cell may both be compromised when higher doses are administered [42, 43]. Both the potential for BrdU to label apoptotic cells and the mutagenic qualities of BrdU are major technical considerations in any BrdU protocol, and virtually any study that does not independently control for both factors is deemed invalid. Furthermore, investigators use both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes for BrdU administration, meaning there may be a significant degree of variation in BrdU access to CNS tissue. I.p. injections are subject to potentially thwarted access to the CNS due to differences in blood brain barrier permeability to BrdU across species [43]. Continuous i.c.v. infusion through the lateral ventricles enhances the likelihood that all relevant cells within the CNS will be labeled, yet the level of exposure may render neuronal cells more susceptible to the mutagenic qualities of BrdU including altered gene expression or even cell death [43]. Ultimately, variations in both the dose and route of BrdU administration reported across groups can significantly alter the outcome of any one experiment and lead to several different interpretations of one set of results.

Considerations in BrdU Staining: Antibody Specificity

As with any immunohistological assay, the specificity and sensitivity of primary and secondary antibodies must be taken into consideration when designing an optimal protocol. Antibody specificity is even more crucial in BrdU staining protocols, considering that false positive staining can lead investigators to make truly profound conclusions from inaccurate results. Optimization of antibody specificity must be applied not only to the actual BrdU label but also to any additional labels used to mark neurons or glial cells. A study by Leuner et al., 2009 evaluated differences in the sensitivities of

BrdU antibodies from such companies as Vector, BD, and Roche, and found significant differences in the number of newborn cells labeled by each separate antibody within the dentate gyrus [47]. Several biotech companies sell BrdU antibodies and the choice of company is highly variable across research groups, meaning the specific choice of BrdU antibody may become a significant confounding factor. More crucial than the choice of BrdU antibody is the use of proper labels that undoubtedly distinguish a neuron from a glial cell, an endothelial cell, and other nascent cells within the CNS. Though there are currently several markers for each distinct stage within neuronal development, the issue of both antibody specificity and background staining also holds true for antibodies chosen to define cell type. Neu-N is the most widely used antibody to identify neurons, and it was not until recently that the Neu-N antigen was identified as the neuron-specific Fox-3 gene, a member of the Fox-1 gene splicing family [48]. Additionally, the authors found that any cross reactivity of Neu-N occurs with the neural-specific synapsin-1 protein, meaning it is still a highly acceptable antibody to chose for neural staining [48]. However, in addition to variations in BrdU antibody binding specificity, Leuner et al., 2009 reported high background staining when using the Neu-N antibody, specifically based on the individual DNA denaturation protocol [47]. Since HCl, HCl and formamide, and steam heating are all common treatments used for DNA denaturation, the study evaluated each technique separately to determine how and if they altered background staining. Each technique resulted in notable differences in the Neu-N signal to noise ratio, a factor that can drastically overestimate or underestimate Neu-N staining in a BrdU protocol [47].

Though the technique of BrdU labeling is currently the gold standard in evaluating adult neurogenesis, there are considerable differences between individual protocols that may greatly affect how a given set of results is interpreted. Changes in both the dose and route of BrdU injections can lead to false positive results by labeling

Deleted: e

old neurons going through apoptotic cell cycling or even cell death. Additionally, variations in primary antibodies to BrdU or neural markers can result in different staining intensities, causing individual investigators to rely on their own intuition and judgment in counting cells. Therefore, it is clear that these specific aspects of a BrdU protocol must be as consistent as possible across research groups when attempting to verify the presence of adult neurogenesis. Most importantly, this is of particular importance in higher order species or in classically non-neurogenic regions where there may only be miniscule, yet extremely notable, quantities of dividing cells.

Controls in BrdU Staining

Despite the aforementioned flaws inherent to the BrdU technique, it remains the most prominent, efficient, and validated method available for detecting adult neurogenesis, especially in mammalian species. The technique of BrdU staining exploits the fundamental phase of DNA synthesis inherent to the process of mitosis that is virtually identical across the phylogenetic tree. Even to this day, the idea of adult neurogenesis is almost beyond belief thus it comes as no surprise when investigators looking into the process must undergo a series of rigorous controls. Notably, with the advent and relative abundance of confocal microscopy, it has become almost a routine process that adult neurogenesis be verified with the use of this 3-dimensional imaging technique, so as to ensure that the full depth of a given cell is uniformly labeled with both BrdU and neuronal markers [23]. For example, a study by Dayer et al., 2005 reported neurogenesis in the adult rat neocortex and striatum, and used confocal microscopy combined with a total of eight different neuronal markers including Neu-N, CRMP4, NG2, DCX, GABA and GAD67 to unquestionably verify that the BrdU label was found in neurons [13]. Confocal imaging to detect the presence of both BrdU and neuronal markers demonstrated the presence of the two labels throughout several z-planes of the

double-labeled cells, further indicating that the BrdU positive cell was not simply a non-neuronal cell layered on top of a neuron [13]. Current investigations in adult neurogenesis must also verify that the BrdU label has not been incorporated into old neurons that have re-entered the cell cycle prior to apoptosis. A study by Zhao et al., 2003 reported the occurrence of new neurons within the substantia nigra of mice, a finding that would clearly have a profound influence on Parkinson's Disease therapeutics [44]. The authors were careful to control for potential BrdU incorporation into degenerating or apoptotic cells by sacrificing a subset of animals 2 days after initiating i.c.v infusions of BrdU, a time point at which apoptotic activity of nigral cells would be at a maximum [44]. BrdU incorporation was not observed at this time point, and incorporation was highest by 21 days, suggesting that staining at the later time points could be attributed to neurogenesis [44].

Comment [S5]: Good example of how brd U was used appropriately

The BrdU technique is currently unparalleled in its ability to detect adult neurogenesis, though it is apparent that issues of antibody specificity, background staining, labeling of apoptotic cells, and incorrect identification of cell type are all legitimate reasons for the variations in data interpretation across research groups. When Gould et al., 1999 first reported adult neurogenesis in the neocortex of primates, the skepticism that ensued was primarily concerned with the methods used in the original study and how the authors interpreted them [22-24]. Critics were skeptical of whether BrdU was labeling new cells or old cells undergoing apoptosis, whether labels used to identify neurons were truly neuron-specific, and whether optical issues with cell identification were taken into consideration [23]. Regardless, the application of BrdU labeling successfully replaced the original ^{3}H-thymidine method and is in continuous use today by most of the leading experts in adult neurogenesis. However it is necessary that every investigator use multiple controls in their individual BrdU staining protocols including confocal microscopy, multiple neuronal markers, and multiple time points after

BrdU injections to rule out BrdU labeling of apoptotic cells. When appropriate controls are used and the aforementioned technical considerations are addressed, adult neurogenesis can be validated in virtually any species and any brain region. Until an alternate, more accurate method arises, BrdU staining will continue to be the prominent method of choice in this profound and growing field.

References

1. Goldman, S.A. and F. Nottebohm, Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A. 1983 Apr;80(8):2390-4.
2. Gahr, M., et al., What is the adaptive role of neurogenesis in adult birds? Prog Brain Res. 2002;138:233-54.
3. Doetsch, F. and C. Scharff, Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav Evol. 2001;58(5):306-22.
4. Nottebohm, F., Neuronal replacement in adulthood. Ann N Y Acad Sci. 1985;457:143-61.
5. Bonfanti, L. and P. Peretto, Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci. 2011 Sep;34(6):930-50.
6. Gould, E., How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007 Jun;8(6):481-8.
7. Kornack, D.R. and P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5768-73.
8. Kaplan, M.S. and J.W. Hinds, Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 1977 Sep 9;197(4308):1092-4.
9. Altman, J. and G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965 Jun;124(3):319-35.
10. Lois, C. and A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.
11. Lois, C. and A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.
12. Kaplan, M.S., Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981 Jan 10;195(2):323-38.
13. Dayer, A.G., et al., New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005 Jan 31;168(3):415-27.
14. Chen, J., S.S. Magavi, and J.D. Macklis, Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A. 2004 Nov 16;101(46):16357-62.
15. Kokoeva, M.V., H. Yin, and J.S. Flier, Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science. 2005 Oct 28;310(5748):679-83.
16. Xu, Y., et al., Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol. 2005 Apr;192(2):251-64.

17. Ahmed, E.I., et al., Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci. 2008 Sep;11(9):995-7.
18. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 Mar 15;16(6):2027-33.
19. Goldman, S.A., Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998 Aug;36(2):267-86.
20. Rakic, P., Limits of neurogenesis in primates. Science. 1985 Mar 1;227(4690):1054-6.
21. Gould, E., et al., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5263-7.
22. Gould, E., et al., Neurogenesis in the neocortex of adult primates. Science. 1999 Oct 15;286(5439):548-52.
23. Rakic, P., Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci. 2002 Jan;3(1):65-71.
24. Nowakowski, R.S. and N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion? Science. 2000 May 5;288(5467):771.
25. Kornack, D.R. and P. Rakic, Cell proliferation without neurogenesis in adult primate neocortex. Science. 2001 Dec 7;294(5549):2127-30.
26. Gould, E., et al., Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10910-7.
27. Bernier, P.J., et al., Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11464-9.
28. Kornack, D.R. and P. Rakic, The generation, migration, and differentiation of olfactory neurons in the adult primate brain. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4752-7.
29. Bayer, S.A., J.W. Yackel, and P.S. Puri, Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science. 1982 May 21;216(4548):890-2.
30. Pereira, A.C., et al., An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5638-43.
31. Leuner, B., E.R. Glasper, and E. Gould, Sexual Experience Promotes Adult Neurogenesis in the Hippocampus Despite an Initial Elevation in Stress Hormones. PLoS ONE. 5(7).
32. Gould, E., et al., Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci. 1999 Mar;2(3):260-5.
33. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.
34. Knoth, R., et al., Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One. 2010 Jan 29;5(1).
35. Sanai, N., et al., Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature. 2004 Feb 19;427(6976):740-4.
36. Bhardwaj, R.D., et al., Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12564-8.
37. Jin, K., et al., Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13198-202. Epub 2006 Aug 21.
38. Liu, Y.W., et al., Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci. 2008 Dec;28(11):2254-65.

39. Miller, M.W. and R.S. Nowakowski, Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 1988 Aug 2;457(1):44-52.
40. Nowakowski, R.S., S.B. Lewin, and M.W. Miller, Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.
41. Burd, G.D. and F. Nottebohm, Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol. 1985 Oct 8;240(2):143-52.
42. Rakic, P., Adult neurogenesis in mammals: an identity crisis. J Neurosci. 2002 Feb 1;22(3):614-8.
43. Cooper-Kuhn, C.M. and H.G. Kuhn, Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):13-21.
44. Zhao, M., et al., Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7925-30.
45. Copani, A., et al., Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci. 2001 Jan;24(1):25-31.
46. Katchanov, J., et al., Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
47. Leuner, B., E.R. Glasper, and E. Gould, Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol. 2009 Nov 10;517(2):123-33.
48. Kim, K.K., R.S. Adelstein, and S. Kawamoto, Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. J Biol Chem. 2009 Nov 6;284(45):31052-61.
49. Shingo, T., et al., Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science. 2003 Jan 3;299(5603):117-20.

Pass. Very thoughtful, critical, and thorough. Good job!

Introduction: Adult Neurogenesis

The initial reports of adult neurogenesis overthrew a roughly one-hundred year old phenomenon stating that neuronal renewal was virtually impossible beyond birth. When it was first reported that neurons were unable to undergo post-natal mitosis, it seemed rather unfortunate that the cell type most critical for normal human functioning was the one type incapable of regeneration after trauma. Yet when both Joseph Altman and Michael Kaplan first discovered adult neurogenesis in the rodent hippocampus, their work was greeted with pure skepticism as it seemed nearly impossible that their scientific predecessors had not uncovered this process earlier. It was not until 40 years later when Frank Nottebohm clearly identified continuously high quantities of neurogenesis in adult canaries and songbirds that the field was re-visited. When combined with the technological advances in biology and immunohistochemistry, evidence for adult neurogenesis was then continually uncovered in species that spanned the length of the phylogenetic tree, including mice, rats, primates, and later humans. Surprisingly, the underlying purpose or benefit for adult neurogenesis, particularly in higher order species, is still rather unknown, and some even postulate that a gradual decline in adult neurogenesis in a given species is evolutionarily advantageous. Regardless, the potential function that adult neurogenesis plays in behavior, learning, and memory as well as its enormous therapeutic implications, render this process one of the most significant discoveries for the future of human health.

I) Adult Neurogenesis across Species: Birds

Frank Nottebohm and his colleagues initially searched for evidence of adult neurogenesis in canaries in the 1980s, despite the fact that initial data for adult

neurogenesis in rodents had been largely dismissed [1]. In the avian brain, neural progenitors are housed within the ventricular zone (VZ) of the lateral ventricles, and successful recruitment and migration of these neural precursors occurs throughout most of the year into much of the avian forebrain [2, 3]. These avian forebrain regions that continuously recruit new neurons are functionally homologous to the mammalian hippocampus, striatum, and, somewhat, to the mammalian cortex [2]. Additionally there exists substantial, year-long neuronal recruitment to distinct nuclei of the vocal control system in the avian forebrain, most notably to the hyperstriatum ventralis pars cadualis (HVC) [2]. The HVC projects to the robust nucleus of the archistriatum (RA) for proper song production in males, hence the male HVC and RA are much larger than those of the female, and females do not sing unless treated with male gonadal hormones [4]. The male HVC experiences dramatic changes in size due to both the seasonal fluctuations in circulating testosterone, such that in non-breeding seasons when testosterone is low, the HVC is reduced to almost half of its normal neuronal number [4]. Surprisingly, within 12 months the HVC replaces over half of the lost neurons, corresponding to the onset of the breeding season and an increase in circulating testosterone [4]. The female HVC also demonstrates a continuous, year-long recruitment of new neurons, though they differentiate into interneurons within the HVC that neither project to the RA nor promote song production. Goldman et al., 1983 clearly demonstrated that new neurons in the female canary HVC were morphologically neurons, incorporated the ^{3}H thymidine label, produced action potentials, and were activated in response to acoustic stimuli [1, 4]. Later studies corroborated the results from this original study and found that roughly 20,000 new and functional neurons were added to several, widespread regions throughout the forebrain, in addition to some new recruitment to parts of the avian midbrain, medulla, and cerebellum [4].

This large quantity and expanse of neurogenesis in the avian forebrain suggests that most regions of the avian brain are capable of supporting both neuronal migration as well as later differentiation, and that neurons are able to survive within the forebrain long enough to function in existing circuits. A major reason for the vast quantities and consistency of neural precursor migration to the avian forebrain is the presence of a supportive system for migration and a supportive environment within the forebrain parenchyma that permits the survival of these new neurons. Radial guide cells extend from the VZ proliferative "hot spots" throughout much of the avian forebrain, thus providing a truly supportive scaffold upon which neural precursor cells can travel to distant sites [2, 3]. Additionally, gonadal hormones are crucial for both neuronal recruitment and survival, as estrogen promotes the recruitment and survival of new HVC neurons and testosterone promotes the survival of new RA-projecting HVC neurons [2, 3]. Additionally brain derived neurotrophic factor (BDNF) circulates within the HVC to support the survival and growth of newly recruited neurons [2]. The supportive roles of estrogen and testosterone explain both 1) the seasonal fluctuations in the size of the male HVC that correlates to the concentration of circulating testosterone, and 2) the presence of HVC neurogenesis in females that yields only HVC interneurons incapable of producing song [2, 4]. Furthermore, the expanse of the migratory network and the level of trophic support within the forebrain parenchyma distinguish the avian forebrain from other mammalian species and may explain why adult neurogenesis declines along the phylogenetic tree.

Adult Neurogenesis across Species: Rodents

Unlike the vast adult neurogenesis throughout much of the avian forebrain, adult neurogenesis in rodents is drastically reduced to the olfactory bulb and the dentate gyrus of the hippocampus [5]. Neural progenitor cells from the subventricular zone (SVZ) of

the lateral ventricles migrate along the rodent rostral migratory stream (RMS), a structure similar to the radial guide cell system of the avian forebrain but devoid of the supporting radial-glial guide cell scaffold that widely projects to the avian forebrain [3, 5, 6]. Within the hippocampus, the subgranular zone (SGZ) of the dentate gyrus acts as a source of neural progenitor cells that can eventually differentiate into mature granule cells and add to the total population of the dentate gyrus [5, 7]. Initial evidence of hippocampal neurogenesis in rodents appeared roughly 50 years ago through the work of Altman and Kaplan who, like Nottebohm, used the ^{3}H thymidine label and the morphological characteristics of neurons to identify adult neurogenesis [8, 9]. Later evidence in support of neural progenitors within the SVZ came from *in vitro* studies showing that explants from mouse SVZ were able to differentiate into both neurons and glia [10]. Additionally, a study by Lois and Alvarez-Buylla in 1994 discovered that rodents indeed possess a migratory path to the olfactory bulb composed of a dense network of astrocytes that guides migrating neuroblasts rostrally to successfully differentiate into olfactory interneurons [11].

There are notable differences between avian and rodent adult neurogenesis, namely the rarity of neurogenesis in non-neurogenic regions of the rodent forebrain and the decline in neurogenesis in aged rodents. Notably, these distinctions indicate a substantial decline in the prevalence of adult neurogenesis from one species to the next. Neurogenesis in the adult rodent brain is regionally constrained such that neurogenesis in non-neurogenic regions is both rare and often transient. Though Kaplan reported evidence of neurogenesis within the rodent visual cortex [12], and Dayer et al., 2005 reported GABAergic neurogenesis within the rodent neocortex [13], the extent of the new neuron recruitment appeared substantially lower than the quantity of daily neural recruitment within the avian forebrain. Furthermore, a study by Chen et al., 2004 is currently one of the only studies that has detected mouse neocortical neurogenesis, a

finding that has yet to be replicated [14]. Select studies have reported neurogenesis in the hypothalamus of mice [15] and rats [16], as well as in sexually dimorphic nuclei of the rat hypothalamus [17]. During adolescence, gonadal hormone activation led to a significant increase in BrdU labeling within the female anteroventral periventricular nucleus and the sexually dimorphic nucleus of the preoptic area in males, suggesting that adolescent neurogenesis is both hormone dependent and crucial for organizing circuits that control adult sexual behavior [17]. Despite the occasional evidence for neurogenesis in these classically non-neurogenic regions, the process of new neuronal recruitment in such regions either occurs only at one distinct developmental time point or is primarily incomplete [5]. Incomplete neurogenesis holds that a vast majority of newly formed neurons in these regions are incapable of surviving long enough to have any functional relevance in existing circuits [5]. Furthermore, the origin and migratory path of these forebrain and midbrain neurons is not always clear, and differs significantly from VZ progenitors and the radial-glial guide cells that clearly allow for the migration of avian neural precursors to a vast majority of the telencephalon.

The second important difference between rodent and avian adult neurogenesis is the gradual decline in the process over time in any given rodent, even in the major neurogenic region of the hippocampus. The frequency of hippocampal neurogenesis is not uniform throughout the adult rodent life, such that in comparison to 6 month old rats, 12 to 27 month old rats have a significant reduction in the number of both new and migrating neurons within the granule cell layer of the dentate gyrus [18]. This is in stark contrast to avian neurogenesis within both the forebrain and hippocampus that demonstrate a continuous recruitment of new neurons throughout much of adulthood [2, 4, 19]. Specifically, the HVC of the canary forebrain recruits between 0.1 and 0.74% new neurons per day, and the hippocampus recruits between 0.15 and 0.37% new neurons per day [2]. Therefore, the decline in rodent neurogenesis over time and the

vast regional constraints on rodent neurogenesis indicate that the continuity and the expanse of this process become continually restricted in more complex species. This may suggest that despite its profound implications to the overall function of the brain, adult neurogenesis may not be crucial for the optimal survival of higher order species.

Adult Neurogenesis across Species: Primates and Humans

The eventual acceptance of adult neurogenesis in rodents led leading investigators in primate neurobiology to search for similar process in non-human primates, as any indication of neurogenesis in any region of the primate brain would hold enormous therapeutic potential. In 1985, Pasko Rakic initially reported that neurogenesis was completely absent throughout the entire primate brain, for injections of the ^{3}H-thymidine label failed to stain any neuron in the neocortex, the hippocampus, the olfactory bulb, the basal ganglia, or any other brain region analyzed [20]. It was postulated that the lack of neurogenesis in such a complex, highly developed species was a purposeful adaptation, such that the continual environmental interactions in primates and humans necessitated a set of stable, persistent synapses for much of the adult life [20]. Yet almost 15 years later, Gould et al., 1998 found convincing evidence for neurogenesis within the dentate gyrus of young, middle-aged and older (23 years) Old World Monkeys [21]. BrdU labeled neurons also co-localized with the distinct marker for immature neurons, TOAD-64, indicating that labeled cells were indeed neurons [21]. Around the same time, despite their initial claims that neurogenesis does not occur within primates, Kornack and Rakic 1999 finally observed and validated neurogenesis in the dentate gyrus of adult macaque monkeys [7]. Taken together, these studies pointed to the presence of *in vivo* neural stem cells within the subgranular zone of the dentate gyrus and their later differentiation into hippocampal granule cells, a

finding that was highly significant in light of previous reports indicating that primate neurogenesis was neither necessary nor feasible beyond birth.

Neurogenesis within the hippocampus and olfactory bulb of the primate did not come as much of a surprise considering the vast amount of support for these two processes in lower order rodents. Similar to adult neurogenesis in rodents, few investigators were able to provide support for adult neurogenesis in any other region of the primate brain. However, Gould et al., 1999 attempted to report evidence of new, BrdU-labeled neurons within the neocortex that had traveled to their final position along a migratory stream projecting from the SVZ to their final cortical destination [22]. Not surprisingly, this study faced harsh criticism, disbelief, and mostly skepticism, with critics primarily questioning her interpretations and the methods used in the study [23-25]. The study faced such criticism, that the investigators completed a second study to evaluate the survival of these new neurons that they had reportedly identified within the primate neocortex [26]. They continued to find evidence for both hippocampal as well as neocortical neurogenesis, though it was clear that several of the BrdU-labeled cells in the cortex were unable to survive past approximately 9 weeks [26]. Moreover, Bernier et al., 2001 reported adult neurogenesis within the primate amygdala and piriform cortex as well as the presence of a temporal migratory stream along which the precursor cells from the SVZ had possibly migrated to the cortex [27]. Yet they too noted that several migrating neurons along this supposed temporal stream were unable to survive long enough to reach their cortical destination [27]. In response to both claims, Kornack and Rakic again searched for evidence of cortical neurogenesis, yet their results showed only the growth of new glial cells within the cortex and no evidence for neurogenesis [25]. To this day, there have been very few additional reports of successful neurogenesis within the cortex of primates, suggesting that any migrating neural

precursors from the SVZ are likely to die within weeks of settling in the cortical parenchyma.

Similar to the restrictions of the rodent brain in comparison to the avian brain, the limitations placed on forebrain and midbrain neurogenesis in primates appears to be primarily caused by a decline in basic anatomical support for the process as noted by the grossly underdeveloped RMS in primates. The migratory stream available to primates is limited in its regional and forebrain extension, much like the decreased complexity of the RMS in rodents compared to birds [5]. In the primate RMS neuroblasts rely on neurophilic interactions between one another rather than radial-glial cell guided or even astrocyte guided migration, and extension of the RMS is strictly limited to the olfactory bulb [28]. Additionally, relative to the rodent brain, the migration rate of primate neuroblasts destined for the olfactory bulb is much slower, requiring at least 75 days for full migration after normalizing for differences in brain size between rodents and primates [28]. Thus it appears as though the lost complexity of the primate migratory stream is again a limiting factor in the prevalence of primate neurogenesis in any area other than the olfactory bulb and the hippocampus. This feature places a large restriction on the potential for neural precursors in the SVZ to successfully migrate to any other region of the forebrain. Additionally, the forebrain is essentially considered to be a non-neurogenic region likely void of proper trophic factors to support neural differentiation and survival [5]. Despite reports of neocortical neurogenesis, the ability for new neurons to survive and integrate into functional circuits in any area other than the hippocampus is drastically reduced in comparison to that seen in avian species.

Additionally, there exist important restrictions on hippocampal neurogenesis within the primate, particularly in comparison to rodents. While granule cells that are newly generated in the rodent hippocampus continually add to the pool of hippocampal neurons in the adult rodent, for example between 30 and 365 days of life, this is likely

not the case in primates [28, 29]. Hippocampal neurogenesis in the adult rodent increases in response to several environmental stimuli including exercise [30], sexual experience [31], and learning [32], all of which lead to an overall increase the total number of functional granule cells. However, the primate brain appears to show no significant accumulation of new hippocampal neuron addition, suggesting that hippocampal neurogenesis correlates to an equal rate of neuronal apoptosis [7]. Thus, hippocampal neurogenesis within rodents may facilitate new learning and synaptic plasticity in the face of constant environmental changes or necessary changes in social behavior throughout the adult life. Considering that both primates and humans extensively interact with the environment, it is possible that the gradual decline in neurogenesis in favor of long-lasting synapses and circuits is necessary for long term memory storage and the overall complexity of the primate and human brain [7].

Humans show virtually an identical pattern of neurogenesis as primates, with neural progenitor cells housed only in the hippocampus and the SVZ lining the ventricular system. The first reports of human neural progenitor cells were rather astounding, as they truly verified that neurogenesis was indeed possible in the human brain. Eriksson et al., 1998 evaluated the brains of cancer patients that had been previously treated with BrdU, a treatment that had been used to follow the proliferative profile of tumor cells [33]. The study found a substantial quantity of BrdU labeled cells positive for neural markers within the dentate gyrus of these individuals, providing official proof that neural precursors can survive in the human brain [33]. Knoth et al., 2010 analyzed hippocampal tissue samples from individuals ranging from childhood to almost 90 years of age, specifically searching for neural precursors and neural proliferation [34]. Surprisingly they found that several cells co-localized with both immature neuronal and cell proliferation markers at almost all ages, though they noted a significant decline the number of labeled cells beyond roughly 40 years of age [34]. Additionally, Sinai et al.,

2004 confirmed the presence of SVZ neural progenitors in postmortem adult human tissue as well as a unique band of astrocytes extending from the SVZ [35]. However they found no evidence in support of neural precursor migration along this band of astrocytes, suggesting that neural precursors in humans are even more restricted to distinct locations within the brain parenchyma than even non-human primates [35]. Additionally, a separate study used an alternate method of detecting proliferating neural cells, the phenomenon of carbon-14 labeling, and found that neurons in the neocortex of humans were the exact age of the individual [36]. This suggested that, like primates, neocortical neurogenesis in humans is largely restricted to embryological development prior to birth. No other studies have successfully reported human neurogenesis or the presence of neural precursors in any other region outside of the hippocampus or the SVZ. Nonetheless, the presence of individual neural precursors in humans warrants great attention, and highlights the necessity to identify an optimal environment that would allow neurons to differentiate and thrive in several regions throughout the human brain.

Avian and mammal adult neurogenesis differ in both the frequency of neural turn over as well as the constraints placed on neural progenitors as to where they can survive and differentiate. More importantly, the regional constraints appear to be more limiting to the expanse of adult neurogenesis in more complex species. The fact that neural precursors of the SVZ and hippocampus can survive well into adulthood of rodents, primates, and humans, indicates that age does not appear to restrict the potential for neurogenesis, but rather their capacity to survive in various regions of the brain is the more significant limiting factor. Despite reports of neurogenesis within the rodent neocortex or hypothalamus as well as the primate neocortex, such accounts are both rare and often conflicting between groups, a finding that is in direct contrast to the widespread and continual neurogenesis throughout the avian forebrain.

II) Temporal vs. Regional Constraints in Adult Neurogenesis

Despite the fact that the total amount of mammalian neurogenesis declines in prevalence both across species and within a given species, adult neurogenesis becomes more regionally constrained when the mammalian brain becomes more complex. Lower order vertebrates such as fish and the aforementioned songbirds experience high quantities of neurogenesis both throughout adulthood and within several higher order forebrain structures. In comparison to birds, mammals as low as rodents have a drastic reduction in the brain regions available for supporting complete neurogenesis. While both avian species and rodents possess a similar pool of neural precursors within the SVZ of the lateral ventricles, their migratory ability and their capability to survive in various forebrain regions is dramatically different. Though both primates and humans still possess distinct pools of neural progenitors that are capable of proliferating well into adulthood, there are even fewer reports of successful integration of such precursors into other brain regions. Additionally, regional constraints on mammalian neurogenesis appears to be the more significant factor in higher order species due to fact that labeling of proliferating and immature neuronal precursors has identified active neural progenitor cells in elderly rodents [18], primates [21], and humans [33, 34]. The fact that any neural precursor exists in the mammalian brain several years after birth indicates that mammalian brains are capable of harvesting and supporting neural stem cells, but that their survival and differentiation requires a distinct environment that appears to be uncharacteristic of higher order brain regions.

It is noteworthy that certain non-neurogenic regions of the mammalian brain, particularly in humans, can suddenly support nascent neurogenesis after trauma due to the change in circulating factors associated with an injured brain region. In a report by

Jin et al., 2006, brain biopsy tissue samples from stroke patients were stained for Ki67, a marker of cell proliferation, as well as DCX and βIII tubulin, two markers expressed in new neurons [37]. Particularly within the penumbra, or the surviving cortical tissue surrounding the ischemic core, the study found that several cells were positive for both Ki67 and either DCX or βIII tubulin [37]. As neurogenesis has been shown to be enhanced in such degenerative diseases as Huntington's and Alzheimer's, the authors noted that it is likely that a similar process was taking place in these stroke patients, such that pre-existing neural precursors were stimulated to begin dividing in order to replace lost neurons after the insult [37]. They noted that the source of the precursors could either be the SVZ where progenitors may have migrated along capillary beds, or even local precursors within the cortex [37]. In a similar study, Liu et al., 2008 evaluated tissue samples from epileptic patients undergoing resection of both the hippocampus and the anterior temporal cortex [38]. By staining for DCX in control and epileptic tissue samples, the authors sought to identify if immature, new neurons were more prevalent in epileptic tissue [38]. They found a notable increase in DCX positive cells in both the granule cell layer of the hippocampus and distinct temporal cortical regions of epileptic patients in comparison to control tissue [38]. The authors noted that *in vitro* cultures of epileptic human tissue housed neural progenitor cells, suggesting that the trauma induced by seizure is able to essentially induce proliferation of quiescent progenitor cells within brain regions that are usually non-neurogenic [38].

In both cases, trauma to the brain induced proliferation of neural precursors near the site of the lesion, likely as a last resort attempt to restore function before the level of trauma became fully detrimental. Yet this phenomenon corroborates the idea that adult neurogenesis in higher order mammals is possible late into adult life but is constrained to distinct regions throughout the brain that appear to have evolved as optimal stem cell niches. Constitutive neurogenesis within the hippocampus is now a well-known,

accepted fact in all mammals, as is the presence of neural progenitor cells throughout the SVZ capable of migrating to the olfactory bulb. Though the overall prevalence of neurogenesis appears to decline gradually in aging mammals, it continues to be an ongoing albeit occasional process, and neural progenitors within the hippocampus or SVZ do not appear to suddenly die off as the brain ages. Rather, the major decline in adult neurogenesis that is seen from birds to rodents to monkeys and to humans is due to the major restrictions placed on neural precursors as to the brain environments in which they are capable of thriving. The discovery of adult neurogenesis in humans was an immense milestone in the field of neurological and neurodegenerative disorders, though the therapeutic potential of individual neural precursors cannot be achieved until investigators uncover a method that allows such precursors to survive and function in more widespread regions of the human brain.

III) Methods for Detecting Adult Neurogenesis

When the first reports of adult neurogenesis surfaced, most individuals within the scientific community were hostile to even evaluate the methods used by such scientists as Michael Kaplan and Joseph Altman who had proposed such a phenomenon. Even decades later when investigations into this process became more widespread, non-believers were more skeptical of the idea rather than the accuracy of the methods used. Yet when both BrdU labeling and immunohistochemistry were incorporated into the study of adult neurogenesis, claims of neurogenesis in higher order primates and in non-neurogenic brain regions called for a re-evaluation of the evidence [23]. Much of the criticism of certain groups concerned the specific methods and BrdU protocols used as well as the individual interpretations of the results. The methodological critiques were both justified and legitimate, considering that the idea of adult neurogenesis in mammals has such profound implications to human health. Though the current use of BrdU is

highly efficient and validated, it requires that investigators undergo rigorous controls and consider several alternate interpretations in order for their results to be accepted by the scientific community.

Old versus New Thymidine Analogs

The first reports of adult neurogenesis in both mammals and birds initially used the analog ^{3}H-thymidine that was efficiently incorporated into dividing cells in the DNA synthesis phase of mitosis, thus indicating a cell's "birthday" [4, 9, 39]. Cells exposed to the ^{3}H-thymidine were later exposed to silver grains that would bind the thymidine analog and that would allow for cell visualization via autoradiography [4, 9, 23, 40]. Investigators delineated stringent criteria for cell labeling, such that a mitotically-active cell was one whose overlying silver grains were at least 50% of the labeling in cells that showed maximal labeling [23]. Frank Nottebohm reported that avian neurons were considered labeled if they possessed 3 silver grains per nucleus, while Michael Kaplan reported that a labeled rodent neuron was one with 5 grains over the nucleus in the olfactory bulb or 19 grains over the nucleus of a granule cell from the dentate gyrus [4, 8]. This initial method in detecting cell proliferation was highly efficient and validated, and it was advantageous in that it allowed for an objective, quantitative analysis of proliferating cells.

Though the potential for quantification of cell division was beneficial for early neurobiologists using this method, major issues arose when attempting to identify cell type without the vast array of neural or glial markers available today. Early reports of adult neurogenesis determined cell type using both ultrathin sections stained with either Nissl or Cresyl violet or electron microscopy to search for morphological clues that would distinguish a neuron from a glial cell [4, 8]. In general, cells were considered to be neurons when they possessed round, compact nuclei with one or two nucleoli when

visualized with standard histology, and if electron microscopy (EM) revealed the presence of synaptic terminals with multiple types of vesicles, smooth membrane contours, and long processes containing microtubules [1, 4, 8, 12, 41]. The use of the 3H thymidine label and the detailed characterization of neuronal morphology initially identified the presence of adult neurogenesis in both avian and mammalian speices, a process that had long been considered impossible. Clearly, the phenomenon was initially dismissed, and not until almost 40 years later did innovations in biological techniques allow for the idea to be more highly reconsidered.

The idea of neurogenesis, particularly in the mammalian hippocampus, was reconsidered in the early 1990s during which both neural and glial-specific antibodies were readily available and when researchers first incorporated the use of bromodeoxyuridine (BrdU) to tag neuronal cell division in adult mammals. The use of BrdU to assess the proliferative nature of growing tumors in cancer patients prompted investigators to apply the same marker to label proliferating and migrating neurons within the mature CNS [40]. Similar to ^{3}H thymidine, BrdU could be incorporated into the cell specifically during the S phase of mitosis after which the label was identified, though using immunohistochemistry [40]. Additionally, BrdU held major advantages over that of ^{3}H thymidine in that visualizing the BrdU label took only 1 to 3 days rather than the 1 to 3 months required for the autoradiography associated with ^{3}H thymidine, and specialized facilities were not required to visualize the BrdU label [39]. Additionally, new antibodies that were specific to surface or cytoskeletal markers of neurons or glial cells allowed neurobiologists to distinguish one cell type from another without relying on subjective morphological criteria or the demanding techniques of EM.

To this day, BrdU has arguably been the most significant contribution to the field of adult neurogenesis, and it is the most prominent method used to detect the process. The transition from the ^{3}H thymidine analog to the BrdU label was an immense move in

the field that made the study of adult neurogenesis both feasible and accessible to virtually any neurobiologist. Unfortunately, the BrdU method is not without inherent flaws rendering it continually susceptible to much scrutiny throughout the scientific community. The rather subjective nature of immunohistochemistry required to detect the BrdU label is the major factor in the BrdU protocol that may cause investigators to evaluate data rather differently. Due to its current prevalence within neurobiology, there are several standard controls and considerations that investigators cannot ignore in order for their studies to be both accepted and validated by their peers.

Considerations in BrdU Staining: Route and Dose of BrdU

Though the use of BrdU dramatically advanced the field of adult neurogenesis, there exist several variations in individual BrdU protocols that can significantly impact how results are interpreted. One of the major differences identified across protocols is the concentration of BrdU used, as well as the route of its injection [42, 43]. It has been proposed that a dose of 50mg/kg BrdU is sufficient to label any and all dividing neurons, though doses ranging from 50 to 500mg/kg have also been used in order to optimize staining and to account for smaller neurons that might not be labeled with protocols using the lower doses [42, 44]. Though lower doses of BrdU may ultimately underestimate the quantity of proliferating cells, administration of higher doses renders the experimenters more likely to stain cells undergoing DNA turnover or even apoptosis [23, 42, 43]. Cells preparing for apoptosis or in the process of degeneration lose Cdk inhibition, leading them to re-enter the cell cycle prior to death [45, 46]. In such cases, BrdU will stain cells undergoing either process leading to erroneous and false positive results. Additionally, as with any radioactive or exogenous agent applied to cells, BrdU is considered to be a mutagen, meaning its successful incorporation into nuclear material and the general health of the cell may both be compromised when higher doses

are administered [42, 43]. Both the potential for BrdU to label apoptotic cells and the mutagenic qualities of BrdU are major technical considerations in any BrdU protocol, and virtually any study that does not independently control for both factors is deemed invalid. Furthermore, investigators use both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes for BrdU administration, meaning there may be a significant degree of variation in BrdU access to CNS tissue. I.p. injections are subject to potentially thwarted access to the CNS due to differences in blood brain barrier permeability to BrdU across species [43]. Continuous i.c.v. infusion through the lateral ventricles enhances the likelihood that all relevant cells within the CNS will be labeled, yet the level of exposure may render neuronal cells more susceptible to the mutagenic qualities of BrdU including altered gene expression or even cell death [43]. Ultimately, variations in both the dose and route of BrdU administration reported across groups can significantly alter the outcome of any one experiment and lead to several different interpretations of one set of results.

Considerations in BrdU Staining: Antibody Specificity

As with any immunohistological assay, the specificity and sensitivity of primary and secondary antibodies must be taken into consideration when designing an optimal protocol. Antibody specificity is even more crucial in BrdU staining protocols, considering that false positive staining can lead investigators to make truly profound conclusions from inaccurate results. Optimization of antibody specificity must be applied not only to the actual BrdU label but also to any additional labels used to mark neurons or glial cells. A study by Leuner et al., 2009 evaluated differences in the sensitivities of BrdU antibodies from such companies as Vector, BD, and Roche, and found significant differences in the number of newborn cells labeled by each separate antibody within the dentate gyrus [47]. Several biotech companies sell BrdU antibodies and the choice of

company is highly variable across research groups, meaning the specific choice of BrdU antibody may become a significant confounding factor. More crucial than the choice of BrdU antibody is the use of proper labels that undoubtedly distinguish a neuron from a glial cell, an endothelial cell, and other nascent cells within the CNS. Though there are currently several markers for each distinct stage within neuronal development, the issue of both antibody specificity and background staining also holds true for antibodies chosen to define cell type. Neu-N is the most widely used antibody to identify neurons, and it was not until recently that the Neu-N antigen was identified as the neuron-specific Fox-3 gene, a member of the Fox-1 gene splicing family [48]. Additionally, the authors found that any cross reactivity of Neu-N occurs with the neural-specific synapsin-1 protein, meaning it is still a highly acceptable antibody to chose for neural staining [48]. However, in addition to variations in BrdU antibody binding specificity, Leuner et al., 2009 reported high background staining when using the Neu-N antibody, specifically based on the individual DNA denaturation protocol [47]. Since HCl, HCl and formamide, and steam heating are all common treatments used for DNA denaturation, the study evaluated each technique separately to determine how and if they altered background staining. Each technique resulted in notable differences in the Neu-N signal to noise ratio, a factor that can drastically overestimate or underestimate Neu-N staining in a BrdU protocol [47].

Though the technique of BrdU labeling is currently the gold standard in evaluating adult neurogenesis, there are considerable differences between individual protocols that may greatly affect how a given set of results is interpreted. Changes in both the does and route of BrdU injections can lead to false positive results by labeling old neurons going through apoptotic cell cycling or even cell death. Additionally, variations in primary antibodies to BrdU or neural markers can result in different staining intensities, causing individual investigators to rely on their own intuition and judgment in

counting cells. Therefore, it is clear that these specific aspects of a BrdU protocol must be as consistent as possible across research groups when attempting to verify the presence of adult neurogenesis. Most importantly, this is of particular importance in higher order species or in classically non-neurogenic regions where there may only be miniscule, yet extremely notable, quantities of dividing cells.

Controls in BrdU Staining

Despite the aforementioned flaws inherent to the BrdU technique, it remains the most prominent, efficient, and validated method available for detecting adult neurogenesis, especially in mammalian species. The technique of BrdU staining exploits the fundamental phase of DNA synthesis inherent to the process of mitosis that is virtually identical across the phylogenetic tree. Even to this day, the idea of adult neurogenesis is almost beyond belief thus it comes as no surprise when investigators looking into the process must undergo a series of rigorous controls. Notably, with the advent and relative abundance of confocal microscopy, it has become almost a routine process that adult neurogenesis be verified with the use of this 3-dimensional imaging technique, so as to ensure that the full depth of a given cell is uniformly labeled with both BrdU and neuronal markers [23]. For example, a study by Dayer et al., 2005 reported neurogenesis in the adult rat neocortex and striatum, and used confocal microscopy combined with a total of eight different neuronal markers including Neu-N, CRMP4, NG2, DCX, GABA and GAD67 to unquestionably verify that the BrdU label was found in neurons [13]. Confocal imaging to detect the presence of both BrdU and neuronal markers demonstrated the presence of the two labels throughout several z-planes of the double-labeled cells, further indicating that the BrdU positive cell was not simply a non-neuronal cell layered on top of a neuron [13]. Current investigations in adult neurogenesis must also verify that the BrdU label has not been incorporated into old

neurons that have re-entered the cell cycle prior to apoptosis. A study by Zhao et al., 2003 reported the occurrence of new neurons within the substantia nigra of mice, a finding that would clearly have a profound influence on Parkinson's Disease therapeutics [44]. The authors were careful to control for potential BrdU incorporation into degenerating or apoptotic cells by sacrificing a subset of animals 2 days after initiating i.c.v infusions of BrdU, a time point at which apoptotic activity of nigral cells would be at a maximum [44]. BrdU incorporation was not observed at this time point, and incorporation was highest by 21 days, suggesting that staining at the later time points could be attributed to neurogenesis [44].

The BrdU technique is currently unparalleled in its ability to detect adult neurogenesis, though it is apparent that issues of antibody specificity, background staining, labeling of apoptotic cells, and incorrect identification of cell type are all legitimate reasons for the variations in data interpretation across research groups. When Gould et al., 1999 first reported adult neurogenesis in the neocortex of primates, the skepticism that ensued was primarily concerned with the methods used in the original study and how the authors interpreted them [22-24]. Critics were skeptical of whether BrdU was labeling new cells or old cells undergoing apoptosis, whether labels used to identify neurons were truly neuron-specific, and whether optical issues with cell identification were taken into consideration [23]. Regardless, the application of BrdU labeling successfully replaced the original ^{3}H-thymidine method and is in continuous use today by most of the leading experts in adult neurogenesis. However it is necessary that every investigator use multiple controls in their individual BrdU staining protocols including confocal microscopy, multiple neuronal markers, and multiple time points after BrdU injections to rule out BrdU labeling of apoptotic cells. When appropriate controls are used and the aforementioned technical considerations are addressed, adult neurogenesis can be validated in virtually any species and any brain region. Until an

alternate, more accurate method arises, BrdU staining will continue to be the prominent method of choice in this profound and growing field.

References

1. Goldman, S.A. and F. Nottebohm, Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A. 1983 Apr;80(8):2390-4.
2. Gahr, M., et al., What is the adaptive role of neurogenesis in adult birds? Prog Brain Res. 2002;138:233-54.
3. Doetsch, F. and C. Scharff, Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav Evol. 2001;58(5):306-22.
4. Nottebohm, F., Neuronal replacement in adulthood. Ann N Y Acad Sci. 1985;457:143-61.
5. Bonfanti, L. and P. Peretto, Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci. 2011 Sep;34(6):930-50.
6. Gould, E., How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007 Jun;8(6):481-8.
7. Kornack, D.R. and P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5768-73.
8. Kaplan, M.S. and J.W. Hinds, Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 1977 Sep 9;197(4308):1092-4.
9. Altman, J. and G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965 Jun;124(3):319-35.
10. Lois, C. and A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.
11. Lois, C. and A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.
12. Kaplan, M.S., Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981 Jan 10;195(2):323-38.
13. Dayer, A.G., et al., New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005 Jan 31;168(3):415-27.
14. Chen, J., S.S. Magavi, and J.D. Macklis, Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A. 2004 Nov 16;101(46):16357-62.
15. Kokoeva, M.V., H. Yin, and J.S. Flier, Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science. 2005 Oct 28;310(5748):679-83.
16. Xu, Y., et al., Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol. 2005 Apr;192(2):251-64.
17. Ahmed, E.I., et al., Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci. 2008 Sep;11(9):995-7.
18. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 Mar 15;16(6):2027-33.

19. Goldman, S.A., Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998 Aug;36(2):267-86.
20. Rakic, P., Limits of neurogenesis in primates. Science. 1985 Mar 1;227(4690):1054-6.
21. Gould, E., et al., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5263-7.
22. Gould, E., et al., Neurogenesis in the neocortex of adult primates. Science. 1999 Oct 15;286(5439):548-52.
23. Rakic, P., Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci. 2002 Jan;3(1):65-71.
24. Nowakowski, R.S. and N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion? Science. 2000 May 5;288(5467):771.
25. Kornack, D.R. and P. Rakic, Cell proliferation without neurogenesis in adult primate neocortex. Science. 2001 Dec 7;294(5549):2127-30.
26. Gould, E., et al., Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10910-7.
27. Bernier, P.J., et al., Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11464-9.
28. Kornack, D.R. and P. Rakic, The generation, migration, and differentiation of olfactory neurons in the adult primate brain. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4752-7.
29. Bayer, S.A., J.W. Yackel, and P.S. Puri, Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science. 1982 May 21;216(4548):890-2.
30. Pereira, A.C., et al., An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5638-43.
31. Leuner, B., E.R. Glasper, and E. Gould, Sexual Experience Promotes Adult Neurogenesis in the Hippocampus Despite an Initial Elevation in Stress Hormones. PLoS ONE. 5(7).
32. Gould, E., et al., Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci. 1999 Mar;2(3):260-5.
33. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.
34. Knoth, R., et al., Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One. 2010 Jan 29;5(1).
35. Sanai, N., et al., Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature. 2004 Feb 19;427(6976):740-4.
36. Bhardwaj, R.D., et al., Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12564-8.
37. Jin, K., et al., Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13198-202. Epub 2006 Aug 21.
38. Liu, Y.W., et al., Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci. 2008 Dec;28(11):2254-65.
39. Miller, M.W. and R.S. Nowakowski, Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 1988 Aug 2;457(1):44-52.
40. Nowakowski, R.S., S.B. Lewin, and M.W. Miller, Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.

41. Burd, G.D. and F. Nottebohm, Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol. 1985 Oct 8;240(2):143-52.
42. Rakic, P., Adult neurogenesis in mammals: an identity crisis. J Neurosci. 2002 Feb 1;22(3):614-8.
43. Cooper-Kuhn, C.M. and H.G. Kuhn, Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):13-21.
44. Zhao, M., et al., Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7925-30.
45. Copani, A., et al., Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci. 2001 Jan;24(1):25-31.
46. Katchanov, J., et al., Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
47. Leuner, B., E.R. Glasper, and E. Gould, Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol. 2009 Nov 10;517(2):123-33.
48. Kim, K.K., R.S. Adelstein, and S. Kawamoto, Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. J Biol Chem. 2009 Nov 6;284(45):31052-61.
49. Shingo, T., et al., Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science. 2003 Jan 3;299(5603):117-20.

Low Pass. Incredibly disorganized, rambling, disjointed. But here and there you see glimpses indicating that the author understands the methodology and controversy.

Introduction: Adult Neurogenesis

The initial reports of adult neurogenesis overthrew a roughly one-hundred year old idea that neuronal renewal was virtually impossible beyond birth. When it was first reported that neurons were unable to undergo post-natal mitosis, it seemed rather unfortunate that the cell type most critical for normal human functioning was the one type incapable of regeneration after trauma. Yet when both Joseph Altman and Michael Kaplan first discovered adult neurogenesis in the rodent hippocampus, their work was greeted with pure skepticism as it seemed nearly impossible that their scientific predecessors had not uncovered this process earlier. It was not until 40 years later when Fernando Nottebohm clearly identified continuous neurogenesis in adult canaries and songbirds that the field was re-visited. When combined with the technological advances in biology and immunohistochemistry, evidence for adult neurogenesis was then uncovered in species that spanned the length of the phylogenetic tree, including mice, rats, primates, and later humans. Surprisingly, the underlying purpose or benefit for adult neurogenesis, particularly in higher order species, is still rather unknown, and some even postulate that a gradual decline in adult neurogenesis in a given species is evolutionarily advantageous. Regardless, the potential function that adult neurogenesis plays in behavior, learning, and memory as well as its enormous therapeutic implications, render this process one of the most significant discoveries for the future of human health.

Deleted: phenomenon stating

Deleted: Frank

Deleted: ly high quantities of

Deleted: continually

I) Adult Neurogenesis across Species: Birds

Fernando Nottebohm and his colleagues initially searched for evidence of adult neurogenesis in canaries in the 1980s, despite the fact that initial data for adult neurogenesis in rodents had been largely dismissed [1]. In the avian brain, neural progenitors are housed within the ventricular zone (VZ) of the lateral ventricles, and successful recruitment and migration of these neural precursors occurs throughout most of the year into much of the avian forebrain [2, 3]. These avian forebrain regions that continuously recruit new neurons are functionally homologous to the mammalian hippocampus, striatum, and, somewhat, to the mammalian cortex [2]. Additionally there exists substantial, year-long neuronal recruitment to distinct nuclei of the vocal control system in the avian forebrain, most notably to the hyperstriatum ventralis pars caudalis (HVC) [2]. The HVC projects to the robust nucleus of the archistriatum (RA) for proper song production in males, hence the male HVC and RA are much larger than those of the female, and females do not sing unless treated with male gonadal hormones [4]. The male HVC experiences dramatic changes in size due to both the seasonal fluctuations in circulating testosterone, such that in non-breeding seasons when testosterone is low, the HVC is reduced to almost half of its normal neuronal number [4]. Surprisingly, within 12 months the HVC replaces over half of the lost neurons, corresponding to the onset of the breeding season and an increase in circulating testosterone [4]. The female HVC also demonstrates a continuous, year-long recruitment of new neurons, though they differentiate into interneurons within the HVC that neither project to the RA nor promote song production. Goldman et al., 1983 clearly demonstrated that new neurons in the female canary HVC were morphologically neurons, incorporated the ^{3}H thymidine label, produced action potentials, and were activated in response to acoustic stimuli [1, 4]. Later studies corroborated the results from this original study and found that roughly 20,000 new and functional neurons were

Deleted: Frank

Comment [SMB1]: Paton, not Goldman

added to several, widespread regions throughout the forebrain, in addition to some new recruitment to parts of the avian midbrain, medulla, and cerebellum [4].

This large quantity and expanse of neurogenesis in the avian forebrain suggests that most regions of the avian brain are capable of supporting both neuronal migration as well as later differentiation, and that neurons are able to survive within the forebrain long enough to function in existing circuits. A major reason for the vast quantities and consistency of neural precursor migration to the avian forebrain is the presence of a supportive system for migration and a supportive environment within the forebrain parenchyma that permits the survival of these new neurons. Radial glial cells extend from the VZ proliferative "hot spots" throughout much of the avian forebrain, thus providing a truly supportive scaffold upon which neural precursor cells can travel to distant sites [2, 3]. Additionally, gonadal hormones are crucial for both neuronal recruitment and survival, as estrogen promotes the recruitment and survival of new HVC neurons and testosterone promotes the survival of new RA-projecting HVC neurons [2, 3]. Additionally brain derived neurotrophic factor (BDNF) circulates within the HVC to support the survival and growth of newly recruited neurons [2]. The supportive roles of estrogen and testosterone explain both 1) the seasonal fluctuations in the size of the male HVC that correlates to the concentration of circulating testosterone, and 2) the presence of HVC neurogenesis in females that yields only HVC interneurons incapable of producing song [2, 4]. Furthermore, the expanse of the migratory network and the level of trophic support within the forebrain parenchyma distinguish the avian forebrain from other mammalian species and may explain why adult neurogenesis declines along the phylogenetic tree.

Deleted: guide

Adult Neurogenesis across Species: Rodents

Unlike the vast adult neurogenesis throughout much of the avian forebrain, adult neurogenesis in rodents is drastically reduced to the olfactory bulb and the dentate gyrus of the hippocampus [5]. Neural progenitor cells from the subventricular zone (SVZ) of the lateral ventricles migrate along the rodent rostral migratory stream (RMS), a structure similar to the radial guide cell system of the avian forebrain but devoid of the supporting radial-glial guide cell scaffold that widely projects to the avian forebrain [3, 5, 6]. Within the hippocampus, the subgranular zone (SGZ) of the dentate gyrus acts as a source of neural progenitor cells that can eventually differentiate into mature granule cells and add to the total population of the dentate gyrus [5, 7]. Initial evidence of hippocampal neurogenesis in rodents appeared roughly 50 years ago through the work of Altman and Kaplan who, like Nottebohm, used the ^{3}H thymidine label and the morphological characteristics of neurons to identify adult neurogenesis [8, 9]. Later evidence in support of neural progenitors within the SVZ came from *in vitro* studies showing that explants from mouse SVZ were able to differentiate into both neurons and glia [10]. Additionally, a study by Lois and Alvarez-Buylla in 1994 discovered that rodents indeed possess a migratory path to the olfactory bulb composed of a dense network of astrocytes that guides migrating neuroblasts rostrally to successfully differentiate into olfactory interneurons [11].

There are notable differences between avian and rodent adult neurogenesis, namely the rarity of neurogenesis in non-neurogenic regions of the rodent forebrain and the decline in neurogenesis in aged rodents. Notably, these distinctions indicate a substantial decline in the prevalence of adult neurogenesis from one species to the next. Neurogenesis in the adult rodent brain is regionally constrained such that neurogenesis in non-neurogenic regions is both rare and often transient. Though Kaplan reported evidence of neurogenesis within the rodent visual cortex [12], and Dayer et al., 2005 reported GABAergic neurogenesis within the rodent neocortex [13], the extent of the

Comment [SMB2]: The controversy today is whether indeed these are the only regions of adult neurogenesis in mammals. Gould reports neurogenesis in neocortex of mammals, right?

Comment [SMB3]: Rarity or absence?

new neuron recruitment appeared substantially lower than the quantity of daily neural recruitment within the avian forebrain. Furthermore, a study by Chen et al., 2004 is currently one of the only studies that has detected mouse neocortical neurogenesis, a finding that has yet to be replicated [14]. Select studies have reported neurogenesis in the hypothalamus of mice [15] and rats [16], as well as in sexually dimorphic nuclei of the rat hypothalamus [17]. During adolescence, gonadal hormone activation led to a significant increase in BrdU labeling within the female anteroventral periventricular nucleus and the sexually dimorphic nucleus of the preoptic area in males, suggesting that adolescent neurogenesis is both hormone dependent and crucial for organizing circuits that control adult sexual behavior [17]. Despite the occasional evidence for neurogenesis in these classically non-neurogenic regions, the process of new neuronal recruitment in such regions either occurs only at one distinct developmental time point or is primarily incomplete [5]. Incomplete neurogenesis holds that a vast majority of newly formed neurons in these regions are incapable of surviving long enough to have any functional relevance in existing circuits [5]. Furthermore, the origin and migratory path of these forebrain and midbrain neurons is not always clear, and differs significantly from VZ progenitors and the radial-glial guide cells that clearly allow for the migration of avian neural precursors to a vast majority of the telencephalon.

The second important difference between rodent and avian adult neurogenesis is the gradual decline in the process overtime in any given rodent, even in the major neurogenic region of the hippocampus. The frequency of hippocampal neurogenesis is not uniform throughout the adult rodent life, such that in comparison to 6 month old rats, 12 to 27 month old rats have a significant reduction in the number of both new and migrating neurons within the granule cell layer of the dentate gyrus [18]. This is in stark contrast to avian neurogenesis within both the forebrain and hippocampus that demonstrate a continuous recruitment of new neurons throughout much of adulthood [2,

Comment [SMB4]: But Gould in primates, right?

Comment [SMB5]: They did not show that these were neurons, a critical point to interpret these results.

Comment [SMB6]: ? No one has looked sequentially in the same rodent. How could they?

4, 19]. Specifically, the HVC of the canary forebrain recruits between 0.1 and 0.74% new neurons per day, and the hippocampus recruits between 0.15 and 0.37% new neurons per day [2]. Therefore, the decline in rodent neurogenesis overtime and the vast regional constraints on rodent neurogenesis indicate that the continuity and the expanse of this process become continually restricted in more complex species. This may suggest that despite its profound implications to the overall function of the brain, adult neurogenesis may not be crucial for the optimal survival of higher order species.

Adult Neurogenesis across Species: Primates and Humans

The eventual acceptance of adult neurogenesis in rodents led investigators in primate neurobiology to search for similar process in non-human primates, as any indication of neurogenesis in any region of the primate brain would hold enormous therapeutic potential. In 1985, Pasko Rakic initially reported that neurogenesis was completely absent throughout the entire primate brain, for injections of the ^{3}H-thymidine label failed to stain any neuron in the neocortex, the hippocampus, the olfactory bulb, the basal ganglia, or any other brain region analyzed [20]. It was postulated that the lack of neurogenesis in such a complex, highly developed species was a purposeful adaptation, such that the continual environmental interactions in primates and humans necessitated a set of stable, persistent synapses for much of the adult life [20]. Yet almost 15 years later, Gould et al., 1998 found convincing evidence for neurogenesis within the dentate gyrus of young, middle-aged and older (23 years) Old World Monkeys [21]. BrdU labeled neurons also co-localized with the distinct marker for immature neurons, TOAD-64, indicating that labeled cells were indeed neurons [21]. Around the same time, despite their initial claims that neurogenesis does not occur within primates, Kornack and Rakic 1999 finally observed and validated neurogenesis in the dentate gyrus of adult macaque monkeys [7]. Taken together, these studies pointed to the presence of *in*

Deleted: leading

Comment [SMB7]: But denied seeing it in neocortex.

You fail to engage the crucial distinctions between these studies, which you must do to understand and address the controversy.

You take this up later, when you reach different conclusions while comparing the same two studies.

vivo neural stem cells within the subgranular zone of the dentate gyrus and their later differentiation into hippocampal granule cells, a finding that was highly significant in light of previous reports indicating that primate neurogenesis was neither necessary nor feasible beyond birth.

Neurogenesis within the hippocampus and olfactory bulb of the primate did not come as much of a surprise considering the vast amount of support for these two processes in lower order rodents. Similar to adult neurogenesis in rodents, few investigators were able to provide support for adult neurogenesis in any other region of the primate brain. However, Gould et al., 1999 reported evidence of new, BrdU-labeled neurons within the neocortex that had traveled to their final position along a migratory stream projecting from the SVZ to their final cortical destination [22]. Not surprisingly, this study faced harsh criticism, disbelief, and mostly skepticism, with critics primarily questioning her interpretations and the methods used in the study [23-25]. The study faced such criticism, that the investigators completed a second study to evaluate the survival of these new neurons that they had reportedly identified within the primate neocortex [26]. They continued to find evidence for both hippocampal as well as neocortical neurogenesis, though it was clear that several of the BrdU-labeled cells in the cortex were unable to survive past approximately 9 weeks [26]. Moreover, Bernier et al., 2001 reported adult neurogenesis within the primate amygdala and piriform cortex as well as the presence of a temporal migratory stream along which the precursor cells from the SVZ had possibly migrated to the cortex [27]. Yet they too noted that several migrating neurons along this supposed temporal stream were unable to survive long enough to reach their cortical destination [27]. In response to both claims, Kornack and Rakic again searched for evidence of cortical neurogenesis, yet their results showed only the growth of new glial cells within the cortex and no evidence for neurogenesis [25]. To this day, there have been very few additional reports of successful

Deleted: attempted to

neurogenesis within the cortex of primates, suggesting that any migrating neural precursors from the SVZ are likely to die within weeks of settling in the cortical parenchyma.

Similar to the restrictions of the rodent brain in comparison to the avian brain, the limitations placed on forebrain and midbrain neurogenesis in primates appears to be primarily caused by a decline in basic anatomical support for the process as noted by the grossly underdeveloped RMS in primates. The migratory stream available to primates is limited in its regional and forebrain extension, much like the decreased complexity of the RMS in rodents compared to birds [5]. In the primate RMS neuroblasts rely on neurophilic interactions between one another rather than radial-glial cell guided or even astrocyte guided migration, and extension of the RMS is strictly limited to the olfactory bulb [28]. Additionally, relative to the rodent brain, the migration rate of primate neuroblasts destined for the olfactory bulb is much slower, requiring at least 75 days for full migration after normalizing for differences in brain size between rodents and primates [28]. Thus it appears as though the lost complexity of the primate migratory stream is again a limiting factor in the prevalence of primate neurogenesis in any area other than the olfactory bulb and the hippocampus. This feature places a large restriction on the potential for neural precursors in the SVZ to successfully migrate to any other region of the forebrain. Additionally, the forebrain is essentially considered to be a non-neurogenic region likely void of proper trophic factors to support neural differentiation and survival [5]. Despite reports of neocortical neurogenesis, the ability for new neurons to survive and integrate into functional circuits in any area other than the hippocampus is drastically reduced in comparison to that seen in avian species.

Additionally, there exist important restrictions on hippocampal neurogenesis within the primate, particularly in comparison to rodents. While granule cells that are newly generated in the rodent hippocampus continually add to the pool of hippocampal

neurons in the adult rodent, for example between 30 and 365 days of life, this is likely not the case in primates [28, 29]. Hippocampal neurogenesis in the adult rodent increases in response to several environmental stimuli including exercise [30], sexual experience [31], and learning [32], all of which lead to an overall increase the total number of functional granule cells. However, the primate brain appears to show no significant accumulation of new hippocampal neuron addition, suggesting that hippocampal neurogenesis correlates to an equal rate of neuronal apoptosis [7]. Thus, hippocampal neurogenesis within rodents may facilitate new learning and synaptic plasticity in the face of constant environmental changes or necessary changes in social behavior throughout the adult life. Considering that both primates and humans extensively interact with the environment, it is possible that the gradual decline in neurogenesis in favor of long-lasting synapses and circuits is necessary for long term memory storage and the overall complexity of the primate and human brain [7].

Humans show virtually an identical pattern of neurogenesis as primates, with neural progenitor cells housed only in the hippocampus and the SVZ lining the ventricular system. The first reports of human neural progenitor cells were rather astounding, as they truly verified that neurogenesis was indeed possible in the human brain. Eriksson et al., 1998 evaluated the brains of cancer patients that had been previously treated with BrdU, a treatment that had been used to follow the proliferative profile of tumor cells [33]. The study found a substantial quantity of BrdU labeled cells positive for neural markers within the dentate gyrus of these individuals, providing official proof that neural precursors can survive in the human brain [33]. Knoth et al., 2010 analyzed hippocampal tissue samples from individuals ranging from childhood to almost 90 years of age, specifically searching for neural precursors and neural proliferation [34]. Surprisingly they found that several cells co-localized with both immature neuronal and cell proliferation markers at almost all ages, though they noted a significant decline the

number of labeled cells beyond roughly 40 years of age [34]. Additionally, Sinai et al., 2004 confirmed the presence of SVZ neural progenitors in postmortem adult human tissue as well as a unique band of astrocytes extending from the SVZ [35]. However they found no evidence in support of neural precursor migration along this band of astrocytes, suggesting that neural precursors in humans are even more restricted to distinct locations within the brain parenchyma than even non-human primates [35]. Additionally, a separate study used an alternate method of detecting proliferating neural cells, the phenomenon of carbon-14 labeling, and found that neurons in the neocortex of humans were the exact age of the individual [36]. This suggested that, like primates, neocortical neurogenesis in humans is largely restricted to embryological development prior to birth. No other studies have successfully reported human neurogenesis or the presence of neural precursors in any other region outside of the hippocampus or the SVZ. Nonetheless, the presence of individual neural precursors in humans warrants great attention, and highlights the necessity to identify an optimal environment that would allow neurons to differentiate and thrive in several regions throughout the human brain.

Avian and mammal adult neurogenesis differ in both the frequency of neural turn over as well as the constraints placed on neural progenitors as to where they can survive and differentiate. More importantly, the regional constraints appear to be more limiting to the expanse of adult neurogenesis in more complex species. The fact that neural precursors of the SVZ and hippocampus can survive well into adulthood of rodents, primates, and humans, indicates that age does not appear to restrict the potential for neurogenesis, but rather their capacity to survive in various regions of the brain is the more significant limiting factor. Despite reports of neurogenesis within the rodent neocortex or hypothalamus as well as the primate neocortex, such accounts are

both rare and often conflicting between groups, a finding that is in direct contrast to the widespread and continual neurogenesis throughout the avian forebrain.

II) Temporal vs. Regional Constraints in Adult Neurogenesis

Despite the fact that the total amount of mammalian neurogenesis declines in prevalence both across species and within a given species, adult neurogenesis becomes more regionally constrained when the mammalian brain becomes more complex. Lower order vertebrates such as fish and the aforementioned songbirds experience high quantities of neurogenesis both throughout adulthood and within several higher order forebrain structures. In comparison to birds, mammals as low as rodents have a drastic reduction in the brain regions available for supporting complete neurogenesis. While both avian species and rodents possess a similar pool of neural precursors within the SVZ of the lateral ventricles, their migratory ability and their capability to survive in various forebrain regions is dramatically different. Though both primates and humans still possess distinct pools of neural progenitors that are capable of proliferating well into adulthood, there are even fewer reports of successful integration of such precursors into other brain regions. Additionally, regional constraints on mammalian neurogenesis appears to be the more significant factor in higher order species due to fact that labeling of proliferating and immature neuronal precursors has identified active neural progenitor cells in elderly rodents [18], primates [21], and humans [33, 34]. The fact that any neural precursor exists in the mammalian brain several years after birth indicates that mammalian brains are capable of harvesting and supporting neural stem cells, but that their survival and differentiation requires a distinct environment that appears to be uncharacteristic of higher order brain regions.

It is noteworthy that certain non-neurogenic regions of the mammalian brain, particularly in humans, can suddenly support nascent neurogenesis after trauma due to the change in circulating factors associated with an injured brain region. In a report by Jin et al., 2006, brain biopsy tissue samples from stroke patients were stained for Ki67, a marker of cell proliferation, as well as DCX and βIII tubulin, two markers expressed in new neurons [37]. Particularly within the penumbra, or the surviving cortical tissue surrounding the ischemic core, the study found that several cells were positive for both Ki67 and either DCX or βIII tubulin [37]. As neurogenesis has been shown to be enhanced in such degenerative diseases as Huntington's and Alzheimer's, the authors noted that it is likely that a similar process was taking place in these stroke patients, such that pre-existing neural precursors were stimulated to begin dividing in order to replace lost neurons after the insult [37]. They noted that the source of the precursors could either be the SVZ where progenitors may have migrated along capillary beds, or even local precursors within the cortex [37]. In a similar study, Liu et al., 2008 evaluated tissue samples from epileptic patients undergoing resection of both the hippocampus and the anterior temporal cortex [38]. By staining for DCX in control and epileptic tissue samples, the authors sought to identify if immature, new neurons were more prevalent in epileptic tissue [38]. They found a notable increase in DCX positive cells in both the granule cell layer of the hippocampus and distinct temporal cortical regions of epileptic patients in comparison to control tissue [38]. The authors noted that *in vitro* cultures of epileptic human tissue housed neural progenitor cells, suggesting that the trauma induced by seizure is able to essentially induce proliferation of quiescent progenitor cells within brain regions that are usually non-neurogenic [38].

In both cases, trauma to the brain induced proliferation of neural precursors near the site of the lesion, likely as a last resort attempt to restore function before the level of trauma became fully detrimental. Yet this phenomenon corroborates the idea that adult

neurogenesis in higher order mammals is possible late into adult life but is constrained to distinct regions throughout the brain that appear to have evolved as optimal stem cell niches. Constitutive neurogenesis within the hippocampus is now a well-known, accepted fact in all mammals, as is the presence of neural progenitor cells throughout the SVZ capable of migrating to the olfactory bulb. Though the overall prevalence of neurogenesis appears to decline gradually in aging mammals, it continues to be an ongoing albeit occasional process, and neural progenitors within the hippocampus or SVZ do not appear to suddenly die off as the brain ages. Rather, the major decline in adult neurogenesis that is seen from birds to rodents to monkeys and to humans is due to the major restrictions placed on neural precursors as to the brain environments in which they are capable of thriving. The discovery of adult neurogenesis in humans was an immense milestone in the field of neurological and neurodegenerative disorders, though the therapeutic potential of individual neural precursors cannot be achieved until investigators uncover a method that allows such precursors to survive and function in more widespread regions of the human brain.

Comment [SMB8]: What? I don't understand what you're trying to say.

III) Methods for Detecting Adult Neurogenesis

When the first reports of adult neurogenesis surfaced, most individuals within the scientific community were hostile to even evaluate the methods used by such scientists as Michael Kaplan and Joseph Altman who had proposed such a phenomenon. Even decades later when investigations into this process became more widespread, non-believers were more skeptical of the idea rather than the accuracy of the methods used. Yet when both BrdU labeling and immunohistochemistry were incorporated into the study of adult neurogenesis, claims of neurogenesis in higher order primates and in non-neurogenic brain regions called for a re-evaluation of the evidence [23]. Much of the criticism of certain groups concerned the specific methods and BrdU protocols used as

well as the individual interpretations of the results. The methodological critiques were both justified and legitimate, considering that the idea of adult neurogenesis in mammals has such profound implications to human health. Though the current use of BrdU is highly efficient and validated, it requires that investigators undergo rigorous controls and consider several alternate interpretations in order for their results to be accepted by the scientific community.

Old versus New Thymidine Analogs

The first reports of adult neurogenesis in both mammals and birds initially used the analog ^{3}H-thymidine that was efficiently incorporated into dividing cells in the DNA synthesis phase of mitosis, thus indicating a cell's "birthday" [4, 9, 39]. Cells exposed to the ^{3}H-thymidine were later exposed to silver grains that would bind the thymidine analog and that would allow for cell visualization via autoradiography [4, 9, 23, 40]. Investigators delineated stringent criteria for cell labeling, such that a mitotically-active cell was one whose overlying silver grains were at least 50% of the labeling in cells that showed maximal labeling [23]. Nottebohm reported that avian neurons were considered labeled if they possessed 3 silver grains per nucleus, while Michael Kaplan reported that a labeled rodent neuron was one with 5 grains over the nucleus in the olfactory bulb or 19 grains over the nucleus of a granule cell from the dentate gyrus [4, 8]. This initial method in detecting cell proliferation was highly efficient and validated, and it was advantageous in that it allowed for an objective, quantitative analysis of proliferating cells.

Deleted: Frank

Though the potential for quantification of cell division was beneficial for early neurobiologists using this method, major issues arose when attempting to identify cell type without the vast array of neural or glial markers available today. Early reports of adult neurogenesis determined cell type using both ultrathin sections stained with either

Nissl or Cresyl violet or electron microscopy to search for morphological clues that would distinguish a neuron from a glial cell [4, 8]. In general, cells were considered to be neurons when they possessed round, compact nuclei with one or two nucleoli when visualized with standard histology, and if electron microscopy (EM) revealed the presence of synaptic terminals with multiple types of vesicles, smooth membrane contours, and long processes containing microtubules [1, 4, 8, 12, 41]. The use of the 3H thymidine label and the detailed characterization of neuronal morphology initially identified the presence of adult neurogenesis in both avian and mammalian speices, a process that had long been considered impossible. Clearly, the phenomenon was initially dismissed, and not until almost 40 years later did innovations in biological techniques allow for the idea to be more highly reconsidered.

The idea of neurogenesis, particularly in the mammalian hippocampus, was reconsidered in the early 1990s during which both neural and glial-specific antibodies were readily available and when researchers first incorporated the use of bromodeoxyuridine (BrdU) to tag neuronal cell division in adult mammals. The use of BrdU to assess the proliferative nature of growing tumors in cancer patients prompted investigators to apply the same marker to label proliferating and migrating neurons within the mature CNS [40]. Similar to ^{3}H thymidine, BrdU could be incorporated into the cell specifically during the S phase of mitosis after which the label was identified, though using immunohistochemistry [40]. Additionally, BrdU held major advantages over that of ^{3}H thymidine in that visualizing the BrdU label took only 1 to 3 days rather than the 1 to 3 months required for the autoradiography associated with ^{3}H thymidine, and specialized facilities were not required to visualize the BrdU label [39]. Additionally, new antibodies that were specific to surface or cytoskeletal markers of neurons or glial cells allowed neurobiologists to distinguish one cell type from another without relying on subjective morphological criteria or the demanding techniques of EM.

Comment [SMB9]: Yes.

To this day, BrdU has arguably been the most significant contribution to the field of adult neurogenesis, and it is the most prominent method used to detect the process. The transition from the ^{3}H thymidine analog to the BrdU label was an immense move in the field that made the study of adult neurogenesis both feasible and accessible to virtually any neurobiologist. Unfortunately, the BrdU method is not without inherent flaws rendering it continually susceptible to much scrutiny throughout the scientific community. The rather subjective nature of immunohistochemistry required to detect the BrdU label is the major factor in the BrdU protocol that may cause investigators to evaluate data rather differently. Due to its current prevalence within neurobiology, there are several standard controls and considerations that investigators cannot ignore in order for their studies to be both accepted and validated by their peers.

Comment [SMB10]: Yes.

Considerations in BrdU Staining: Route and Dose of BrdU

Though the use of BrdU dramatically advanced the field of adult neurogenesis, there exist several variations in individual BrdU protocols that can significantly impact how results are interpreted. One of the major differences identified across protocols is the concentration of BrdU used, as well as the route of its injection [42, 43]. It has been proposed that a dose of 50mg/kg BrdU is sufficient to label any and all dividing neurons, though doses ranging from 50 to 500mg/kg have also been used in order to optimize staining and to account for smaller neurons that might not be labeled with protocols using the lower doses [42, 44]. Though lower doses of BrdU may ultimately underestimate the quantity of proliferating cells, administration of higher doses renders the experimenters more likely to stain cells undergoing DNA turnover or even apoptosis [23, 42, 43]. Cells preparing for apoptosis or in the process of degeneration lose Cdk inhibition, leading them to re-enter the cell cycle prior to death [45, 46]. In such cases, BrdU will stain cells undergoing either process leading to erroneous and false positive

results. Additionally, as with any radioactive or exogenous agent applied to cells, BrdU is considered to be a mutagen, meaning its successful incorporation into nuclear material and the general health of the cell may both be compromised when higher doses are administered [42, 43]. Both the potential for BrdU to label apoptotic cells and the mutagenic qualities of BrdU are major technical considerations in any BrdU protocol, and virtually any study that does not independently control for both factors is deemed invalid. Furthermore, investigators use both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes for BrdU administration, meaning there may be a significant degree of variation in BrdU access to CNS tissue. I.p. injections are subject to potentially thwarted access to the CNS due to differences in blood brain barrier permeability to BrdU across species [43]. Continuous i.c.v. infusion through the lateral ventricles enhances the likelihood that all relevant cells within the CNS will be labeled, yet the level of exposure may render neuronal cells more susceptible to the mutagenic qualities of BrdU including altered gene expression or even cell death [43]. Ultimately, variations in both the dose and route of BrdU administration reported across groups can significantly alter the outcome of any one experiment and lead to several different interpretations of one set of results.

Considerations in BrdU Staining: Antibody Specificity

As with any immunohistological assay, the specificity and sensitivity of primary and secondary antibodies must be taken into consideration when designing an optimal protocol. Antibody specificity is even more crucial in BrdU staining protocols, considering that false positive staining can lead investigators to make truly profound conclusions from inaccurate results. Optimization of antibody specificity must be applied not only to the actual BrdU label but also to any additional labels used to mark neurons or glial cells. A study by Leuner et al., 2009 evaluated differences in the sensitivities of

BrdU antibodies from such companies as Vector, BD, and Roche, and found significant differences in the number of newborn cells labeled by each separate antibody within the dentate gyrus [47]. Several biotech companies sell BrdU antibodies and the choice of company is highly variable across research groups, meaning the specific choice of BrdU antibody may become a significant confounding factor. More crucial than the choice of BrdU antibody is the use of proper labels that undoubtedly distinguish a neuron from a glial cell, an endothelial cell, and other nascent cells within the CNS. Though there are currently several markers for each distinct stage within neuronal development, the issue of both antibody specificity and background staining also holds true for antibodies chosen to define cell type. Neu-N is the most widely used antibody to identify neurons, and it was not until recently that the Neu-N antigen was identified as the neuron-specific Fox-3 gene, a member of the Fox-1 gene splicing family [48]. Additionally, the authors found that any cross reactivity of Neu-N occurs with the neural-specific synapsin-1 protein, meaning it is still a highly acceptable antibody to chose for neural staining [48]. However, in addition to variations in BrdU antibody binding specificity, Leuner et al., 2009 reported high background staining when using the Neu-N antibody, specifically based on the individual DNA denaturation protocol [47]. Since HCl, HCl and formamide, and steam heating are all common treatments used for DNA denaturation, the study evaluated each technique separately to determine how and if they altered background staining. Each technique resulted in notable differences in the Neu-N signal to noise ratio, a factor that can drastically overestimate or underestimate Neu-N staining in a BrdU protocol [47].

Though the technique of BrdU labeling is currently the gold standard in evaluating adult neurogenesis, there are considerable differences between individual protocols that may greatly affect how a given set of results is interpreted. Changes in both the does and route of BrdU injections can lead to false positive results by labeling

old neurons going through apoptotic cell cycling or even cell death. Additionally, variations in primary antibodies to BrdU or neural markers can result in different staining intensities, causing individual investigators to rely on their own intuition and judgment in counting cells. Therefore, it is clear that these specific aspects of a BrdU protocol must be as consistent as possible across research groups when attempting to verify the presence of adult neurogenesis. Most importantly, this is of particular importance in higher order species or in classically non-neurogenic regions where there may only be miniscule, yet extremely notable, quantities of dividing cells.

Controls in BrdU Staining

Despite the aforementioned flaws inherent to the BrdU technique, it remains the most prominent, efficient, and validated method available for detecting adult neurogenesis, especially in mammalian species. The technique of BrdU staining exploits the fundamental phase of DNA synthesis inherent to the process of mitosis that is virtually identical across the phylogenetic tree. Even to this day, the idea of adult neurogenesis is almost beyond belief thus it comes as no surprise when investigators looking into the process must undergo a series of rigorous controls. Notably, with the advent and relative abundance of confocal microscopy, it has become almost a routine process that adult neurogenesis be verified with the use of this 3-dimensional imaging technique, so as to ensure that the full depth of a given cell is uniformly labeled with both BrdU and neuronal markers [23]. For example, a study by Dayer et al., 2005 reported neurogenesis in the adult rat neocortex and striatum, and used confocal microscopy combined with a total of eight different neuronal markers including Neu-N, CRMP4, NG2, DCX, GABA and GAD67 to unquestionably verify that the BrdU label was found in neurons [13]. Confocal imaging to detect the presence of both BrdU and neuronal markers demonstrated the presence of the two labels throughout several z-planes of the

double-labeled cells, further indicating that the BrdU positive cell was not simply a non-neuronal cell layered on top of a neuron [13]. Current investigations in adult neurogenesis must also verify that the BrdU label has not been incorporated into old neurons that have re-entered the cell cycle prior to apoptosis. A study by Zhao et al., 2003 reported the occurrence of new neurons within the substantia nigra of mice, a finding that would clearly have a profound influence on Parkinson's Disease therapeutics [44]. The authors were careful to control for potential BrdU incorporation into degenerating or apoptotic cells by sacrificing a subset of animals 2 days after initiating i.c.v infusions of BrdU, a time point at which apoptotic activity of nigral cells would be at a maximum [44]. BrdU incorporation was not observed at this time point, and incorporation was highest 21 days after infusion, suggesting that staining at the later time points could be attributed to neurogenesis [44].

Deleted: by

The BrdU technique is currently unparalleled in its ability to detect adult neurogenesis, though it is apparent that issues of antibody specificity, background staining, labeling of apoptotic cells, and incorrect identification of cell type are all legitimate reasons for the variations in data interpretation across research groups. When Gould et al., 1999 first reported adult neurogenesis in the neocortex of primates, the skepticism that ensued was primarily concerned with the methods used in the original study and how the authors interpreted them [22-24]. Critics were skeptical of whether BrdU was labeling new cells or old cells undergoing apoptosis, whether labels used to identify neurons were truly neuron-specific, and whether optical issues with cell identification were taken into consideration [23]. Regardless, the application of BrdU labeling successfully replaced the original ^{3}H-thymidine method and is in continuous use today by most of the leading experts in adult neurogenesis. However it is necessary that every investigator use multiple controls in their individual BrdU staining protocols including confocal microscopy, multiple neuronal markers, and multiple time points after

BrdU injections to rule out BrdU labeling of apoptotic cells. When appropriate controls are used and the aforementioned technical considerations are addressed, adult neurogenesis can be validated in virtually any species and any brain region. Until an alternate, more accurate method arises, BrdU staining will continue to be the prominent method of choice in this profound and growing field.

References

1. Goldman, S.A. and F. Nottebohm, Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A. 1983 Apr;80(8):2390-4.
2. Gahr, M., et al., What is the adaptive role of neurogenesis in adult birds? Prog Brain Res. 2002;138:233-54.
3. Doetsch, F. and C. Scharff, Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav Evol. 2001;58(5):306-22.
4. Nottebohm, F., Neuronal replacement in adulthood. Ann N Y Acad Sci. 1985;457:143-61.
5. Bonfanti, L. and P. Peretto, Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci. 2011 Sep;34(6):930-50.
6. Gould, E., How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007 Jun;8(6):481-8.
7. Kornack, D.R. and P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5768-73.
8. Kaplan, M.S. and J.W. Hinds, Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 1977 Sep 9;197(4308):1092-4.
9. Altman, J. and G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965 Jun;124(3):319-35.
10. Lois, C. and A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.
11. Lois, C. and A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.
12. Kaplan, M.S., Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981 Jan 10;195(2):323-38.
13. Dayer, A.G., et al., New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005 Jan 31;168(3):415-27.
14. Chen, J., S.S. Magavi, and J.D. Macklis, Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A. 2004 Nov 16;101(46):16357-62.
15. Kokoeva, M.V., H. Yin, and J.S. Flier, Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science. 2005 Oct 28;310(5748):679-83.
16. Xu, Y., et al., Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol. 2005 Apr;192(2):251-64.

17. Ahmed, E.I., et al., Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci. 2008 Sep;11(9):995-7.
18. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 Mar 15;16(6):2027-33.
19. Goldman, S.A., Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998 Aug;36(2):267-86.
20. Rakic, P., Limits of neurogenesis in primates. Science. 1985 Mar 1;227(4690):1054-6.
21. Gould, E., et al., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5263-7.
22. Gould, E., et al., Neurogenesis in the neocortex of adult primates. Science. 1999 Oct 15;286(5439):548-52.
23. Rakic, P., Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci. 2002 Jan;3(1):65-71.
24. Nowakowski, R.S. and N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion? Science. 2000 May 5;288(5467):771.
25. Kornack, D.R. and P. Rakic, Cell proliferation without neurogenesis in adult primate neocortex. Science. 2001 Dec 7;294(5549):2127-30.
26. Gould, E., et al., Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10910-7.
27. Bernier, P.J., et al., Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11464-9.
28. Kornack, D.R. and P. Rakic, The generation, migration, and differentiation of olfactory neurons in the adult primate brain. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4752-7.
29. Bayer, S.A., J.W. Yackel, and P.S. Puri, Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science. 1982 May 21;216(4548):890-2.
30. Pereira, A.C., et al., An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5638-43.
31. Leuner, B., E.R. Glasper, and E. Gould, Sexual Experience Promotes Adult Neurogenesis in the Hippocampus Despite an Initial Elevation in Stress Hormones. PLoS ONE. 5(7).
32. Gould, E., et al., Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci. 1999 Mar;2(3):260-5.
33. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.
34. Knoth, R., et al., Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One. 2010 Jan 29;5(1).
35. Sanai, N., et al., Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature. 2004 Feb 19;427(6976):740-4.
36. Bhardwaj, R.D., et al., Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12564-8.
37. Jin, K., et al., Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13198-202. Epub 2006 Aug 21.
38. Liu, Y.W., et al., Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci. 2008 Dec;28(11):2254-65.

39. Miller, M.W. and R.S. Nowakowski, Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 1988 Aug 2;457(1):44-52.
40. Nowakowski, R.S., S.B. Lewin, and M.W. Miller, Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.
41. Burd, G.D. and F. Nottebohm, Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol. 1985 Oct 8;240(2):143-52.
42. Rakic, P., Adult neurogenesis in mammals: an identity crisis. J Neurosci. 2002 Feb 1;22(3):614-8.
43. Cooper-Kuhn, C.M. and H.G. Kuhn, Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):13-21.
44. Zhao, M., et al., Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7925-30.
45. Copani, A., et al., Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci. 2001 Jan;24(1):25-31.
46. Katchanov, J., et al., Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
47. Leuner, B., E.R. Glasper, and E. Gould, Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol. 2009 Nov 10;517(2):123-33.
48. Kim, K.K., R.S. Adelstein, and S. Kawamoto, Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. J Biol Chem. 2009 Nov 6;284(45):31052-61.
49. Shingo, T., et al., Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science. 2003 Jan 3;299(5603):117-20.

Controversy Question

Student 5

Pass, with considerable range of opinions on writing style and organization of the answer.

PASS PLUS: Excellent essay – well-written and informative. Explictly contrasts neurogenesis across species rather than just giving evidence. Temporal constraints was glossed over when comparing species. Could have given more of an opinion about Part III and what we can conclude about neurogenesis given the methods at our disposal.

Introduction: Adult Neurogenesis

The initial reports of adult neurogenesis overthrew a roughly one-hundred year old phenomenon stating that neuronal renewal was virtually impossible beyond birth. When it was first reported that neurons were unable to undergo post-natal mitosis, it seemed rather unfortunate that the cell type most critical for normal human functioning was the one type incapable of regeneration after trauma. Yet when both Joseph Altman and Michael Kaplan first discovered adult neurogenesis in the rodent hippocampus, their work was greeted with pure skepticism as it seemed nearly impossible that their scientific predecessors had not uncovered this process earlier. It was not until 40 years later when Frank Nottebohm clearly identified continuously high quantities of neurogenesis in adult canaries and songbirds that the field was re-visited. When combined with the technological advances in biology and immunohistochemistry, evidence for adult neurogenesis was then continually uncovered in species that spanned the length of the phylogenetic tree, including mice, rats, primates, and later humans. Surprisingly, the underlying purpose or benefit for adult neurogenesis, particularly in higher order species, is still rather unknown, and some even postulate that a gradual decline in adult neurogenesis in a given species is evolutionarily advantageous. Regardless, the potential function that adult neurogenesis plays in behavior, learning, and memory as well as its enormous therapeutic implications, render this process one of the most significant discoveries for the future of human health.

Comment [S1]: Really well written introduction!

I) Adult Neurogenesis across Species: Birds

Frank Nottebohm and his colleagues initially searched for evidence of adult neurogenesis in canaries in the 1980s, despite the fact that initial data for adult neurogenesis in rodents had been largely dismissed [1]. In the avian brain, neural progenitors are housed within the ventricular zone (VZ) of the lateral ventricles, and successful recruitment and migration of these neural precursors occurs throughout most of the year into much of the avian forebrain [2, 3]. These avian forebrain regions that continuously recruit new neurons are functionally homologous to the mammalian hippocampus, striatum, and, somewhat, to the mammalian cortex [2]. Additionally there exists substantial, year-long neuronal recruitment to distinct nuclei of the vocal control system in the avian forebrain, most notably to the hyperstriatum ventralis pars caudalis (HVC) [2]. The HVC projects to the robust nucleus of the archistriatum (RA) for proper song production in males, hence the male HVC and RA are much larger than those of the female, and females do not sing unless treated with male gonadal hormones [4]. The male HVC experiences dramatic changes in size due to both the seasonal fluctuations in circulating testosterone, such that in non-breeding seasons when testosterone is low, the HVC is reduced to almost half of its normal neuronal number [4]. Surprisingly, within 12 months the HVC replaces over half of the lost neurons, corresponding to the onset of the breeding season and an increase in circulating testosterone [4]. The female HVC also demonstrates a continuous, year-long recruitment of new neurons, though they differentiate into interneurons within the HVC that neither project to the RA nor promote song production. Goldman et al., 1983 clearly demonstrated that new neurons in the female canary HVC were morphologically neurons, incorporated the ^{3}H thymidine label, produced action potentials, and were activated in response to acoustic stimuli [1, 4]. Later studies corroborated the results from this original study and found that roughly 20,000 new and functional neurons were

added to several, widespread regions throughout the forebrain, in addition to some new recruitment to parts of the avian midbrain, medulla, and cerebellum [4].

This large quantity and expanse of neurogenesis in the avian forebrain suggests that most regions of the avian brain are capable of supporting both neuronal migration as well as later differentiation, and that neurons are able to survive within the forebrain long enough to function in existing circuits. A major reason for the vast quantities and consistency of neural precursor migration to the avian forebrain is the presence of a supportive system for migration and a supportive environment within the forebrain parenchyma that permits the survival of these new neurons. Radial guide cells extend from the VZ proliferative "hot spots" throughout much of the avian forebrain, thus providing a truly supportive scaffold upon which neural precursor cells can travel to distant sites [2, 3]. Additionally, gonadal hormones are crucial for both neuronal recruitment and survival, as estrogen promotes the recruitment and survival of new HVC neurons and testosterone promotes the survival of new RA-projecting HVC neurons [2, 3]. Additionally brain derived neurotrophic factor (BDNF) circulates within the HVC to support the survival and growth of newly recruited neurons [2]. The supportive roles of estrogen and testosterone explain both 1) the seasonal fluctuations in the size of the male HVC that correlates to the concentration of circulating testosterone, and 2) the presence of HVC neurogenesis in females that yields only HVC interneurons incapable of producing song [2, 4]. Furthermore, the expanse of the migratory network and the level of trophic support within the forebrain parenchyma distinguish the avian forebrain from other mammalian species and may explain why adult neurogenesis declines along the phylogenetic tree.

Adult Neurogenesis across Species: Rodents

Unlike the vast adult neurogenesis throughout much of the avian forebrain, adult neurogenesis in rodents is drastically reduced to the olfactory bulb and the dentate gyrus of the hippocampus [5]. Neural progenitor cells from the subventricular zone (SVZ) of the lateral ventricles migrate along the rodent rostral migratory stream (RMS), a structure similar to the radial guide cell system of the avian forebrain but devoid of the supporting radial-glial guide cell scaffold that widely projects to the avian forebrain [3, 5, 6]. Within the hippocampus, the subgranular zone (SGZ) of the dentate gyrus acts as a source of neural progenitor cells that can eventually differentiate into mature granule cells and add to the total population of the dentate gyrus [5, 7]. Initial evidence of hippocampal neurogenesis in rodents appeared roughly 50 years ago through the work of Altman and Kaplan who, like Nottebohm, used the ^{3}H thymidine label and the morphological characteristics of neurons to identify adult neurogenesis [8, 9]. Later evidence in support of neural progenitors within the SVZ came from *in vitro* studies showing that explants from mouse SVZ were able to differentiate into both neurons and glia [10]. Additionally, a study by Lois and Alvarez-Buylla in 1994 discovered that rodents indeed possess a migratory path to the olfactory bulb composed of a dense network of astrocytes that guides migrating neuroblasts rostrally to successfully differentiate into olfactory interneurons [11].

There are notable differences between avian and rodent adult neurogenesis, namely the rarity of neurogenesis in non-neurogenic regions of the rodent forebrain and the decline in neurogenesis in aged rodents. Notably, these distinctions indicate a substantial decline in the prevalence of adult neurogenesis from one species to the next. Neurogenesis in the adult rodent brain is regionally constrained such that neurogenesis in non-neurogenic regions is both rare and often transient. Though Kaplan reported evidence of neurogenesis within the rodent visual cortex [12], and Dayer al., 2005 reported GABAergic neurogenesis within the rodent neocortex [13], the extent of the

Deleted: et

new neuron recruitment appeared substantially lower than the quantity of daily neural recruitment within the avian forebrain. Furthermore, a study by Chen et al., 2004 is currently one of the only studies that has detected mouse neocortical neurogenesis, a finding that has yet to be replicated [14]. Select studies have reported neurogenesis in the hypothalamus of mice [15] and rats [16], as well as in sexually dimorphic nuclei of the rat hypothalamus [17]. During adolescence, gonadal hormone activation led to a significant increase in BrdU labeling within the female anteroventral periventricular nucleus and the sexually dimorphic nucleus of the preoptic area in males, suggesting that adolescent neurogenesis is both hormone dependent and crucial for organizing circuits that control adult sexual behavior [17]. Despite the occasional evidence for neurogenesis in these classically non-neurogenic regions, the process of new neuronal recruitment in such regions either occurs only at one distinct developmental time point or is primarily incomplete [5]. Incomplete neurogenesis holds that a vast majority of newly formed neurons in these regions are incapable of surviving long enough to have any functional relevance in existing circuits [5]. Furthermore, the origin and migratory path of these forebrain and midbrain neurons is not always clear, and differs significantly from VZ progenitors and the radial-glial guide cells that clearly allow for the migration of avian neural precursors to a vast majority of the telencephalon.

The second important difference between rodent and avian adult neurogenesis is the gradual decline in the process overtime in any given rodent, even in the major neurogenic region of the hippocampus. The frequency of hippocampal neurogenesis is not uniform throughout the adult rodent life, such that in comparison to 6 month old rats, 12 to 27 month old rats have a significant reduction in the number of both new and migrating neurons within the granule cell layer of the dentate gyrus [18]. This is in stark contrast to avian neurogenesis within both the forebrain and hippocampus that demonstrate a continuous recruitment of new neurons throughout much of adulthood [2,

4, 19]. Specifically, the HVC of the canary forebrain recruits between 0.1 and 0.74% new neurons per day, and the hippocampus recruits between 0.15 and 0.37% new neurons per day [2]. Therefore, the decline in rodent neurogenesis overtime and the vast regional constraints on rodent neurogenesis indicate that the continuity and the expanse of this process become continually restricted in more complex species. This may suggest that despite its profound implications to the overall function of the brain, adult neurogenesis may not be crucial for the optimal survival of higher order species.

Adult Neurogenesis across Species: Primates and Humans

The eventual acceptance of adult neurogenesis in rodents led investigators in primate neurobiology to search for similar process in non-human primates, as any indication of neurogenesis in any region of the primate brain would hold enormous therapeutic potential. In 1985, Pasko Rakic initially reported that neurogenesis was completely absent throughout the entire primate brain, for injections of the ^{3}H-thymidine label failed to stain any neuron in the neocortex, the hippocampus, the olfactory bulb, the basal ganglia, or any other brain region analyzed [20]. It was postulated that the lack of neurogenesis in such a complex, highly developed species was a purposeful adaptation, such that the continual environmental interactions in primates and humans necessitated a set of stable, persistent synapses for much of the adult life [20]. Yet almost 15 years later, Gould et al., 1998 found convincing evidence for neurogenesis within the dentate gyrus of young, middle-aged and older (23 years) Old World Monkeys [21]. BrdU labeled neurons also co-localized with the distinct marker for immature neurons, TOAD-64, indicating that labeled cells were indeed neurons [21]. Around the same time, despite their initial claims that neurogenesis does not occur within primates, Kornack and Rakic 1999 finally observed and validated neurogenesis in the dentate gyrus of adult macaque monkeys [7]. Taken together, these studies pointed to the presence of *in*

Deleted: leading

vivo neural stem cells within the subgranular zone of the dentate gyrus and their later differentiation into hippocampal granule cells, a finding that was highly significant in light of previous reports indicating that primate neurogenesis was neither necessary nor feasible beyond birth.

Neurogenesis within the hippocampus and olfactory bulb of the primate did not come as much of a surprise considering the vast amount of support for these two processes in lower order rodents. Similar to adult neurogenesis in rodents, few investigators were able to provide support for adult neurogenesis in any other region of the primate brain. However, Gould et al., 1999 attempted to report evidence of new, BrdU-labeled neurons within the neocortex that had traveled to their final position along a migratory stream projecting from the SVZ to their final cortical destination [22]. Not surprisingly, this study faced harsh criticism, disbelief, and mostly skepticism, with critics primarily questioning her interpretations and the methods used in the study [23-25]. The study faced such criticism, that the investigators completed a second study to evaluate the survival of these new neurons that they had reportedly identified within the primate neocortex [26]. They continued to find evidence for both hippocampal as well as neocortical neurogenesis, though it was clear that several of the BrdU-labeled cells in the cortex were unable to survive past approximately 9 weeks [26]. Moreover, Bernier et al., 2001 reported adult neurogenesis within the primate amygdala and piriform cortex as well as the presence of a temporal migratory stream along which the precursor cells from the SVZ had possibly migrated to the cortex [27]. Yet they too noted that several migrating neurons along this supposed temporal stream were unable to survive long enough to reach their cortical destination [27]. In response to both claims, Kornack and Rakic again searched for evidence of cortical neurogenesis, yet their results showed only the growth of new glial cells within the cortex and no evidence for neurogenesis [25]. To this day, there have been very few additional reports of successful

neurogenesis within the cortex of primates, suggesting that any migrating neural precursors from the SVZ are likely to die within weeks of settling in the cortical parenchyma.

Similar to the restrictions of the rodent brain in comparison to the avian brain, the limitations placed on forebrain and midbrain neurogenesis in primates appears to be primarily caused by a decline in basic anatomical support for the process as noted by the grossly underdeveloped RMS in primates. The migratory stream available to primates is limited in its regional and forebrain extension, much like the decreased complexity of the RMS in rodents compared to birds [5]. In the primate RMS neuroblasts rely on neurophilic interactions between one another rather than radial-glial cell guided or even astrocyte guided migration, and extension of the RMS is strictly limited to the olfactory bulb [28]. Additionally, relative to the rodent brain, the migration rate of primate neuroblasts destined for the olfactory bulb is much slower, requiring at least 75 days for full migration after normalizing for differences in brain size between rodents and primates [28]. Thus it appears as though the lost complexity of the primate migratory stream is again a limiting factor in the prevalence of primate neurogenesis in any area other than the olfactory bulb and the hippocampus. This feature places a large restriction on the potential for neural precursors in the SVZ to successfully migrate to any other region of the forebrain. Additionally, the forebrain is essentially considered to be a non-neurogenic region likely void of proper trophic factors to support neural differentiation and survival [5]. Despite reports of neocortical neurogenesis, the ability for new neurons to survive and integrate into functional circuits in any area other than the hippocampus is drastically reduced in comparison to that seen in avian species.

Additionally, there exist important restrictions on hippocampal neurogenesis within the primate, particularly in comparison to rodents. While granule cells that are newly generated in the rodent hippocampus continually add to the pool of hippocampal

neurons in the adult rodent, for example between 30 and 365 days of life, this is likely not the case in primates [28, 29]. Hippocampal neurogenesis in the adult rodent increases in response to several environmental stimuli including exercise [30], sexual experience [31], and learning [32], all of which lead to an overall increase the total number of functional granule cells. However, the primate brain appears to show no significant accumulation of new hippocampal neuron addition, suggesting that hippocampal neurogenesis correlates to an equal rate of neuronal apoptosis [7]. Thus, hippocampal neurogenesis within rodents may facilitate new learning and synaptic plasticity in the face of constant environmental changes or necessary changes in social behavior throughout the adult life. Considering that both primates and humans extensively interact with the environment, it is possible that the gradual decline in neurogenesis in favor of long-lasting synapses and circuits is necessary for long term memory storage and the overall complexity of the primate and human brain [7].

Comment [S2]: Great integration with functional implications of neurogenesis.

Humans show virtually an identical pattern of neurogenesis as primates, with neural progenitor cells housed only in the hippocampus and the SVZ lining the ventricular system. The first reports of human neural progenitor cells were rather astounding, as they truly verified that neurogenesis was indeed possible in the human brain. Eriksson et al., 1998 evaluated the brains of cancer patients that had been previously treated with BrdU, a treatment that had been used to follow the proliferative profile of tumor cells [33]. The study found a substantial quantity of BrdU labeled cells positive for neural markers within the dentate gyrus of these individuals, providing official proof that neural precursors can survive in the human brain [33]. Knoth et al., 2010 analyzed hippocampal tissue samples from individuals ranging from childhood to almost 90 years of age, specifically searching for neural precursors and neural proliferation [34]. Surprisingly they found that several cells co-localized with both immature neuronal and cell proliferation markers at almost all ages, though they noted a significant decline the

number of labeled cells beyond roughly 40 years of age [34]. Additionally, Sinai et al., 2004 confirmed the presence of SVZ neural progenitors in postmortem adult human tissue as well as a unique band of astrocytes extending from the SVZ [35]. However they found no evidence in support of neural precursor migration along this band of astrocytes, suggesting that neural precursors in humans are even more restricted to distinct locations within the brain parenchyma than even non-human primates [35]. Additionally, a separate study used an alternate method of detecting proliferating neural cells, the phenomenon of carbon-14 labeling, and found that neurons in the neocortex of humans were the exact age of the individual [36]. This suggested that, like primates, neocortical neurogenesis in humans is largely restricted to embryological development prior to birth. No other studies have successfully reported human neurogenesis or the presence of neural precursors in any other region outside of the hippocampus or the SVZ. Nonetheless, the presence of individual neural precursors in humans warrants great attention, and highlights the necessity to identify an optimal environment that would allow neurons to differentiate and thrive in several regions throughout the human brain.

Avian and mammal adult neurogenesis differ in both the frequency of neural turn over as well as the constraints placed on neural progenitors as to where they can survive and differentiate. More importantly, the regional constraints appear to be more limiting to the expanse of adult neurogenesis in more complex species. The fact that neural precursors of the SVZ and hippocampus can survive well into adulthood of rodents, primates, and humans, indicates that age does not appear to restrict the potential for neurogenesis, but rather their capacity to survive in various regions of the brain is the more significant limiting factor. Despite reports of neurogenesis within the rodent neocortex or hypothalamus as well as the primate neocortex, such accounts are

Comment [S3]: Good summary

both rare and often conflicting between groups, a finding that is in direct contrast to the widespread and continual neurogenesis throughout the avian forebrain.

II) Temporal vs. Regional Constraints in Adult Neurogenesis

Despite the fact that the total amount of mammalian neurogenesis declines in prevalence both across species and within a given species, adult neurogenesis becomes more regionally constrained when the mammalian brain becomes more complex. Lower order vertebrates such as fish and the aforementioned songbirds experience high quantities of neurogenesis both throughout adulthood and within several higher order forebrain structures. In comparison to birds, mammals as low as rodents have a drastic reduction in the brain regions available for supporting complete neurogenesis. While both avian species and rodents possess a similar pool of neural precursors within the SVZ of the lateral ventricles, their migratory ability and their capability to survive in various forebrain regions is dramatically different. Though both primates and humans still possess distinct pools of neural progenitors that are capable of proliferating well into adulthood, there are even fewer reports of successful integration of such precursors into other brain regions. Additionally, regional constraints on mammalian neurogenesis appears to be the more significant factor in higher order species due to fact that labeling of proliferating and immature neuronal precursors has identified active neural progenitor cells in elderly rodents [18], primates [21], and humans [33, 34]. The fact that any neural precursor exists in the mammalian brain several years after birth indicates that mammalian brains are capable of harvesting and supporting neural stem cells, but that their survival and differentiation requires a distinct environment that appears to be uncharacteristic of higher order brain regions.

Comment [S4]: Needs to answer about whether temporal restrictions differ among species.

It is noteworthy that certain non-neurogenic regions of the mammalian brain, particularly in humans, can suddenly support nascent neurogenesis after trauma due to the change in circulating factors associated with an injured brain region. In a report by Jin et al., 2006, brain biopsy tissue samples from stroke patients were stained for Ki67, a marker of cell proliferation, as well as DCX and βIII tubulin, two markers expressed in new neurons [37]. Particularly within the penumbra, or the surviving cortical tissue surrounding the ischemic core, the study found that several cells were positive for both Ki67 and either DCX or βIII tubulin [37]. As neurogenesis has been shown to be enhanced in such degenerative diseases as Huntington's and Alzheimer's, the authors noted that it is likely that a similar process was taking place in these stroke patients, such that pre-existing neural precursors were stimulated to begin dividing in order to replace lost neurons after the insult [37]. They noted that the source of the precursors could either be the SVZ where progenitors may have migrated along capillary beds, or even local precursors within the cortex [37]. In a similar study, Liu et al., 2008 evaluated tissue samples from epileptic patients undergoing resection of both the hippocampus and the anterior temporal cortex [38]. By staining for DCX in control and epileptic tissue samples, the authors sought to identify if immature, new neurons were more prevalent in epileptic tissue [38]. They found a notable increase in DCX positive cells in both the granule cell layer of the hippocampus and distinct temporal cortical regions of epileptic patients in comparison to control tissue [38]. The authors noted that *in vitro* cultures of epileptic human tissue housed neural progenitor cells, suggesting that the trauma induced by seizure is able to essentially induce proliferation of quiescent progenitor cells within brain regions that are usually non-neurogenic [38].

In both cases, trauma to the brain induced proliferation of neural precursors near the site of the lesion, likely as a last resort attempt to restore function before the level of trauma became fully detrimental. Yet this phenomenon corroborates the idea that adult

neurogenesis in higher order mammals is possible late into adult life but is constrained to distinct regions throughout the brain that appear to have evolved as optimal stem cell niches. Constitutive neurogenesis within the hippocampus is now a well-known, accepted fact in all mammals, as is the presence of neural progenitor cells throughout the SVZ capable of migrating to the olfactory bulb. Though the overall prevalence of neurogenesis appears to decline gradually in aging mammals, it continues to be an ongoing albeit occasional process, and neural progenitors within the hippocampus or SVZ do not appear to suddenly die off as the brain ages. Rather, the major decline in adult neurogenesis that is seen from birds to rodents to monkeys and to humans is due to the major restrictions placed on neural precursors as to the brain environments in which they are capable of thriving. The discovery of adult neurogenesis in humans was an immense milestone in the field of neurological and neurodegenerative disorders, though the therapeutic potential of individual neural precursors cannot be achieved until investigators uncover a method that allows such precursors to survive and function in more widespread regions of the human brain.

III) Methods for Detecting Adult Neurogenesis

When the first reports of adult neurogenesis surfaced, most individuals within the scientific community were hostile to even evaluate the methods used by such scientists as Michael Kaplan and Joseph Altman who had proposed such a phenomenon. Even decades later when investigations into this process became more widespread, non-believers were more skeptical of the idea rather than the accuracy of the methods used. Yet when both BrdU labeling and immunohistochemistry were incorporated into the study of adult neurogenesis, claims of neurogenesis in higher order primates and in non-neurogenic brain regions called for a re-evaluation of the evidence [23]. Much of the criticism of certain groups concerned the specific methods and BrdU protocols used as

well as the individual interpretations of the results. The methodological critiques were both justified and legitimate, considering that the idea of adult neurogenesis in mammals has such profound implications to human health. Though the current use of BrdU is highly efficient and validated, it requires that investigators undergo rigorous controls and consider several alternate interpretations in order for their results to be accepted by the scientific community.

Old versus New Thymidine Analogs

The first reports of adult neurogenesis in both mammals and birds initially used the analog ^{3}H-thymidine that was efficiently incorporated into dividing cells in the DNA synthesis phase of mitosis, thus indicating a cell's "birthday" [4, 9, 39]. Cells exposed to the ^{3}H-thymidine were later exposed to silver grains that would bind the thymidine analog and that would allow for cell visualization via autoradiography [4, 9, 23, 40]. Investigators delineated stringent criteria for cell labeling, such that a mitotically-active cell was one whose overlying silver grains were at least 50% of the labeling in cells that showed maximal labeling [23]. Frank Nottebohm reported that avian neurons were considered labeled if they possessed 3 silver grains per nucleus, while Michael Kaplan reported that a labeled rodent neuron was one with 5 grains over the nucleus in the olfactory bulb or 19 grains over the nucleus of a granule cell from the dentate gyrus [4, 8]. This initial method in detecting cell proliferation was highly efficient and validated, and it was advantageous in that it allowed for an objective, quantitative analysis of proliferating cells.

Though the potential for quantification of cell division was beneficial for early neurobiologists using this method, major issues arose when attempting to identify cell type without the vast array of neural or glial markers available today. Early reports of adult neurogenesis determined cell type using both ultrathin sections stained with either

Nissl or Cresyl violet or electron microscopy to search for morphological clues that would distinguish a neuron from a glial cell [4, 8]. In general, cells were considered to be neurons when they possessed round, compact nuclei with one or two nucleoli when visualized with standard histology, and if electron microscopy (EM) revealed the presence of synaptic terminals with multiple types of vesicles, smooth membrane contours, and long processes containing microtubules [1, 4, 8, 12, 41]. The use of the 3H thymidine label and the detailed characterization of neuronal morphology initially identified the presence of adult neurogenesis in both avian and mammalian speices, a process that had long been considered impossible. Clearly, the phenomenon was initially dismissed, and not until almost 40 years later did innovations in biological techniques allow for the idea to be more highly reconsidered.

The idea of neurogenesis, particularly in the mammalian hippocampus, was reconsidered in the early 1990s during which both neural and glial-specific antibodies were readily available and when researchers first incorporated the use of bromodeoxyuridine (BrdU) to tag neuronal cell division in adult mammals. The use of BrdU to assess the proliferative nature of growing tumors in cancer patients prompted investigators to apply the same marker to label proliferating and migrating neurons within the mature CNS [40]. Similar to ^{3}H thymidine, BrdU could be incorporated into the cell specifically during the S phase of mitosis after which the label was identified, though using immunohistochemistry [40]. Additionally, BrdU held major advantages over that of ^{3}H thymidine in that visualizing the BrdU label took only 1 to 3 days rather than the 1 to 3 months required for the autoradiography associated with ^{3}H thymidine, and specialized facilities were not required to visualize the BrdU label [39]. Additionally, new antibodies that were specific to surface or cytoskeletal markers of neurons or glial cells allowed neurobiologists to distinguish one cell type from another without relying on subjective morphological criteria or the demanding techniques of EM.

To this day, BrdU has arguably been the most significant contribution to the field of adult neurogenesis, and it is the most prominent method used to detect the process. The transition from the ^{3}H thymidine analog to the BrdU label was an immense move in the field that made the study of adult neurogenesis both feasible and accessible to virtually any neurobiologist. Unfortunately, the BrdU method is not without inherent flaws rendering it continually susceptible to much scrutiny throughout the scientific community. The rather subjective nature of immunohistochemistry required to detect the BrdU label is the major factor in the BrdU protocol that may cause investigators to evaluate data rather differently. Due to its current prevalence within neurobiology, there are several standard controls and considerations that investigators cannot ignore in order for their studies to be both accepted and validated by their peers.

Considerations in BrdU Staining: Route and Dose of BrdU

Though the use of BrdU dramatically advanced the field of adult neurogenesis, there exist several variations in individual BrdU protocols that can significantly impact how results are interpreted. One of the major differences identified across protocols is the concentration of BrdU used, as well as the route of its injection [42, 43]. It has been proposed that a dose of 50mg/kg BrdU is sufficient to label any and all dividing neurons, though doses ranging from 50 to 500mg/kg have also been used in order to optimize staining and to account for smaller neurons that might not be labeled with protocols using the lower doses [42, 44]. Though lower doses of BrdU may ultimately underestimate the quantity of proliferating cells, administration of higher doses renders the experimenters more likely to stain cells undergoing DNA turnover or even apoptosis [23, 42, 43]. Cells preparing for apoptosis or in the process of degeneration lose Cdk inhibition, leading them to re-enter the cell cycle prior to death [45, 46]. In such cases, BrdU will stain cells undergoing either process leading to erroneous and false positive

results. Additionally, as with any radioactive or exogenous agent applied to cells, BrdU is considered to be a mutagen, meaning its successful incorporation into nuclear material and the general health of the cell may both be compromised when higher doses are administered [42, 43]. Both the potential for BrdU to label apoptotic cells and the mutagenic qualities of BrdU are major technical considerations in any BrdU protocol, and virtually any study that does not independently control for both factors is deemed invalid. Furthermore, investigators use both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes for BrdU administration, meaning there may be a significant degree of variation in BrdU access to CNS tissue. I.p. injections are subject to potentially thwarted access to the CNS due to differences in blood brain barrier permeability to BrdU across species [43]. Continuous i.c.v. infusion through the lateral ventricles enhances the likelihood that all relevant cells within the CNS will be labeled, yet the level of exposure may render neuronal cells more susceptible to the mutagenic qualities of BrdU including altered gene expression or even cell death [43]. Ultimately, variations in both the dose and route of BrdU administration reported across groups can significantly alter the outcome of any one experiment and lead to several different interpretations of one set of results.

Considerations in BrdU Staining: Antibody Specificity

As with any immunohistological assay, the specificity and sensitivity of primary and secondary antibodies must be taken into consideration when designing an optimal protocol. Antibody specificity is even more crucial in BrdU staining protocols, considering that false positive staining can lead investigators to make truly profound conclusions from inaccurate results. Optimization of antibody specificity must be applied not only to the actual BrdU label but also to any additional labels used to mark neurons or glial cells. A study by Leuner et al., 2009 evaluated differences in the sensitivities of

BrdU antibodies from such companies as Vector, BD, and Roche, and found significant differences in the number of newborn cells labeled by each separate antibody within the dentate gyrus [47]. Several biotech companies sell BrdU antibodies and the choice of company is highly variable across research groups, meaning the specific choice of BrdU antibody may become a significant confounding factor. More crucial than the choice of BrdU antibody is the use of proper labels that undoubtedly distinguish a neuron from a glial cell, an endothelial cell, and other nascent cells within the CNS. Though there are currently several markers for each distinct stage within neuronal development, the issue of both antibody specificity and background staining also holds true for antibodies chosen to define cell type. Neu-N is the most widely used antibody to identify neurons, and it was not until recently that the Neu-N antigen was identified as the neuron-specific Fox-3 gene, a member of the Fox-1 gene splicing family [48]. Additionally, the authors found that any cross reactivity of Neu-N occurs with the neural-specific synapsin-1 protein, meaning it is still a highly acceptable antibody to chose for neural staining [48]. However, in addition to variations in BrdU antibody binding specificity, Leuner et al., 2009 reported high background staining when using the Neu-N antibody, specifically based on the individual DNA denaturation protocol [47]. Since HCl, HCl and formamide, and steam heating are all common treatments used for DNA denaturation, the study evaluated each technique separately to determine how and if they altered background staining. Each technique resulted in notable differences in the Neu-N signal to noise ratio, a factor that can drastically overestimate or underestimate Neu-N staining in a BrdU protocol [47].

Though the technique of BrdU labeling is currently the gold standard in evaluating adult neurogenesis, there are considerable differences between individual protocols that may greatly affect how a given set of results is interpreted. Changes in both the dose and route of BrdU injections can lead to false positive results by labeling

Deleted: e

old neurons going through apoptotic cell cycling or even cell death. Additionally, variations in primary antibodies to BrdU or neural markers can result in different staining intensities, causing individual investigators to rely on their own intuition and judgment in counting cells. Therefore, it is clear that these specific aspects of a BrdU protocol must be as consistent as possible across research groups when attempting to verify the presence of adult neurogenesis. Most importantly, this is of particular importance in higher order species or in classically non-neurogenic regions where there may only be miniscule, yet extremely notable, quantities of dividing cells.

Controls in BrdU Staining

Despite the aforementioned flaws inherent to the BrdU technique, it remains the most prominent, efficient, and validated method available for detecting adult neurogenesis, especially in mammalian species. The technique of BrdU staining exploits the fundamental phase of DNA synthesis inherent to the process of mitosis that is virtually identical across the phylogenetic tree. Even to this day, the idea of adult neurogenesis is almost beyond belief thus it comes as no surprise when investigators looking into the process must undergo a series of rigorous controls. Notably, with the advent and relative abundance of confocal microscopy, it has become almost a routine process that adult neurogenesis be verified with the use of this 3-dimensional imaging technique, so as to ensure that the full depth of a given cell is uniformly labeled with both BrdU and neuronal markers [23]. For example, a study by Dayer et al., 2005 reported neurogenesis in the adult rat neocortex and striatum, and used confocal microscopy combined with a total of eight different neuronal markers including Neu-N, CRMP4, NG2, DCX, GABA and GAD67 to unquestionably verify that the BrdU label was found in neurons [13]. Confocal imaging to detect the presence of both BrdU and neuronal markers demonstrated the presence of the two labels throughout several z-planes of the

double-labeled cells, further indicating that the BrdU positive cell was not simply a non-neuronal cell layered on top of a neuron [13]. Current investigations in adult neurogenesis must also verify that the BrdU label has not been incorporated into old neurons that have re-entered the cell cycle prior to apoptosis. A study by Zhao et al., 2003 reported the occurrence of new neurons within the substantia nigra of mice, a finding that would clearly have a profound influence on Parkinson's Disease therapeutics [44]. The authors were careful to control for potential BrdU incorporation into degenerating or apoptotic cells by sacrificing a subset of animals 2 days after initiating i.c.v infusions of BrdU, a time point at which apoptotic activity of nigral cells would be at a maximum [44]. BrdU incorporation was not observed at this time point, and incorporation was highest by 21 days, suggesting that staining at the later time points could be attributed to neurogenesis [44].

Comment [S5]: Good example of how brd U was used appropriately

The BrdU technique is currently unparalleled in its ability to detect adult neurogenesis, though it is apparent that issues of antibody specificity, background staining, labeling of apoptotic cells, and incorrect identification of cell type are all legitimate reasons for the variations in data interpretation across research groups. When Gould et al., 1999 first reported adult neurogenesis in the neocortex of primates, the skepticism that ensued was primarily concerned with the methods used in the original study and how the authors interpreted them [22-24]. Critics were skeptical of whether BrdU was labeling new cells or old cells undergoing apoptosis, whether labels used to identify neurons were truly neuron-specific, and whether optical issues with cell identification were taken into consideration [23]. Regardless, the application of BrdU labeling successfully replaced the original ^{3}H-thymidine method and is in continuous use today by most of the leading experts in adult neurogenesis. However it is necessary that every investigator use multiple controls in their individual BrdU staining protocols including confocal microscopy, multiple neuronal markers, and multiple time points after

BrdU injections to rule out BrdU labeling of apoptotic cells. When appropriate controls are used and the aforementioned technical considerations are addressed, adult neurogenesis can be validated in virtually any species and any brain region. Until an alternate, more accurate method arises, BrdU staining will continue to be the prominent method of choice in this profound and growing field.

References

1. Goldman, S.A. and F. Nottebohm, Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A. 1983 Apr;80(8):2390-4.
2. Gahr, M., et al., What is the adaptive role of neurogenesis in adult birds? Prog Brain Res. 2002;138:233-54.
3. Doetsch, F. and C. Scharff, Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav Evol. 2001;58(5):306-22.
4. Nottebohm, F., Neuronal replacement in adulthood. Ann N Y Acad Sci. 1985;457:143-61.
5. Bonfanti, L. and P. Peretto, Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci. 2011 Sep;34(6):930-50.
6. Gould, E., How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007 Jun;8(6):481-8.
7. Kornack, D.R. and P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5768-73.
8. Kaplan, M.S. and J.W. Hinds, Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 1977 Sep 9;197(4308):1092-4.
9. Altman, J. and G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965 Jun;124(3):319-35.
10. Lois, C. and A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.
11. Lois, C. and A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.
12. Kaplan, M.S., Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981 Jan 10;195(2):323-38.
13. Dayer, A.G., et al., New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005 Jan 31;168(3):415-27.
14. Chen, J., S.S. Magavi, and J.D. Macklis, Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A. 2004 Nov 16;101(46):16357-62.
15. Kokoeva, M.V., H. Yin, and J.S. Flier, Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science. 2005 Oct 28;310(5748):679-83.
16. Xu, Y., et al., Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol. 2005 Apr;192(2):251-64.

17. Ahmed, E.I., et al., Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci. 2008 Sep;11(9):995-7.
18. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 Mar 15;16(6):2027-33.
19. Goldman, S.A., Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998 Aug;36(2):267-86.
20. Rakic, P., Limits of neurogenesis in primates. Science. 1985 Mar 1;227(4690):1054-6.
21. Gould, E., et al., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5263-7.
22. Gould, E., et al., Neurogenesis in the neocortex of adult primates. Science. 1999 Oct 15;286(5439):548-52.
23. Rakic, P., Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci. 2002 Jan;3(1):65-71.
24. Nowakowski, R.S. and N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion? Science. 2000 May 5;288(5467):771.
25. Kornack, D.R. and P. Rakic, Cell proliferation without neurogenesis in adult primate neocortex. Science. 2001 Dec 7;294(5549):2127-30.
26. Gould, E., et al., Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10910-7.
27. Bernier, P.J., et al., Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11464-9.
28. Kornack, D.R. and P. Rakic, The generation, migration, and differentiation of olfactory neurons in the adult primate brain. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4752-7.
29. Bayer, S.A., J.W. Yackel, and P.S. Puri, Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science. 1982 May 21;216(4548):890-2.
30. Pereira, A.C., et al., An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5638-43.
31. Leuner, B., E.R. Glasper, and E. Gould, Sexual Experience Promotes Adult Neurogenesis in the Hippocampus Despite an Initial Elevation in Stress Hormones. PLoS ONE. 5(7).
32. Gould, E., et al., Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci. 1999 Mar;2(3):260-5.
33. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.
34. Knoth, R., et al., Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One. 2010 Jan 29;5(1).
35. Sanai, N., et al., Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature. 2004 Feb 19;427(6976):740-4.
36. Bhardwaj, R.D., et al., Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12564-8.
37. Jin, K., et al., Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13198-202. Epub 2006 Aug 21.
38. Liu, Y.W., et al., Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci. 2008 Dec;28(11):2254-65.

39. Miller, M.W. and R.S. Nowakowski, Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 1988 Aug 2;457(1):44-52.
40. Nowakowski, R.S., S.B. Lewin, and M.W. Miller, Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.
41. Burd, G.D. and F. Nottebohm, Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol. 1985 Oct 8;240(2):143-52.
42. Rakic, P., Adult neurogenesis in mammals: an identity crisis. J Neurosci. 2002 Feb 1;22(3):614-8.
43. Cooper-Kuhn, C.M. and H.G. Kuhn, Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):13-21.
44. Zhao, M., et al., Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7925-30.
45. Copani, A., et al., Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci. 2001 Jan;24(1):25-31.
46. Katchanov, J., et al., Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
47. Leuner, B., E.R. Glasper, and E. Gould, Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol. 2009 Nov 10;517(2):123-33.
48. Kim, K.K., R.S. Adelstein, and S. Kawamoto, Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. J Biol Chem. 2009 Nov 6;284(45):31052-61.
49. Shingo, T., et al., Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science. 2003 Jan 3;299(5603):117-20.

Pass. Very thoughtful, critical, and thorough. Good job!

Introduction: Adult Neurogenesis

The initial reports of adult neurogenesis overthrew a roughly one-hundred year old phenomenon stating that neuronal renewal was virtually impossible beyond birth. When it was first reported that neurons were unable to undergo post-natal mitosis, it seemed rather unfortunate that the cell type most critical for normal human functioning was the one type incapable of regeneration after trauma. Yet when both Joseph Altman and Michael Kaplan first discovered adult neurogenesis in the rodent hippocampus, their work was greeted with pure skepticism as it seemed nearly impossible that their scientific predecessors had not uncovered this process earlier. It was not until 40 years later when Frank Nottebohm clearly identified continuously high quantities of neurogenesis in adult canaries and songbirds that the field was re-visited. When combined with the technological advances in biology and immunohistochemistry, evidence for adult neurogenesis was then continually uncovered in species that spanned the length of the phylogenetic tree, including mice, rats, primates, and later humans. Surprisingly, the underlying purpose or benefit for adult neurogenesis, particularly in higher order species, is still rather unknown, and some even postulate that a gradual decline in adult neurogenesis in a given species is evolutionarily advantageous. Regardless, the potential function that adult neurogenesis plays in behavior, learning, and memory as well as its enormous therapeutic implications, render this process one of the most significant discoveries for the future of human health.

I) Adult Neurogenesis across Species: Birds

Frank Nottebohm and his colleagues initially searched for evidence of adult neurogenesis in canaries in the 1980s, despite the fact that initial data for adult

neurogenesis in rodents had been largely dismissed [1]. In the avian brain, neural progenitors are housed within the ventricular zone (VZ) of the lateral ventricles, and successful recruitment and migration of these neural precursors occurs throughout most of the year into much of the avian forebrain [2, 3]. These avian forebrain regions that continuously recruit new neurons are functionally homologous to the mammalian hippocampus, striatum, and, somewhat, to the mammalian cortex [2]. Additionally there exists substantial, year-long neuronal recruitment to distinct nuclei of the vocal control system in the avian forebrain, most notably to the hyperstriatum ventralis pars cadualis (HVC) [2]. The HVC projects to the robust nucleus of the archistriatum (RA) for proper song production in males, hence the male HVC and RA are much larger than those of the female, and females do not sing unless treated with male gonadal hormones [4]. The male HVC experiences dramatic changes in size due to both the seasonal fluctuations in circulating testosterone, such that in non-breeding seasons when testosterone is low, the HVC is reduced to almost half of its normal neuronal number [4]. Surprisingly, within 12 months the HVC replaces over half of the lost neurons, corresponding to the onset of the breeding season and an increase in circulating testosterone [4]. The female HVC also demonstrates a continuous, year-long recruitment of new neurons, though they differentiate into interneurons within the HVC that neither project to the RA nor promote song production. Goldman et al., 1983 clearly demonstrated that new neurons in the female canary HVC were morphologically neurons, incorporated the ^{3}H thymidine label, produced action potentials, and were activated in response to acoustic stimuli [1, 4]. Later studies corroborated the results from this original study and found that roughly 20,000 new and functional neurons were added to several, widespread regions throughout the forebrain, in addition to some new recruitment to parts of the avian midbrain, medulla, and cerebellum [4].

This large quantity and expanse of neurogenesis in the avian forebrain suggests that most regions of the avian brain are capable of supporting both neuronal migration as well as later differentiation, and that neurons are able to survive within the forebrain long enough to function in existing circuits. A major reason for the vast quantities and consistency of neural precursor migration to the avian forebrain is the presence of a supportive system for migration and a supportive environment within the forebrain parenchyma that permits the survival of these new neurons. Radial guide cells extend from the VZ proliferative "hot spots" throughout much of the avian forebrain, thus providing a truly supportive scaffold upon which neural precursor cells can travel to distant sites [2, 3]. Additionally, gonadal hormones are crucial for both neuronal recruitment and survival, as estrogen promotes the recruitment and survival of new HVC neurons and testosterone promotes the survival of new RA-projecting HVC neurons [2, 3]. Additionally brain derived neurotrophic factor (BDNF) circulates within the HVC to support the survival and growth of newly recruited neurons [2]. The supportive roles of estrogen and testosterone explain both 1) the seasonal fluctuations in the size of the male HVC that correlates to the concentration of circulating testosterone, and 2) the presence of HVC neurogenesis in females that yields only HVC interneurons incapable of producing song [2, 4]. Furthermore, the expanse of the migratory network and the level of trophic support within the forebrain parenchyma distinguish the avian forebrain from other mammalian species and may explain why adult neurogenesis declines along the phylogenetic tree.

Adult Neurogenesis across Species: Rodents

Unlike the vast adult neurogenesis throughout much of the avian forebrain, adult neurogenesis in rodents is drastically reduced to the olfactory bulb and the dentate gyrus of the hippocampus [5]. Neural progenitor cells from the subventricular zone (SVZ) of

the lateral ventricles migrate along the rodent rostral migratory stream (RMS), a structure similar to the radial guide cell system of the avian forebrain but devoid of the supporting radial-glial guide cell scaffold that widely projects to the avian forebrain [3, 5, 6]. Within the hippocampus, the subgranular zone (SGZ) of the dentate gyrus acts as a source of neural progenitor cells that can eventually differentiate into mature granule cells and add to the total population of the dentate gyrus [5, 7]. Initial evidence of hippocampal neurogenesis in rodents appeared roughly 50 years ago through the work of Altman and Kaplan who, like Nottebohm, used the ^{3}H thymidine label and the morphological characteristics of neurons to identify adult neurogenesis [8, 9]. Later evidence in support of neural progenitors within the SVZ came from *in vitro* studies showing that explants from mouse SVZ were able to differentiate into both neurons and glia [10]. Additionally, a study by Lois and Alvarez-Buylla in 1994 discovered that rodents indeed possess a migratory path to the olfactory bulb composed of a dense network of astrocytes that guides migrating neuroblasts rostrally to successfully differentiate into olfactory interneurons [11].

There are notable differences between avian and rodent adult neurogenesis, namely the rarity of neurogenesis in non-neurogenic regions of the rodent forebrain and the decline in neurogenesis in aged rodents. Notably, these distinctions indicate a substantial decline in the prevalence of adult neurogenesis from one species to the next. Neurogenesis in the adult rodent brain is regionally constrained such that neurogenesis in non-neurogenic regions is both rare and often transient. Though Kaplan reported evidence of neurogenesis within the rodent visual cortex [12], and Dayer et al., 2005 reported GABAergic neurogenesis within the rodent neocortex [13], the extent of the new neuron recruitment appeared substantially lower than the quantity of daily neural recruitment within the avian forebrain. Furthermore, a study by Chen et al., 2004 is currently one of the only studies that has detected mouse neocortical neurogenesis, a

finding that has yet to be replicated [14]. Select studies have reported neurogenesis in the hypothalamus of mice [15] and rats [16], as well as in sexually dimorphic nuclei of the rat hypothalamus [17]. During adolescence, gonadal hormone activation led to a significant increase in BrdU labeling within the female anteroventral periventricular nucleus and the sexually dimorphic nucleus of the preoptic area in males, suggesting that adolescent neurogenesis is both hormone dependent and crucial for organizing circuits that control adult sexual behavior [17]. Despite the occasional evidence for neurogenesis in these classically non-neurogenic regions, the process of new neuronal recruitment in such regions either occurs only at one distinct developmental time point or is primarily incomplete [5]. Incomplete neurogenesis holds that a vast majority of newly formed neurons in these regions are incapable of surviving long enough to have any functional relevance in existing circuits [5]. Furthermore, the origin and migratory path of these forebrain and midbrain neurons is not always clear, and differs significantly from VZ progenitors and the radial-glial guide cells that clearly allow for the migration of avian neural precursors to a vast majority of the telencephalon.

The second important difference between rodent and avian adult neurogenesis is the gradual decline in the process over time in any given rodent, even in the major neurogenic region of the hippocampus. The frequency of hippocampal neurogenesis is not uniform throughout the adult rodent life, such that in comparison to 6 month old rats, 12 to 27 month old rats have a significant reduction in the number of both new and migrating neurons within the granule cell layer of the dentate gyrus [18]. This is in stark contrast to avian neurogenesis within both the forebrain and hippocampus that demonstrate a continuous recruitment of new neurons throughout much of adulthood [2, 4, 19]. Specifically, the HVC of the canary forebrain recruits between 0.1 and 0.74% new neurons per day, and the hippocampus recruits between 0.15 and 0.37% new neurons per day [2]. Therefore, the decline in rodent neurogenesis over time and the

vast regional constraints on rodent neurogenesis indicate that the continuity and the expanse of this process become continually restricted in more complex species. This may suggest that despite its profound implications to the overall function of the brain, adult neurogenesis may not be crucial for the optimal survival of higher order species.

Adult Neurogenesis across Species: Primates and Humans

The eventual acceptance of adult neurogenesis in rodents led leading investigators in primate neurobiology to search for similar process in non-human primates, as any indication of neurogenesis in any region of the primate brain would hold enormous therapeutic potential. In 1985, Pasko Rakic initially reported that neurogenesis was completely absent throughout the entire primate brain, for injections of the ^{3}H-thymidine label failed to stain any neuron in the neocortex, the hippocampus, the olfactory bulb, the basal ganglia, or any other brain region analyzed [20]. It was postulated that the lack of neurogenesis in such a complex, highly developed species was a purposeful adaptation, such that the continual environmental interactions in primates and humans necessitated a set of stable, persistent synapses for much of the adult life [20]. Yet almost 15 years later, Gould et al., 1998 found convincing evidence for neurogenesis within the dentate gyrus of young, middle-aged and older (23 years) Old World Monkeys [21]. BrdU labeled neurons also co-localized with the distinct marker for immature neurons, TOAD-64, indicating that labeled cells were indeed neurons [21]. Around the same time, despite their initial claims that neurogenesis does not occur within primates, Kornack and Rakic 1999 finally observed and validated neurogenesis in the dentate gyrus of adult macaque monkeys [7]. Taken together, these studies pointed to the presence of *in vivo* neural stem cells within the subgranular zone of the dentate gyrus and their later differentiation into hippocampal granule cells, a

finding that was highly significant in light of previous reports indicating that primate neurogenesis was neither necessary nor feasible beyond birth.

Neurogenesis within the hippocampus and olfactory bulb of the primate did not come as much of a surprise considering the vast amount of support for these two processes in lower order rodents. Similar to adult neurogenesis in rodents, few investigators were able to provide support for adult neurogenesis in any other region of the primate brain. However, Gould et al., 1999 attempted to report evidence of new, BrdU-labeled neurons within the neocortex that had traveled to their final position along a migratory stream projecting from the SVZ to their final cortical destination [22]. Not surprisingly, this study faced harsh criticism, disbelief, and mostly skepticism, with critics primarily questioning her interpretations and the methods used in the study [23-25]. The study faced such criticism, that the investigators completed a second study to evaluate the survival of these new neurons that they had reportedly identified within the primate neocortex [26]. They continued to find evidence for both hippocampal as well as neocortical neurogenesis, though it was clear that several of the BrdU-labeled cells in the cortex were unable to survive past approximately 9 weeks [26]. Moreover, Bernier et al., 2001 reported adult neurogenesis within the primate amygdala and piriform cortex as well as the presence of a temporal migratory stream along which the precursor cells from the SVZ had possibly migrated to the cortex [27]. Yet they too noted that several migrating neurons along this supposed temporal stream were unable to survive long enough to reach their cortical destination [27]. In response to both claims, Kornack and Rakic again searched for evidence of cortical neurogenesis, yet their results showed only the growth of new glial cells within the cortex and no evidence for neurogenesis [25]. To this day, there have been very few additional reports of successful neurogenesis within the cortex of primates, suggesting that any migrating neural

precursors from the SVZ are likely to die within weeks of settling in the cortical parenchyma.

Similar to the restrictions of the rodent brain in comparison to the avian brain, the limitations placed on forebrain and midbrain neurogenesis in primates appears to be primarily caused by a decline in basic anatomical support for the process as noted by the grossly underdeveloped RMS in primates. The migratory stream available to primates is limited in its regional and forebrain extension, much like the decreased complexity of the RMS in rodents compared to birds [5]. In the primate RMS neuroblasts rely on neurophilic interactions between one another rather than radial-glial cell guided or even astrocyte guided migration, and extension of the RMS is strictly limited to the olfactory bulb [28]. Additionally, relative to the rodent brain, the migration rate of primate neuroblasts destined for the olfactory bulb is much slower, requiring at least 75 days for full migration after normalizing for differences in brain size between rodents and primates [28]. Thus it appears as though the lost complexity of the primate migratory stream is again a limiting factor in the prevalence of primate neurogenesis in any area other than the olfactory bulb and the hippocampus. This feature places a large restriction on the potential for neural precursors in the SVZ to successfully migrate to any other region of the forebrain. Additionally, the forebrain is essentially considered to be a non-neurogenic region likely void of proper trophic factors to support neural differentiation and survival [5]. Despite reports of neocortical neurogenesis, the ability for new neurons to survive and integrate into functional circuits in any area other than the hippocampus is drastically reduced in comparison to that seen in avian species.

Additionally, there exist important restrictions on hippocampal neurogenesis within the primate, particularly in comparison to rodents. While granule cells that are newly generated in the rodent hippocampus continually add to the pool of hippocampal neurons in the adult rodent, for example between 30 and 365 days of life, this is likely

not the case in primates [28, 29]. Hippocampal neurogenesis in the adult rodent increases in response to several environmental stimuli including exercise [30], sexual experience [31], and learning [32], all of which lead to an overall increase the total number of functional granule cells. However, the primate brain appears to show no significant accumulation of new hippocampal neuron addition, suggesting that hippocampal neurogenesis correlates to an equal rate of neuronal apoptosis [7]. Thus, hippocampal neurogenesis within rodents may facilitate new learning and synaptic plasticity in the face of constant environmental changes or necessary changes in social behavior throughout the adult life. Considering that both primates and humans extensively interact with the environment, it is possible that the gradual decline in neurogenesis in favor of long-lasting synapses and circuits is necessary for long term memory storage and the overall complexity of the primate and human brain [7].

Humans show virtually an identical pattern of neurogenesis as primates, with neural progenitor cells housed only in the hippocampus and the SVZ lining the ventricular system. The first reports of human neural progenitor cells were rather astounding, as they truly verified that neurogenesis was indeed possible in the human brain. Eriksson et al., 1998 evaluated the brains of cancer patients that had been previously treated with BrdU, a treatment that had been used to follow the proliferative profile of tumor cells [33]. The study found a substantial quantity of BrdU labeled cells positive for neural markers within the dentate gyrus of these individuals, providing official proof that neural precursors can survive in the human brain [33]. Knoth et al., 2010 analyzed hippocampal tissue samples from individuals ranging from childhood to almost 90 years of age, specifically searching for neural precursors and neural proliferation [34]. Surprisingly they found that several cells co-localized with both immature neuronal and cell proliferation markers at almost all ages, though they noted a significant decline the number of labeled cells beyond roughly 40 years of age [34]. Additionally, Sinai et al.,

2004 confirmed the presence of SVZ neural progenitors in postmortem adult human tissue as well as a unique band of astrocytes extending from the SVZ [35]. However they found no evidence in support of neural precursor migration along this band of astrocytes, suggesting that neural precursors in humans are even more restricted to distinct locations within the brain parenchyma than even non-human primates [35]. Additionally, a separate study used an alternate method of detecting proliferating neural cells, the phenomenon of carbon-14 labeling, and found that neurons in the neocortex of humans were the exact age of the individual [36]. This suggested that, like primates, neocortical neurogenesis in humans is largely restricted to embryological development prior to birth. No other studies have successfully reported human neurogenesis or the presence of neural precursors in any other region outside of the hippocampus or the SVZ. Nonetheless, the presence of individual neural precursors in humans warrants great attention, and highlights the necessity to identify an optimal environment that would allow neurons to differentiate and thrive in several regions throughout the human brain.

Avian and mammal adult neurogenesis differ in both the frequency of neural turn over as well as the constraints placed on neural progenitors as to where they can survive and differentiate. More importantly, the regional constraints appear to be more limiting to the expanse of adult neurogenesis in more complex species. The fact that neural precursors of the SVZ and hippocampus can survive well into adulthood of rodents, primates, and humans, indicates that age does not appear to restrict the potential for neurogenesis, but rather their capacity to survive in various regions of the brain is the more significant limiting factor. Despite reports of neurogenesis within the rodent neocortex or hypothalamus as well as the primate neocortex, such accounts are both rare and often conflicting between groups, a finding that is in direct contrast to the widespread and continual neurogenesis throughout the avian forebrain.

II) Temporal vs. Regional Constraints in Adult Neurogenesis

Despite the fact that the total amount of mammalian neurogenesis declines in prevalence both across species and within a given species, adult neurogenesis becomes more regionally constrained when the mammalian brain becomes more complex. Lower order vertebrates such as fish and the aforementioned songbirds experience high quantities of neurogenesis both throughout adulthood and within several higher order forebrain structures. In comparison to birds, mammals as low as rodents have a drastic reduction in the brain regions available for supporting complete neurogenesis. While both avian species and rodents possess a similar pool of neural precursors within the SVZ of the lateral ventricles, their migratory ability and their capability to survive in various forebrain regions is dramatically different. Though both primates and humans still possess distinct pools of neural progenitors that are capable of proliferating well into adulthood, there are even fewer reports of successful integration of such precursors into other brain regions. Additionally, regional constraints on mammalian neurogenesis appears to be the more significant factor in higher order species due to fact that labeling of proliferating and immature neuronal precursors has identified active neural progenitor cells in elderly rodents [18], primates [21], and humans [33, 34]. The fact that any neural precursor exists in the mammalian brain several years after birth indicates that mammalian brains are capable of harvesting and supporting neural stem cells, but that their survival and differentiation requires a distinct environment that appears to be uncharacteristic of higher order brain regions.

It is noteworthy that certain non-neurogenic regions of the mammalian brain, particularly in humans, can suddenly support nascent neurogenesis after trauma due to the change in circulating factors associated with an injured brain region. In a report by

Jin et al., 2006, brain biopsy tissue samples from stroke patients were stained for Ki67, a marker of cell proliferation, as well as DCX and βIII tubulin, two markers expressed in new neurons [37]. Particularly within the penumbra, or the surviving cortical tissue surrounding the ischemic core, the study found that several cells were positive for both Ki67 and either DCX or βIII tubulin [37]. As neurogenesis has been shown to be enhanced in such degenerative diseases as Huntington's and Alzheimer's, the authors noted that it is likely that a similar process was taking place in these stroke patients, such that pre-existing neural precursors were stimulated to begin dividing in order to replace lost neurons after the insult [37]. They noted that the source of the precursors could either be the SVZ where progenitors may have migrated along capillary beds, or even local precursors within the cortex [37]. In a similar study, Liu et al., 2008 evaluated tissue samples from epileptic patients undergoing resection of both the hippocampus and the anterior temporal cortex [38]. By staining for DCX in control and epileptic tissue samples, the authors sought to identify if immature, new neurons were more prevalent in epileptic tissue [38]. They found a notable increase in DCX positive cells in both the granule cell layer of the hippocampus and distinct temporal cortical regions of epileptic patients in comparison to control tissue [38]. The authors noted that *in vitro* cultures of epileptic human tissue housed neural progenitor cells, suggesting that the trauma induced by seizure is able to essentially induce proliferation of quiescent progenitor cells within brain regions that are usually non-neurogenic [38].

In both cases, trauma to the brain induced proliferation of neural precursors near the site of the lesion, likely as a last resort attempt to restore function before the level of trauma became fully detrimental. Yet this phenomenon corroborates the idea that adult neurogenesis in higher order mammals is possible late into adult life but is constrained to distinct regions throughout the brain that appear to have evolved as optimal stem cell niches. Constitutive neurogenesis within the hippocampus is now a well-known,

accepted fact in all mammals, as is the presence of neural progenitor cells throughout the SVZ capable of migrating to the olfactory bulb. Though the overall prevalence of neurogenesis appears to decline gradually in aging mammals, it continues to be an ongoing albeit occasional process, and neural progenitors within the hippocampus or SVZ do not appear to suddenly die off as the brain ages. Rather, the major decline in adult neurogenesis that is seen from birds to rodents to monkeys and to humans is due to the major restrictions placed on neural precursors as to the brain environments in which they are capable of thriving. The discovery of adult neurogenesis in humans was an immense milestone in the field of neurological and neurodegenerative disorders, though the therapeutic potential of individual neural precursors cannot be achieved until investigators uncover a method that allows such precursors to survive and function in more widespread regions of the human brain.

III) Methods for Detecting Adult Neurogenesis

When the first reports of adult neurogenesis surfaced, most individuals within the scientific community were hostile to even evaluate the methods used by such scientists as Michael Kaplan and Joseph Altman who had proposed such a phenomenon. Even decades later when investigations into this process became more widespread, non-believers were more skeptical of the idea rather than the accuracy of the methods used. Yet when both BrdU labeling and immunohistochemistry were incorporated into the study of adult neurogenesis, claims of neurogenesis in higher order primates and in non-neurogenic brain regions called for a re-evaluation of the evidence [23]. Much of the criticism of certain groups concerned the specific methods and BrdU protocols used as well as the individual interpretations of the results. The methodological critiques were both justified and legitimate, considering that the idea of adult neurogenesis in mammals has such profound implications to human health. Though the current use of BrdU is

highly efficient and validated, it requires that investigators undergo rigorous controls and consider several alternate interpretations in order for their results to be accepted by the scientific community.

Old versus New Thymidine Analogs

The first reports of adult neurogenesis in both mammals and birds initially used the analog ^{3}H-thymidine that was efficiently incorporated into dividing cells in the DNA synthesis phase of mitosis, thus indicating a cell's "birthday" [4, 9, 39]. Cells exposed to the ^{3}H-thymidine were later exposed to silver grains that would bind the thymidine analog and that would allow for cell visualization via autoradiography [4, 9, 23, 40]. Investigators delineated stringent criteria for cell labeling, such that a mitotically-active cell was one whose overlying silver grains were at least 50% of the labeling in cells that showed maximal labeling [23]. Frank Nottebohm reported that avian neurons were considered labeled if they possessed 3 silver grains per nucleus, while Michael Kaplan reported that a labeled rodent neuron was one with 5 grains over the nucleus in the olfactory bulb or 19 grains over the nucleus of a granule cell from the dentate gyrus [4, 8]. This initial method in detecting cell proliferation was highly efficient and validated, and it was advantageous in that it allowed for an objective, quantitative analysis of proliferating cells.

Though the potential for quantification of cell division was beneficial for early neurobiologists using this method, major issues arose when attempting to identify cell type without the vast array of neural or glial markers available today. Early reports of adult neurogenesis determined cell type using both ultrathin sections stained with either Nissl or Cresyl violet or electron microscopy to search for morphological clues that would distinguish a neuron from a glial cell [4, 8]. In general, cells were considered to be neurons when they possessed round, compact nuclei with one or two nucleoli when

visualized with standard histology, and if electron microscopy (EM) revealed the presence of synaptic terminals with multiple types of vesicles, smooth membrane contours, and long processes containing microtubules [1, 4, 8, 12, 41]. The use of the 3H thymidine label and the detailed characterization of neuronal morphology initially identified the presence of adult neurogenesis in both avian and mammalian speices, a process that had long been considered impossible. Clearly, the phenomenon was initially dismissed, and not until almost 40 years later did innovations in biological techniques allow for the idea to be more highly reconsidered.

The idea of neurogenesis, particularly in the mammalian hippocampus, was reconsidered in the early 1990s during which both neural and glial-specific antibodies were readily available and when researchers first incorporated the use of bromodeoxyuridine (BrdU) to tag neuronal cell division in adult mammals. The use of BrdU to assess the proliferative nature of growing tumors in cancer patients prompted investigators to apply the same marker to label proliferating and migrating neurons within the mature CNS [40]. Similar to ^{3}H thymidine, BrdU could be incorporated into the cell specifically during the S phase of mitosis after which the label was identified, though using immunohistochemistry [40]. Additionally, BrdU held major advantages over that of ^{3}H thymidine in that visualizing the BrdU label took only 1 to 3 days rather than the 1 to 3 months required for the autoradiography associated with ^{3}H thymidine, and specialized facilities were not required to visualize the BrdU label [39]. Additionally, new antibodies that were specific to surface or cytoskeletal markers of neurons or glial cells allowed neurobiologists to distinguish one cell type from another without relying on subjective morphological criteria or the demanding techniques of EM.

To this day, BrdU has arguably been the most significant contribution to the field of adult neurogenesis, and it is the most prominent method used to detect the process. The transition from the ^{3}H thymidine analog to the BrdU label was an immense move in

the field that made the study of adult neurogenesis both feasible and accessible to virtually any neurobiologist. Unfortunately, the BrdU method is not without inherent flaws rendering it continually susceptible to much scrutiny throughout the scientific community. The rather subjective nature of immunohistochemistry required to detect the BrdU label is the major factor in the BrdU protocol that may cause investigators to evaluate data rather differently. Due to its current prevalence within neurobiology, there are several standard controls and considerations that investigators cannot ignore in order for their studies to be both accepted and validated by their peers.

Considerations in BrdU Staining: Route and Dose of BrdU

Though the use of BrdU dramatically advanced the field of adult neurogenesis, there exist several variations in individual BrdU protocols that can significantly impact how results are interpreted. One of the major differences identified across protocols is the concentration of BrdU used, as well as the route of its injection [42, 43]. It has been proposed that a dose of 50mg/kg BrdU is sufficient to label any and all dividing neurons, though doses ranging from 50 to 500mg/kg have also been used in order to optimize staining and to account for smaller neurons that might not be labeled with protocols using the lower doses [42, 44]. Though lower doses of BrdU may ultimately underestimate the quantity of proliferating cells, administration of higher doses renders the experimenters more likely to stain cells undergoing DNA turnover or even apoptosis [23, 42, 43]. Cells preparing for apoptosis or in the process of degeneration lose Cdk inhibition, leading them to re-enter the cell cycle prior to death [45, 46]. In such cases, BrdU will stain cells undergoing either process leading to erroneous and false positive results. Additionally, as with any radioactive or exogenous agent applied to cells, BrdU is considered to be a mutagen, meaning its successful incorporation into nuclear material and the general health of the cell may both be compromised when higher doses

are administered [42, 43]. Both the potential for BrdU to label apoptotic cells and the mutagenic qualities of BrdU are major technical considerations in any BrdU protocol, and virtually any study that does not independently control for both factors is deemed invalid. Furthermore, investigators use both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes for BrdU administration, meaning there may be a significant degree of variation in BrdU access to CNS tissue. I.p. injections are subject to potentially thwarted access to the CNS due to differences in blood brain barrier permeability to BrdU across species [43]. Continuous i.c.v. infusion through the lateral ventricles enhances the likelihood that all relevant cells within the CNS will be labeled, yet the level of exposure may render neuronal cells more susceptible to the mutagenic qualities of BrdU including altered gene expression or even cell death [43]. Ultimately, variations in both the dose and route of BrdU administration reported across groups can significantly alter the outcome of any one experiment and lead to several different interpretations of one set of results.

Considerations in BrdU Staining: Antibody Specificity

As with any immunohistological assay, the specificity and sensitivity of primary and secondary antibodies must be taken into consideration when designing an optimal protocol. Antibody specificity is even more crucial in BrdU staining protocols, considering that false positive staining can lead investigators to make truly profound conclusions from inaccurate results. Optimization of antibody specificity must be applied not only to the actual BrdU label but also to any additional labels used to mark neurons or glial cells. A study by Leuner et al., 2009 evaluated differences in the sensitivities of BrdU antibodies from such companies as Vector, BD, and Roche, and found significant differences in the number of newborn cells labeled by each separate antibody within the dentate gyrus [47]. Several biotech companies sell BrdU antibodies and the choice of

company is highly variable across research groups, meaning the specific choice of BrdU antibody may become a significant confounding factor. More crucial than the choice of BrdU antibody is the use of proper labels that undoubtedly distinguish a neuron from a glial cell, an endothelial cell, and other nascent cells within the CNS. Though there are currently several markers for each distinct stage within neuronal development, the issue of both antibody specificity and background staining also holds true for antibodies chosen to define cell type. Neu-N is the most widely used antibody to identify neurons, and it was not until recently that the Neu-N antigen was identified as the neuron-specific Fox-3 gene, a member of the Fox-1 gene splicing family [48]. Additionally, the authors found that any cross reactivity of Neu-N occurs with the neural-specific synapsin-1 protein, meaning it is still a highly acceptable antibody to chose for neural staining [48]. However, in addition to variations in BrdU antibody binding specificity, Leuner et al., 2009 reported high background staining when using the Neu-N antibody, specifically based on the individual DNA denaturation protocol [47]. Since HCl, HCl and formamide, and steam heating are all common treatments used for DNA denaturation, the study evaluated each technique separately to determine how and if they altered background staining. Each technique resulted in notable differences in the Neu-N signal to noise ratio, a factor that can drastically overestimate or underestimate Neu-N staining in a BrdU protocol [47].

Though the technique of BrdU labeling is currently the gold standard in evaluating adult neurogenesis, there are considerable differences between individual protocols that may greatly affect how a given set of results is interpreted. Changes in both the does and route of BrdU injections can lead to false positive results by labeling old neurons going through apoptotic cell cycling or even cell death. Additionally, variations in primary antibodies to BrdU or neural markers can result in different staining intensities, causing individual investigators to rely on their own intuition and judgment in

counting cells. Therefore, it is clear that these specific aspects of a BrdU protocol must be as consistent as possible across research groups when attempting to verify the presence of adult neurogenesis. Most importantly, this is of particular importance in higher order species or in classically non-neurogenic regions where there may only be miniscule, yet extremely notable, quantities of dividing cells.

Controls in BrdU Staining

Despite the aforementioned flaws inherent to the BrdU technique, it remains the most prominent, efficient, and validated method available for detecting adult neurogenesis, especially in mammalian species. The technique of BrdU staining exploits the fundamental phase of DNA synthesis inherent to the process of mitosis that is virtually identical across the phylogenetic tree. Even to this day, the idea of adult neurogenesis is almost beyond belief thus it comes as no surprise when investigators looking into the process must undergo a series of rigorous controls. Notably, with the advent and relative abundance of confocal microscopy, it has become almost a routine process that adult neurogenesis be verified with the use of this 3-dimensional imaging technique, so as to ensure that the full depth of a given cell is uniformly labeled with both BrdU and neuronal markers [23]. For example, a study by Dayer et al., 2005 reported neurogenesis in the adult rat neocortex and striatum, and used confocal microscopy combined with a total of eight different neuronal markers including Neu-N, CRMP4, NG2, DCX, GABA and GAD67 to unquestionably verify that the BrdU label was found in neurons [13]. Confocal imaging to detect the presence of both BrdU and neuronal markers demonstrated the presence of the two labels throughout several z-planes of the double-labeled cells, further indicating that the BrdU positive cell was not simply a non-neuronal cell layered on top of a neuron [13]. Current investigations in adult neurogenesis must also verify that the BrdU label has not been incorporated into old

neurons that have re-entered the cell cycle prior to apoptosis. A study by Zhao et al., 2003 reported the occurrence of new neurons within the substantia nigra of mice, a finding that would clearly have a profound influence on Parkinson's Disease therapeutics [44]. The authors were careful to control for potential BrdU incorporation into degenerating or apoptotic cells by sacrificing a subset of animals 2 days after initiating i.c.v infusions of BrdU, a time point at which apoptotic activity of nigral cells would be at a maximum [44]. BrdU incorporation was not observed at this time point, and incorporation was highest by 21 days, suggesting that staining at the later time points could be attributed to neurogenesis [44].

The BrdU technique is currently unparalleled in its ability to detect adult neurogenesis, though it is apparent that issues of antibody specificity, background staining, labeling of apoptotic cells, and incorrect identification of cell type are all legitimate reasons for the variations in data interpretation across research groups. When Gould et al., 1999 first reported adult neurogenesis in the neocortex of primates, the skepticism that ensued was primarily concerned with the methods used in the original study and how the authors interpreted them [22-24]. Critics were skeptical of whether BrdU was labeling new cells or old cells undergoing apoptosis, whether labels used to identify neurons were truly neuron-specific, and whether optical issues with cell identification were taken into consideration [23]. Regardless, the application of BrdU labeling successfully replaced the original ^{3}H-thymidine method and is in continuous use today by most of the leading experts in adult neurogenesis. However it is necessary that every investigator use multiple controls in their individual BrdU staining protocols including confocal microscopy, multiple neuronal markers, and multiple time points after BrdU injections to rule out BrdU labeling of apoptotic cells. When appropriate controls are used and the aforementioned technical considerations are addressed, adult neurogenesis can be validated in virtually any species and any brain region. Until an

alternate, more accurate method arises, BrdU staining will continue to be the prominent method of choice in this profound and growing field.

References

1. Goldman, S.A. and F. Nottebohm, Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A. 1983 Apr;80(8):2390-4.
2. Gahr, M., et al., What is the adaptive role of neurogenesis in adult birds? Prog Brain Res. 2002;138:233-54.
3. Doetsch, F. and C. Scharff, Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav Evol. 2001;58(5):306-22.
4. Nottebohm, F., Neuronal replacement in adulthood. Ann N Y Acad Sci. 1985;457:143-61.
5. Bonfanti, L. and P. Peretto, Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci. 2011 Sep;34(6):930-50.
6. Gould, E., How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007 Jun;8(6):481-8.
7. Kornack, D.R. and P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5768-73.
8. Kaplan, M.S. and J.W. Hinds, Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 1977 Sep 9;197(4308):1092-4.
9. Altman, J. and G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965 Jun;124(3):319-35.
10. Lois, C. and A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.
11. Lois, C. and A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.
12. Kaplan, M.S., Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981 Jan 10;195(2):323-38.
13. Dayer, A.G., et al., New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005 Jan 31;168(3):415-27.
14. Chen, J., S.S. Magavi, and J.D. Macklis, Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A. 2004 Nov 16;101(46):16357-62.
15. Kokoeva, M.V., H. Yin, and J.S. Flier, Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science. 2005 Oct 28;310(5748):679-83.
16. Xu, Y., et al., Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol. 2005 Apr;192(2):251-64.
17. Ahmed, E.I., et al., Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci. 2008 Sep;11(9):995-7.
18. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 Mar 15;16(6):2027-33.

19. Goldman, S.A., Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998 Aug;36(2):267-86.
20. Rakic, P., Limits of neurogenesis in primates. Science. 1985 Mar 1;227(4690):1054-6.
21. Gould, E., et al., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5263-7.
22. Gould, E., et al., Neurogenesis in the neocortex of adult primates. Science. 1999 Oct 15;286(5439):548-52.
23. Rakic, P., Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci. 2002 Jan;3(1):65-71.
24. Nowakowski, R.S. and N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion? Science. 2000 May 5;288(5467):771.
25. Kornack, D.R. and P. Rakic, Cell proliferation without neurogenesis in adult primate neocortex. Science. 2001 Dec 7;294(5549):2127-30.
26. Gould, E., et al., Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10910-7.
27. Bernier, P.J., et al., Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11464-9.
28. Kornack, D.R. and P. Rakic, The generation, migration, and differentiation of olfactory neurons in the adult primate brain. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4752-7.
29. Bayer, S.A., J.W. Yackel, and P.S. Puri, Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science. 1982 May 21;216(4548):890-2.
30. Pereira, A.C., et al., An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5638-43.
31. Leuner, B., E.R. Glasper, and E. Gould, Sexual Experience Promotes Adult Neurogenesis in the Hippocampus Despite an Initial Elevation in Stress Hormones. PLoS ONE. 5(7).
32. Gould, E., et al., Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci. 1999 Mar;2(3):260-5.
33. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.
34. Knoth, R., et al., Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One. 2010 Jan 29;5(1).
35. Sanai, N., et al., Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature. 2004 Feb 19;427(6976):740-4.
36. Bhardwaj, R.D., et al., Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12564-8.
37. Jin, K., et al., Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13198-202. Epub 2006 Aug 21.
38. Liu, Y.W., et al., Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci. 2008 Dec;28(11):2254-65.
39. Miller, M.W. and R.S. Nowakowski, Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 1988 Aug 2;457(1):44-52.
40. Nowakowski, R.S., S.B. Lewin, and M.W. Miller, Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.

41. Burd, G.D. and F. Nottebohm, Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol. 1985 Oct 8;240(2):143-52.
42. Rakic, P., Adult neurogenesis in mammals: an identity crisis. J Neurosci. 2002 Feb 1;22(3):614-8.
43. Cooper-Kuhn, C.M. and H.G. Kuhn, Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):13-21.
44. Zhao, M., et al., Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7925-30.
45. Copani, A., et al., Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci. 2001 Jan;24(1):25-31.
46. Katchanov, J., et al., Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
47. Leuner, B., E.R. Glasper, and E. Gould, Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol. 2009 Nov 10;517(2):123-33.
48. Kim, K.K., R.S. Adelstein, and S. Kawamoto, Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. J Biol Chem. 2009 Nov 6;284(45):31052-61.
49. Shingo, T., et al., Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science. 2003 Jan 3;299(5603):117-20.

Low Pass. Incredibly disorganized, rambling, disjointed. But here and there you see glimpses indicating that the author understands the methodology and controversy.

Introduction: Adult Neurogenesis

The initial reports of adult neurogenesis overthrew a roughly one-hundred year old idea that neuronal renewal was virtually impossible beyond birth. When it was first reported that neurons were unable to undergo post-natal mitosis, it seemed rather unfortunate that the cell type most critical for normal human functioning was the one type incapable of regeneration after trauma. Yet when both Joseph Altman and Michael Kaplan first discovered adult neurogenesis in the rodent hippocampus, their work was greeted with pure skepticism as it seemed nearly impossible that their scientific predecessors had not uncovered this process earlier. It was not until 40 years later when Fernando Nottebohm clearly identified continuous neurogenesis in adult canaries and songbirds that the field was re-visited. When combined with the technological advances in biology and immunohistochemistry, evidence for adult neurogenesis was then uncovered in species that spanned the length of the phylogenetic tree, including mice, rats, primates, and later humans. Surprisingly, the underlying purpose or benefit for adult neurogenesis, particularly in higher order species, is still rather unknown, and some even postulate that a gradual decline in adult neurogenesis in a given species is evolutionarily advantageous. Regardless, the potential function that adult neurogenesis plays in behavior, learning, and memory as well as its enormous therapeutic implications, render this process one of the most significant discoveries for the future of human health.

Deleted: phenomenon stating

Deleted: Frank

Deleted: ly high quantities of

Deleted: continually

I) Adult Neurogenesis across Species: Birds

Fernando Nottebohm and his colleagues initially searched for evidence of adult neurogenesis in canaries in the 1980s, despite the fact that initial data for adult neurogenesis in rodents had been largely dismissed [1]. In the avian brain, neural progenitors are housed within the ventricular zone (VZ) of the lateral ventricles, and successful recruitment and migration of these neural precursors occurs throughout most of the year into much of the avian forebrain [2, 3]. These avian forebrain regions that continuously recruit new neurons are functionally homologous to the mammalian hippocampus, striatum, and, somewhat, to the mammalian cortex [2]. Additionally there exists substantial, year-long neuronal recruitment to distinct nuclei of the vocal control system in the avian forebrain, most notably to the hyperstriatum ventralis pars caudalis (HVC) [2]. The HVC projects to the robust nucleus of the archistriatum (RA) for proper song production in males, hence the male HVC and RA are much larger than those of the female, and females do not sing unless treated with male gonadal hormones [4]. The male HVC experiences dramatic changes in size due to both the seasonal fluctuations in circulating testosterone, such that in non-breeding seasons when testosterone is low, the HVC is reduced to almost half of its normal neuronal number [4]. Surprisingly, within 12 months the HVC replaces over half of the lost neurons, corresponding to the onset of the breeding season and an increase in circulating testosterone [4]. The female HVC also demonstrates a continuous, year-long recruitment of new neurons, though they differentiate into interneurons within the HVC that neither project to the RA nor promote song production. Goldman et al., 1983 clearly demonstrated that new neurons in the female canary HVC were morphologically neurons, incorporated the ^{3}H thymidine label, produced action potentials, and were activated in response to acoustic stimuli [1, 4]. Later studies corroborated the results from this original study and found that roughly 20,000 new and functional neurons were

Deleted: Frank

Comment [SMB1]: Paton, not Goldman

added to several, widespread regions throughout the forebrain, in addition to some new recruitment to parts of the avian midbrain, medulla, and cerebellum [4].

This large quantity and expanse of neurogenesis in the avian forebrain suggests that most regions of the avian brain are capable of supporting both neuronal migration as well as later differentiation, and that neurons are able to survive within the forebrain long enough to function in existing circuits. A major reason for the vast quantities and consistency of neural precursor migration to the avian forebrain is the presence of a supportive system for migration and a supportive environment within the forebrain parenchyma that permits the survival of these new neurons. Radial glial cells extend from the VZ proliferative "hot spots" throughout much of the avian forebrain, thus providing a truly supportive scaffold upon which neural precursor cells can travel to distant sites [2, 3]. Additionally, gonadal hormones are crucial for both neuronal recruitment and survival, as estrogen promotes the recruitment and survival of new HVC neurons and testosterone promotes the survival of new RA-projecting HVC neurons [2, 3]. Additionally brain derived neurotrophic factor (BDNF) circulates within the HVC to support the survival and growth of newly recruited neurons [2]. The supportive roles of estrogen and testosterone explain both 1) the seasonal fluctuations in the size of the male HVC that correlates to the concentration of circulating testosterone, and 2) the presence of HVC neurogenesis in females that yields only HVC interneurons incapable of producing song [2, 4]. Furthermore, the expanse of the migratory network and the level of trophic support within the forebrain parenchyma distinguish the avian forebrain from other mammalian species and may explain why adult neurogenesis declines along the phylogenetic tree.

Deleted: guide

Adult Neurogenesis across Species: Rodents

Unlike the vast adult neurogenesis throughout much of the avian forebrain, adult neurogenesis in rodents is drastically reduced to the olfactory bulb and the dentate gyrus of the hippocampus [5]. Neural progenitor cells from the subventricular zone (SVZ) of the lateral ventricles migrate along the rodent rostral migratory stream (RMS), a structure similar to the radial guide cell system of the avian forebrain but devoid of the supporting radial-glial guide cell scaffold that widely projects to the avian forebrain [3, 5, 6]. Within the hippocampus, the subgranular zone (SGZ) of the dentate gyrus acts as a source of neural progenitor cells that can eventually differentiate into mature granule cells and add to the total population of the dentate gyrus [5, 7]. Initial evidence of hippocampal neurogenesis in rodents appeared roughly 50 years ago through the work of Altman and Kaplan who, like Nottebohm, used the ^{3}H thymidine label and the morphological characteristics of neurons to identify adult neurogenesis [8, 9]. Later evidence in support of neural progenitors within the SVZ came from *in vitro* studies showing that explants from mouse SVZ were able to differentiate into both neurons and glia [10]. Additionally, a study by Lois and Alvarez-Buylla in 1994 discovered that rodents indeed possess a migratory path to the olfactory bulb composed of a dense network of astrocytes that guides migrating neuroblasts rostrally to successfully differentiate into olfactory interneurons [11].

There are notable differences between avian and rodent adult neurogenesis, namely the rarity of neurogenesis in non-neurogenic regions of the rodent forebrain and the decline in neurogenesis in aged rodents. Notably, these distinctions indicate a substantial decline in the prevalence of adult neurogenesis from one species to the next. Neurogenesis in the adult rodent brain is regionally constrained such that neurogenesis in non-neurogenic regions is both rare and often transient. Though Kaplan reported evidence of neurogenesis within the rodent visual cortex [12], and Dayer et al., 2005 reported GABAergic neurogenesis within the rodent neocortex [13], the extent of the

Comment [SMB2]: The controversy today is whether indeed these are the only regions of adult neurogenesis in mammals. Gould reports neurogenesis in neocortex of mammals, right?

Comment [SMB3]: Rarity or absence?

new neuron recruitment appeared substantially lower than the quantity of daily neural recruitment within the avian forebrain. Furthermore, a study by Chen et al., 2004 is currently one of the only studies that has detected mouse neocortical neurogenesis, a finding that has yet to be replicated [14]. Select studies have reported neurogenesis in the hypothalamus of mice [15] and rats [16], as well as in sexually dimorphic nuclei of the rat hypothalamus [17]. During adolescence, gonadal hormone activation led to a significant increase in BrdU labeling within the female anteroventral periventricular nucleus and the sexually dimorphic nucleus of the preoptic area in males, suggesting that adolescent neurogenesis is both hormone dependent and crucial for organizing circuits that control adult sexual behavior [17]. Despite the occasional evidence for neurogenesis in these classically non-neurogenic regions, the process of new neuronal recruitment in such regions either occurs only at one distinct developmental time point or is primarily incomplete [5]. Incomplete neurogenesis holds that a vast majority of newly formed neurons in these regions are incapable of surviving long enough to have any functional relevance in existing circuits [5]. Furthermore, the origin and migratory path of these forebrain and midbrain neurons is not always clear, and differs significantly from VZ progenitors and the radial-glial guide cells that clearly allow for the migration of avian neural precursors to a vast majority of the telencephalon.

The second important difference between rodent and avian adult neurogenesis is the gradual decline in the process overtime in any given rodent, even in the major neurogenic region of the hippocampus. The frequency of hippocampal neurogenesis is not uniform throughout the adult rodent life, such that in comparison to 6 month old rats, 12 to 27 month old rats have a significant reduction in the number of both new and migrating neurons within the granule cell layer of the dentate gyrus [18]. This is in stark contrast to avian neurogenesis within both the forebrain and hippocampus that demonstrate a continuous recruitment of new neurons throughout much of adulthood [2,

Comment [SMB4]: But Gould in primates, right?

Comment [SMB5]: They did not show that these were neurons, a critical point to interpret these results.

Comment [SMB6]: ? No one has looked sequentially in the same rodent. How could they?

4, 19]. Specifically, the HVC of the canary forebrain recruits between 0.1 and 0.74% new neurons per day, and the hippocampus recruits between 0.15 and 0.37% new neurons per day [2]. Therefore, the decline in rodent neurogenesis overtime and the vast regional constraints on rodent neurogenesis indicate that the continuity and the expanse of this process become continually restricted in more complex species. This may suggest that despite its profound implications to the overall function of the brain, adult neurogenesis may not be crucial for the optimal survival of higher order species.

Adult Neurogenesis across Species: Primates and Humans

The eventual acceptance of adult neurogenesis in rodents led investigators in primate neurobiology to search for similar process in non-human primates, as any indication of neurogenesis in any region of the primate brain would hold enormous therapeutic potential. In 1985, Pasko Rakic initially reported that neurogenesis was completely absent throughout the entire primate brain, for injections of the ^{3}H-thymidine label failed to stain any neuron in the neocortex, the hippocampus, the olfactory bulb, the basal ganglia, or any other brain region analyzed [20]. It was postulated that the lack of neurogenesis in such a complex, highly developed species was a purposeful adaptation, such that the continual environmental interactions in primates and humans necessitated a set of stable, persistent synapses for much of the adult life [20]. Yet almost 15 years later, Gould et al., 1998 found convincing evidence for neurogenesis within the dentate gyrus of young, middle-aged and older (23 years) Old World Monkeys [21]. BrdU labeled neurons also co-localized with the distinct marker for immature neurons, TOAD-64, indicating that labeled cells were indeed neurons [21]. Around the same time, despite their initial claims that neurogenesis does not occur within primates, Kornack and Rakic 1999 finally observed and validated neurogenesis in the dentate gyrus of adult macaque monkeys [7]. Taken together, these studies pointed to the presence of *in*

Deleted: leading

Comment [SMB7]: But denied seeing it in neocortex.

You fail to engage the crucial distinctions between these studies, which you must do to understand and address the controversy.

You take this up later, when you reach different conclusions while comparing the same two studies.

vivo neural stem cells within the subgranular zone of the dentate gyrus and their later differentiation into hippocampal granule cells, a finding that was highly significant in light of previous reports indicating that primate neurogenesis was neither necessary nor feasible beyond birth.

Neurogenesis within the hippocampus and olfactory bulb of the primate did not come as much of a surprise considering the vast amount of support for these two processes in lower order rodents. Similar to adult neurogenesis in rodents, few investigators were able to provide support for adult neurogenesis in any other region of the primate brain. However, Gould et al., 1999 reported evidence of new, BrdU-labeled neurons within the neocortex that had traveled to their final position along a migratory stream projecting from the SVZ to their final cortical destination [22]. Not surprisingly, this study faced harsh criticism, disbelief, and mostly skepticism, with critics primarily questioning her interpretations and the methods used in the study [23-25]. The study faced such criticism, that the investigators completed a second study to evaluate the survival of these new neurons that they had reportedly identified within the primate neocortex [26]. They continued to find evidence for both hippocampal as well as neocortical neurogenesis, though it was clear that several of the BrdU-labeled cells in the cortex were unable to survive past approximately 9 weeks [26]. Moreover, Bernier et al., 2001 reported adult neurogenesis within the primate amygdala and piriform cortex as well as the presence of a temporal migratory stream along which the precursor cells from the SVZ had possibly migrated to the cortex [27]. Yet they too noted that several migrating neurons along this supposed temporal stream were unable to survive long enough to reach their cortical destination [27]. In response to both claims, Kornack and Rakic again searched for evidence of cortical neurogenesis, yet their results showed only the growth of new glial cells within the cortex and no evidence for neurogenesis [25]. To this day, there have been very few additional reports of successful

Deleted: attempted to

neurogenesis within the cortex of primates, suggesting that any migrating neural precursors from the SVZ are likely to die within weeks of settling in the cortical parenchyma.

Similar to the restrictions of the rodent brain in comparison to the avian brain, the limitations placed on forebrain and midbrain neurogenesis in primates appears to be primarily caused by a decline in basic anatomical support for the process as noted by the grossly underdeveloped RMS in primates. The migratory stream available to primates is limited in its regional and forebrain extension, much like the decreased complexity of the RMS in rodents compared to birds [5]. In the primate RMS neuroblasts rely on neurophilic interactions between one another rather than radial-glial cell guided or even astrocyte guided migration, and extension of the RMS is strictly limited to the olfactory bulb [28]. Additionally, relative to the rodent brain, the migration rate of primate neuroblasts destined for the olfactory bulb is much slower, requiring at least 75 days for full migration after normalizing for differences in brain size between rodents and primates [28]. Thus it appears as though the lost complexity of the primate migratory stream is again a limiting factor in the prevalence of primate neurogenesis in any area other than the olfactory bulb and the hippocampus. This feature places a large restriction on the potential for neural precursors in the SVZ to successfully migrate to any other region of the forebrain. Additionally, the forebrain is essentially considered to be a non-neurogenic region likely void of proper trophic factors to support neural differentiation and survival [5]. Despite reports of neocortical neurogenesis, the ability for new neurons to survive and integrate into functional circuits in any area other than the hippocampus is drastically reduced in comparison to that seen in avian species.

Additionally, there exist important restrictions on hippocampal neurogenesis within the primate, particularly in comparison to rodents. While granule cells that are newly generated in the rodent hippocampus continually add to the pool of hippocampal

neurons in the adult rodent, for example between 30 and 365 days of life, this is likely not the case in primates [28, 29]. Hippocampal neurogenesis in the adult rodent increases in response to several environmental stimuli including exercise [30], sexual experience [31], and learning [32], all of which lead to an overall increase the total number of functional granule cells. However, the primate brain appears to show no significant accumulation of new hippocampal neuron addition, suggesting that hippocampal neurogenesis correlates to an equal rate of neuronal apoptosis [7]. Thus, hippocampal neurogenesis within rodents may facilitate new learning and synaptic plasticity in the face of constant environmental changes or necessary changes in social behavior throughout the adult life. Considering that both primates and humans extensively interact with the environment, it is possible that the gradual decline in neurogenesis in favor of long-lasting synapses and circuits is necessary for long term memory storage and the overall complexity of the primate and human brain [7].

Humans show virtually an identical pattern of neurogenesis as primates, with neural progenitor cells housed only in the hippocampus and the SVZ lining the ventricular system. The first reports of human neural progenitor cells were rather astounding, as they truly verified that neurogenesis was indeed possible in the human brain. Eriksson et al., 1998 evaluated the brains of cancer patients that had been previously treated with BrdU, a treatment that had been used to follow the proliferative profile of tumor cells [33]. The study found a substantial quantity of BrdU labeled cells positive for neural markers within the dentate gyrus of these individuals, providing official proof that neural precursors can survive in the human brain [33]. Knoth et al., 2010 analyzed hippocampal tissue samples from individuals ranging from childhood to almost 90 years of age, specifically searching for neural precursors and neural proliferation [34]. Surprisingly they found that several cells co-localized with both immature neuronal and cell proliferation markers at almost all ages, though they noted a significant decline the

number of labeled cells beyond roughly 40 years of age [34]. Additionally, Sinai et al., 2004 confirmed the presence of SVZ neural progenitors in postmortem adult human tissue as well as a unique band of astrocytes extending from the SVZ [35]. However they found no evidence in support of neural precursor migration along this band of astrocytes, suggesting that neural precursors in humans are even more restricted to distinct locations within the brain parenchyma than even non-human primates [35]. Additionally, a separate study used an alternate method of detecting proliferating neural cells, the phenomenon of carbon-14 labeling, and found that neurons in the neocortex of humans were the exact age of the individual [36]. This suggested that, like primates, neocortical neurogenesis in humans is largely restricted to embryological development prior to birth. No other studies have successfully reported human neurogenesis or the presence of neural precursors in any other region outside of the hippocampus or the SVZ. Nonetheless, the presence of individual neural precursors in humans warrants great attention, and highlights the necessity to identify an optimal environment that would allow neurons to differentiate and thrive in several regions throughout the human brain.

Avian and mammal adult neurogenesis differ in both the frequency of neural turn over as well as the constraints placed on neural progenitors as to where they can survive and differentiate. More importantly, the regional constraints appear to be more limiting to the expanse of adult neurogenesis in more complex species. The fact that neural precursors of the SVZ and hippocampus can survive well into adulthood of rodents, primates, and humans, indicates that age does not appear to restrict the potential for neurogenesis, but rather their capacity to survive in various regions of the brain is the more significant limiting factor. Despite reports of neurogenesis within the rodent neocortex or hypothalamus as well as the primate neocortex, such accounts are

both rare and often conflicting between groups, a finding that is in direct contrast to the widespread and continual neurogenesis throughout the avian forebrain.

II) Temporal vs. Regional Constraints in Adult Neurogenesis

Despite the fact that the total amount of mammalian neurogenesis declines in prevalence both across species and within a given species, adult neurogenesis becomes more regionally constrained when the mammalian brain becomes more complex. Lower order vertebrates such as fish and the aforementioned songbirds experience high quantities of neurogenesis both throughout adulthood and within several higher order forebrain structures. In comparison to birds, mammals as low as rodents have a drastic reduction in the brain regions available for supporting complete neurogenesis. While both avian species and rodents possess a similar pool of neural precursors within the SVZ of the lateral ventricles, their migratory ability and their capability to survive in various forebrain regions is dramatically different. Though both primates and humans still possess distinct pools of neural progenitors that are capable of proliferating well into adulthood, there are even fewer reports of successful integration of such precursors into other brain regions. Additionally, regional constraints on mammalian neurogenesis appears to be the more significant factor in higher order species due to fact that labeling of proliferating and immature neuronal precursors has identified active neural progenitor cells in elderly rodents [18], primates [21], and humans [33, 34]. The fact that any neural precursor exists in the mammalian brain several years after birth indicates that mammalian brains are capable of harvesting and supporting neural stem cells, but that their survival and differentiation requires a distinct environment that appears to be uncharacteristic of higher order brain regions.

It is noteworthy that certain non-neurogenic regions of the mammalian brain, particularly in humans, can suddenly support nascent neurogenesis after trauma due to the change in circulating factors associated with an injured brain region. In a report by Jin et al., 2006, brain biopsy tissue samples from stroke patients were stained for Ki67, a marker of cell proliferation, as well as DCX and βIII tubulin, two markers expressed in new neurons [37]. Particularly within the penumbra, or the surviving cortical tissue surrounding the ischemic core, the study found that several cells were positive for both Ki67 and either DCX or βIII tubulin [37]. As neurogenesis has been shown to be enhanced in such degenerative diseases as Huntington's and Alzheimer's, the authors noted that it is likely that a similar process was taking place in these stroke patients, such that pre-existing neural precursors were stimulated to begin dividing in order to replace lost neurons after the insult [37]. They noted that the source of the precursors could either be the SVZ where progenitors may have migrated along capillary beds, or even local precursors within the cortex [37]. In a similar study, Liu et al., 2008 evaluated tissue samples from epileptic patients undergoing resection of both the hippocampus and the anterior temporal cortex [38]. By staining for DCX in control and epileptic tissue samples, the authors sought to identify if immature, new neurons were more prevalent in epileptic tissue [38]. They found a notable increase in DCX positive cells in both the granule cell layer of the hippocampus and distinct temporal cortical regions of epileptic patients in comparison to control tissue [38]. The authors noted that *in vitro* cultures of epileptic human tissue housed neural progenitor cells, suggesting that the trauma induced by seizure is able to essentially induce proliferation of quiescent progenitor cells within brain regions that are usually non-neurogenic [38].

In both cases, trauma to the brain induced proliferation of neural precursors near the site of the lesion, likely as a last resort attempt to restore function before the level of trauma became fully detrimental. Yet this phenomenon corroborates the idea that adult

neurogenesis in higher order mammals is possible late into adult life but is constrained to distinct regions throughout the brain that appear to have evolved as optimal stem cell niches. Constitutive neurogenesis within the hippocampus is now a well-known, accepted fact in all mammals, as is the presence of neural progenitor cells throughout the SVZ capable of migrating to the olfactory bulb. Though the overall prevalence of neurogenesis appears to decline gradually in aging mammals, it continues to be an ongoing albeit occasional process, and neural progenitors within the hippocampus or SVZ do not appear to suddenly die off as the brain ages. Rather, the major decline in adult neurogenesis that is seen from birds to rodents to monkeys and to humans is due to the major restrictions placed on neural precursors as to the brain environments in which they are capable of thriving. The discovery of adult neurogenesis in humans was an immense milestone in the field of neurological and neurodegenerative disorders, though the therapeutic potential of individual neural precursors cannot be achieved until investigators uncover a method that allows such precursors to survive and function in more widespread regions of the human brain.

Comment [SMB8]: What? I don't understand what you're trying to say.

III) Methods for Detecting Adult Neurogenesis

When the first reports of adult neurogenesis surfaced, most individuals within the scientific community were hostile to even evaluate the methods used by such scientists as Michael Kaplan and Joseph Altman who had proposed such a phenomenon. Even decades later when investigations into this process became more widespread, non-believers were more skeptical of the idea rather than the accuracy of the methods used. Yet when both BrdU labeling and immunohistochemistry were incorporated into the study of adult neurogenesis, claims of neurogenesis in higher order primates and in non-neurogenic brain regions called for a re-evaluation of the evidence [23]. Much of the criticism of certain groups concerned the specific methods and BrdU protocols used as

well as the individual interpretations of the results. The methodological critiques were both justified and legitimate, considering that the idea of adult neurogenesis in mammals has such profound implications to human health. Though the current use of BrdU is highly efficient and validated, it requires that investigators undergo rigorous controls and consider several alternate interpretations in order for their results to be accepted by the scientific community.

Old versus New Thymidine Analogs

The first reports of adult neurogenesis in both mammals and birds initially used the analog ^{3}H-thymidine that was efficiently incorporated into dividing cells in the DNA synthesis phase of mitosis, thus indicating a cell's "birthday" [4, 9, 39]. Cells exposed to the ^{3}H-thymidine were later exposed to silver grains that would bind the thymidine analog and that would allow for cell visualization via autoradiography [4, 9, 23, 40]. Investigators delineated stringent criteria for cell labeling, such that a mitotically-active cell was one whose overlying silver grains were at least 50% of the labeling in cells that showed maximal labeling [23]. Nottebohm reported that avian neurons were considered labeled if they possessed 3 silver grains per nucleus, while Michael Kaplan reported that a labeled rodent neuron was one with 5 grains over the nucleus in the olfactory bulb or 19 grains over the nucleus of a granule cell from the dentate gyrus [4, 8]. This initial method in detecting cell proliferation was highly efficient and validated, and it was advantageous in that it allowed for an objective, quantitative analysis of proliferating cells.

Deleted: Frank

Though the potential for quantification of cell division was beneficial for early neurobiologists using this method, major issues arose when attempting to identify cell type without the vast array of neural or glial markers available today. Early reports of adult neurogenesis determined cell type using both ultrathin sections stained with either

Nissl or Cresyl violet or electron microscopy to search for morphological clues that would distinguish a neuron from a glial cell [4, 8]. In general, cells were considered to be neurons when they possessed round, compact nuclei with one or two nucleoli when visualized with standard histology, and if electron microscopy (EM) revealed the presence of synaptic terminals with multiple types of vesicles, smooth membrane contours, and long processes containing microtubules [1, 4, 8, 12, 41]. The use of the 3H thymidine label and the detailed characterization of neuronal morphology initially identified the presence of adult neurogenesis in both avian and mammalian speices, a process that had long been considered impossible. Clearly, the phenomenon was initially dismissed, and not until almost 40 years later did innovations in biological techniques allow for the idea to be more highly reconsidered.

The idea of neurogenesis, particularly in the mammalian hippocampus, was reconsidered in the early 1990s during which both neural and glial-specific antibodies were readily available and when researchers first incorporated the use of bromodeoxyuridine (BrdU) to tag neuronal cell division in adult mammals. The use of BrdU to assess the proliferative nature of growing tumors in cancer patients prompted investigators to apply the same marker to label proliferating and migrating neurons within the mature CNS [40]. Similar to ^{3}H thymidine, BrdU could be incorporated into the cell specifically during the S phase of mitosis after which the label was identified, though using immunohistochemistry [40]. Additionally, BrdU held major advantages over that of ^{3}H thymidine in that visualizing the BrdU label took only 1 to 3 days rather than the 1 to 3 months required for the autoradiography associated with ^{3}H thymidine, and specialized facilities were not required to visualize the BrdU label [39]. Additionally, new antibodies that were specific to surface or cytoskeletal markers of neurons or glial cells allowed neurobiologists to distinguish one cell type from another without relying on subjective morphological criteria or the demanding techniques of EM.

Comment [SMB9]: Yes.

To this day, BrdU has arguably been the most significant contribution to the field of adult neurogenesis, and it is the most prominent method used to detect the process. The transition from the ^{3}H thymidine analog to the BrdU label was an immense move in the field that made the study of adult neurogenesis both feasible and accessible to virtually any neurobiologist. Unfortunately, the BrdU method is not without inherent flaws rendering it continually susceptible to much scrutiny throughout the scientific community. The rather subjective nature of immunohistochemistry required to detect the BrdU label is the major factor in the BrdU protocol that may cause investigators to evaluate data rather differently. Due to its current prevalence within neurobiology, there are several standard controls and considerations that investigators cannot ignore in order for their studies to be both accepted and validated by their peers.

Comment [SMB10]: Yes.

Considerations in BrdU Staining: Route and Dose of BrdU

Though the use of BrdU dramatically advanced the field of adult neurogenesis, there exist several variations in individual BrdU protocols that can significantly impact how results are interpreted. One of the major differences identified across protocols is the concentration of BrdU used, as well as the route of its injection [42, 43]. It has been proposed that a dose of 50mg/kg BrdU is sufficient to label any and all dividing neurons, though doses ranging from 50 to 500mg/kg have also been used in order to optimize staining and to account for smaller neurons that might not be labeled with protocols using the lower doses [42, 44]. Though lower doses of BrdU may ultimately underestimate the quantity of proliferating cells, administration of higher doses renders the experimenters more likely to stain cells undergoing DNA turnover or even apoptosis [23, 42, 43]. Cells preparing for apoptosis or in the process of degeneration lose Cdk inhibition, leading them to re-enter the cell cycle prior to death [45, 46]. In such cases, BrdU will stain cells undergoing either process leading to erroneous and false positive

results. Additionally, as with any radioactive or exogenous agent applied to cells, BrdU is considered to be a mutagen, meaning its successful incorporation into nuclear material and the general health of the cell may both be compromised when higher doses are administered [42, 43]. Both the potential for BrdU to label apoptotic cells and the mutagenic qualities of BrdU are major technical considerations in any BrdU protocol, and virtually any study that does not independently control for both factors is deemed invalid. Furthermore, investigators use both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) routes for BrdU administration, meaning there may be a significant degree of variation in BrdU access to CNS tissue. I.p. injections are subject to potentially thwarted access to the CNS due to differences in blood brain barrier permeability to BrdU across species [43]. Continuous i.c.v. infusion through the lateral ventricles enhances the likelihood that all relevant cells within the CNS will be labeled, yet the level of exposure may render neuronal cells more susceptible to the mutagenic qualities of BrdU including altered gene expression or even cell death [43]. Ultimately, variations in both the dose and route of BrdU administration reported across groups can significantly alter the outcome of any one experiment and lead to several different interpretations of one set of results.

Considerations in BrdU Staining: Antibody Specificity

As with any immunohistological assay, the specificity and sensitivity of primary and secondary antibodies must be taken into consideration when designing an optimal protocol. Antibody specificity is even more crucial in BrdU staining protocols, considering that false positive staining can lead investigators to make truly profound conclusions from inaccurate results. Optimization of antibody specificity must be applied not only to the actual BrdU label but also to any additional labels used to mark neurons or glial cells. A study by Leuner et al., 2009 evaluated differences in the sensitivities of

BrdU antibodies from such companies as Vector, BD, and Roche, and found significant differences in the number of newborn cells labeled by each separate antibody within the dentate gyrus [47]. Several biotech companies sell BrdU antibodies and the choice of company is highly variable across research groups, meaning the specific choice of BrdU antibody may become a significant confounding factor. More crucial than the choice of BrdU antibody is the use of proper labels that undoubtedly distinguish a neuron from a glial cell, an endothelial cell, and other nascent cells within the CNS. Though there are currently several markers for each distinct stage within neuronal development, the issue of both antibody specificity and background staining also holds true for antibodies chosen to define cell type. Neu-N is the most widely used antibody to identify neurons, and it was not until recently that the Neu-N antigen was identified as the neuron-specific Fox-3 gene, a member of the Fox-1 gene splicing family [48]. Additionally, the authors found that any cross reactivity of Neu-N occurs with the neural-specific synapsin-1 protein, meaning it is still a highly acceptable antibody to chose for neural staining [48]. However, in addition to variations in BrdU antibody binding specificity, Leuner et al., 2009 reported high background staining when using the Neu-N antibody, specifically based on the individual DNA denaturation protocol [47]. Since HCl, HCl and formamide, and steam heating are all common treatments used for DNA denaturation, the study evaluated each technique separately to determine how and if they altered background staining. Each technique resulted in notable differences in the Neu-N signal to noise ratio, a factor that can drastically overestimate or underestimate Neu-N staining in a BrdU protocol [47].

Though the technique of BrdU labeling is currently the gold standard in evaluating adult neurogenesis, there are considerable differences between individual protocols that may greatly affect how a given set of results is interpreted. Changes in both the does and route of BrdU injections can lead to false positive results by labeling

old neurons going through apoptotic cell cycling or even cell death. Additionally, variations in primary antibodies to BrdU or neural markers can result in different staining intensities, causing individual investigators to rely on their own intuition and judgment in counting cells. Therefore, it is clear that these specific aspects of a BrdU protocol must be as consistent as possible across research groups when attempting to verify the presence of adult neurogenesis. Most importantly, this is of particular importance in higher order species or in classically non-neurogenic regions where there may only be miniscule, yet extremely notable, quantities of dividing cells.

Controls in BrdU Staining

Despite the aforementioned flaws inherent to the BrdU technique, it remains the most prominent, efficient, and validated method available for detecting adult neurogenesis, especially in mammalian species. The technique of BrdU staining exploits the fundamental phase of DNA synthesis inherent to the process of mitosis that is virtually identical across the phylogenetic tree. Even to this day, the idea of adult neurogenesis is almost beyond belief thus it comes as no surprise when investigators looking into the process must undergo a series of rigorous controls. Notably, with the advent and relative abundance of confocal microscopy, it has become almost a routine process that adult neurogenesis be verified with the use of this 3-dimensional imaging technique, so as to ensure that the full depth of a given cell is uniformly labeled with both BrdU and neuronal markers [23]. For example, a study by Dayer et al., 2005 reported neurogenesis in the adult rat neocortex and striatum, and used confocal microscopy combined with a total of eight different neuronal markers including Neu-N, CRMP4, NG2, DCX, GABA and GAD67 to unquestionably verify that the BrdU label was found in neurons [13]. Confocal imaging to detect the presence of both BrdU and neuronal markers demonstrated the presence of the two labels throughout several z-planes of the

double-labeled cells, further indicating that the BrdU positive cell was not simply a non-neuronal cell layered on top of a neuron [13]. Current investigations in adult neurogenesis must also verify that the BrdU label has not been incorporated into old neurons that have re-entered the cell cycle prior to apoptosis. A study by Zhao et al., 2003 reported the occurrence of new neurons within the substantia nigra of mice, a finding that would clearly have a profound influence on Parkinson's Disease therapeutics [44]. The authors were careful to control for potential BrdU incorporation into degenerating or apoptotic cells by sacrificing a subset of animals 2 days after initiating i.c.v infusions of BrdU, a time point at which apoptotic activity of nigral cells would be at a maximum [44]. BrdU incorporation was not observed at this time point, and incorporation was highest 21 days after infusion, suggesting that staining at the later time points could be attributed to neurogenesis [44].

Deleted: by

The BrdU technique is currently unparalleled in its ability to detect adult neurogenesis, though it is apparent that issues of antibody specificity, background staining, labeling of apoptotic cells, and incorrect identification of cell type are all legitimate reasons for the variations in data interpretation across research groups. When Gould et al., 1999 first reported adult neurogenesis in the neocortex of primates, the skepticism that ensued was primarily concerned with the methods used in the original study and how the authors interpreted them [22-24]. Critics were skeptical of whether BrdU was labeling new cells or old cells undergoing apoptosis, whether labels used to identify neurons were truly neuron-specific, and whether optical issues with cell identification were taken into consideration [23]. Regardless, the application of BrdU labeling successfully replaced the original ^{3}H-thymidine method and is in continuous use today by most of the leading experts in adult neurogenesis. However it is necessary that every investigator use multiple controls in their individual BrdU staining protocols including confocal microscopy, multiple neuronal markers, and multiple time points after

BrdU injections to rule out BrdU labeling of apoptotic cells. When appropriate controls are used and the aforementioned technical considerations are addressed, adult neurogenesis can be validated in virtually any species and any brain region. Until an alternate, more accurate method arises, BrdU staining will continue to be the prominent method of choice in this profound and growing field.

References

1. Goldman, S.A. and F. Nottebohm, Neuronal production, migration, and differentiation in a vocal control nucleus of the adult female canary brain. Proc Natl Acad Sci U S A. 1983 Apr;80(8):2390-4.
2. Gahr, M., et al., What is the adaptive role of neurogenesis in adult birds? Prog Brain Res. 2002;138:233-54.
3. Doetsch, F. and C. Scharff, Challenges for brain repair: insights from adult neurogenesis in birds and mammals. Brain Behav Evol. 2001;58(5):306-22.
4. Nottebohm, F., Neuronal replacement in adulthood. Ann N Y Acad Sci. 1985;457:143-61.
5. Bonfanti, L. and P. Peretto, Adult neurogenesis in mammals--a theme with many variations. Eur J Neurosci. 2011 Sep;34(6):930-50.
6. Gould, E., How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007 Jun;8(6):481-8.
7. Kornack, D.R. and P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey. Proc Natl Acad Sci U S A. 1999 May 11;96(10):5768-73.
8. Kaplan, M.S. and J.W. Hinds, Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science. 1977 Sep 9;197(4308):1092-4.
9. Altman, J. and G.D. Das, Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol. 1965 Jun;124(3):319-35.
10. Lois, C. and A. Alvarez-Buylla, Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993 Mar 1;90(5):2074-7.
11. Lois, C. and A. Alvarez-Buylla, Long-distance neuronal migration in the adult mammalian brain. Science. 1994 May 20;264(5162):1145-8.
12. Kaplan, M.S., Neurogenesis in the 3-month-old rat visual cortex. J Comp Neurol. 1981 Jan 10;195(2):323-38.
13. Dayer, A.G., et al., New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 2005 Jan 31;168(3):415-27.
14. Chen, J., S.S. Magavi, and J.D. Macklis, Neurogenesis of corticospinal motor neurons extending spinal projections in adult mice. Proc Natl Acad Sci U S A. 2004 Nov 16;101(46):16357-62.
15. Kokoeva, M.V., H. Yin, and J.S. Flier, Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science. 2005 Oct 28;310(5748):679-83.
16. Xu, Y., et al., Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol. 2005 Apr;192(2):251-64.

17. Ahmed, E.I., et al., Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. Nat Neurosci. 2008 Sep;11(9):995-7.
18. Kuhn, H.G., H. Dickinson-Anson, and F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996 Mar 15;16(6):2027-33.
19. Goldman, S.A., Adult neurogenesis: from canaries to the clinic. J Neurobiol. 1998 Aug;36(2):267-86.
20. Rakic, P., Limits of neurogenesis in primates. Science. 1985 Mar 1;227(4690):1054-6.
21. Gould, E., et al., Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A. 1999 Apr 27;96(9):5263-7.
22. Gould, E., et al., Neurogenesis in the neocortex of adult primates. Science. 1999 Oct 15;286(5439):548-52.
23. Rakic, P., Neurogenesis in adult primate neocortex: an evaluation of the evidence. Nat Rev Neurosci. 2002 Jan;3(1):65-71.
24. Nowakowski, R.S. and N.L. Hayes, New neurons: extraordinary evidence or extraordinary conclusion? Science. 2000 May 5;288(5467):771.
25. Kornack, D.R. and P. Rakic, Cell proliferation without neurogenesis in adult primate neocortex. Science. 2001 Dec 7;294(5549):2127-30.
26. Gould, E., et al., Adult-generated hippocampal and neocortical neurons in macaques have a transient existence. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10910-7.
27. Bernier, P.J., et al., Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11464-9.
28. Kornack, D.R. and P. Rakic, The generation, migration, and differentiation of olfactory neurons in the adult primate brain. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4752-7.
29. Bayer, S.A., J.W. Yackel, and P.S. Puri, Neurons in the rat dentate gyrus granular layer substantially increase during juvenile and adult life. Science. 1982 May 21;216(4548):890-2.
30. Pereira, A.C., et al., An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A. 2007 Mar 27;104(13):5638-43.
31. Leuner, B., E.R. Glasper, and E. Gould, Sexual Experience Promotes Adult Neurogenesis in the Hippocampus Despite an Initial Elevation in Stress Hormones. PLoS ONE. 5(7).
32. Gould, E., et al., Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci. 1999 Mar;2(3):260-5.
33. Eriksson, P.S., et al., Neurogenesis in the adult human hippocampus. Nat Med. 1998 Nov;4(11):1313-7.
34. Knoth, R., et al., Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. PLoS One. 2010 Jan 29;5(1).
35. Sanai, N., et al., Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature. 2004 Feb 19;427(6976):740-4.
36. Bhardwaj, R.D., et al., Neocortical neurogenesis in humans is restricted to development. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12564-8.
37. Jin, K., et al., Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006 Aug 29;103(35):13198-202. Epub 2006 Aug 21.
38. Liu, Y.W., et al., Doublecortin expression in the normal and epileptic adult human brain. Eur J Neurosci. 2008 Dec;28(11):2254-65.

39. Miller, M.W. and R.S. Nowakowski, Use of bromodeoxyuridine-immunohistochemistry to examine the proliferation, migration and time of origin of cells in the central nervous system. Brain Res. 1988 Aug 2;457(1):44-52.
40. Nowakowski, R.S., S.B. Lewin, and M.W. Miller, Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. J Neurocytol. 1989 Jun;18(3):311-8.
41. Burd, G.D. and F. Nottebohm, Ultrastructural characterization of synaptic terminals formed on newly generated neurons in a song control nucleus of the adult canary forebrain. J Comp Neurol. 1985 Oct 8;240(2):143-52.
42. Rakic, P., Adult neurogenesis in mammals: an identity crisis. J Neurosci. 2002 Feb 1;22(3):614-8.
43. Cooper-Kuhn, C.M. and H.G. Kuhn, Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res Dev Brain Res. 2002 Mar 31;134(1-2):13-21.
44. Zhao, M., et al., Evidence for neurogenesis in the adult mammalian substantia nigra. Proc Natl Acad Sci U S A. 2003 Jun 24;100(13):7925-30.
45. Copani, A., et al., Activation of cell-cycle-associated proteins in neuronal death: a mandatory or dispensable path? Trends Neurosci. 2001 Jan;24(1):25-31.
46. Katchanov, J., et al., Mild cerebral ischemia induces loss of cyclin-dependent kinase inhibitors and activation of cell cycle machinery before delayed neuronal cell death. J Neurosci. 2001 Jul 15;21(14):5045-53.
47. Leuner, B., E.R. Glasper, and E. Gould, Thymidine analog methods for studies of adult neurogenesis are not equally sensitive. J Comp Neurol. 2009 Nov 10;517(2):123-33.
48. Kim, K.K., R.S. Adelstein, and S. Kawamoto, Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. J Biol Chem. 2009 Nov 6;284(45):31052-61.
49. Shingo, T., et al., Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science. 2003 Jan 3;299(5603):117-20.