UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Cell Birthdate Effects on Cell Layering and Function in the Mouse Dentate Gyrus

A Dissertation submitted in partial satisfaction of the

Requirements for the Degree of Doctor of Philosophy

in

Neurosciences

by

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The Dissertation of Emily Anne Mathews is approved, and it is acceptable in quality and form for publication on microfilm:

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# DEDICATION

In recognition of all those who have helped me to reach this day:

- my early school science and research teachers, Donna from the Science Center, St. Petersburg, FL; Lynne Douglas; Sheila Wallace
- my research mentors: Dr. Hugo Fernandez, Dr. Michael Davis, Dr. Fred Gage
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# EPIGRAPH

"The soundest fact may fail or prevail in the style of its telling"
-Ursula K. LeGuin, *The Left Hand of Darkness*

"I wanted to discover lovely basic things and I wanted to listen to the music of the birds. But there is so much suffering out there, and it would be so nice to have a solution."
-Fernando Nottebohm

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LIST OF ABBREVIATIONS

**BrdU** – 5'-bromo-2'-deoxyuridine, a halogenated nucleotide analog used to label dividing cells

**CldU** – 5'-chloro-2'-deoxyuridine, a halogenated nucleotide analog used to label dividing cells

**CNS** – central nervous system

**DAPI**– 4',6-diamidino-2-phenylindole; intercalates between nucleotide bases to stain cell nuclei

**DG** – dentate gyrus of the hippocampus

**E** – day of embryonic development

**GCL** – granule cell layer of the dentate gyrus of the hippocampus

**GCV** – gancyclovir

**3H-T** – tritiated thymidine, a radioactive nucleotide analog used to label dividing cells

**IdU** – 5'-iodo-2'-deoxyuridine, a halogenated nucleotide analog used to label dividing cells

**LTP** – long-term potentiation; enhancement of neuronal signaling thought to contribute to memory

**MWM** – Morris water maze

**NTK** – Nestin Thymidine Kinase; a line of transgenic mice generated by Wei Deng (The Salk Institute) in which the nestin promoter drives constitutive expression of herpes simplex virus thymidine kinase.

**P** – day of postnatal development

**PFA** – paraformaldehyde

**PPI** – prepulse inhibition; the startle reflex is inhibited in response to a tone (prepulse) presented just before the startle-eliciting stimulus (pulse)

**Prox1** – Prospero-like protein 1, a homeobox transcription factor and marker of granule cells

**PTSD** – post-traumatic stress disorder

**SGZ** – subgranular zone

**Sox2** – the transcription factor Sry-related homeobox (HMG-box) 2, used as a marker of neural progenitor cells

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Chapter 2 will be submitted for publication: Mathews EA, Zhao C, Morgenstern N, Piatti V, Jessberger S, Schinder AS, Gage FH . "A Distinctive Layering Pattern for Dentate Granule Cells Generated by Developmental and Adult Neurogenesis". The dissertation author was the primary author and investigator of this paper.

Chapter 3 will also be submitted for publication: Mathews EA, Deng W, Saxe M, Gage FH. "Decreasing Postnatal Neurogenesis Has Chronic Effects on Dentate Structure, Adult Neurogenesis and Behavior". The dissertation author was the primary author and investigator of this paper.

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PUBLICATIONS

Bossy-Wetzel E, Talantova MV, Lee WD, Scholzke MN, Harrop A, Mathews E, Gotz T, Han J, Ellisman MH, Perkins GA, Lipton SA. (2004) Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K+ channels. Neuron. Feb 5;41(3):351-65.

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ABSTRACT OF THE DISSERTATION

Cell Birthdate Effects on Cell Layering and Function in the Mouse Dentate Gyrus

by

Emily Anne Mathews

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2008

Professor Fred H. Gage, Chair
Professor Nicholas Spitzer, Co-chair

This work explores unique features of early developmental neurogenesis in the dentate gyrus of the rodent hippocampus. It characterizes an outside-in layering of early- to late-born neurons in the dentate granule cell layer. It demonstrates that cells dividing during embryonic and postnatal life continue to divide infrequently through adult life. In fact, these early-dividing cells constitute most of the adult dividing population. Finally, it describes the ability of the postnatal brain to recover from an insult to neurogenesis (gancyclovir administered postnatally to Nestin-thymidine kinase transgenic animals). Mice with approximately 50% decreased dentate proliferation during postnatal days 5-9 sustain permanent anatomic deficits: both total granule cell number and adult neurogenesis are still decreased by about half at 13 weeks of age. Further, males only show corresponding behavior deficits in memory retention tasks. These findings contribute to the understanding of the origin and

function of the adult dentate gyrus. They underscore that there are unique features of the postnatal brain that set the stage for lifetime learning and neurogenic ability. They also suggest future research into early clinical interventions to restore normal neurogenesis after experiences that may interfere with neurogenesis early in life.

CHAPTER 1

# INTRODUCTION

About 10 years ago, adult neurogenesis was accepted in the dentate gyrus (DG) of the mammalian hippocampus.[1] This discovery presents both the challenge of uncovering the function of new neurons introduced throughout life, and the opportunity to harness an incredible untapped source of brain plasticity, if we could learn to use this lifelong fount of neurons in a regulated fashion.

To understand the function of adult hippocampal neurogenesis, it is important to elucidate what distinguishes cells born during adulthood from those born developmentally. The strongest evidence for such a distinction is that for a birthdate-based layering within the DG. This layering is somewhat analogous to the inside-out developmental layering of the cerebral cortex. However, it occurs in the reverse direction (outside-in), and it involves far less cell-type stratification, because the DG granule cell layer (GCL) consists mostly of granule cells. A similar layering phenomenon also characterizes olfactory bulb neurons – another group of granule cells that layer by birthdate and are continually generated through life.[2]

Early studies have examined developmental layering within the DG, and qualitatively this phenomenon has been accepted since the 1960s and 70s.[3, 4] From these studies, we know the rodent DG forms in 3 stages. During embryogenesis, an initial proliferative wave of cells migrating from the edges of the lateral ventricles initiates DG formation around P10 (mouse)-14 (rat). Prenatal proliferation peaks P15-18 (mouse). Around birth, neurogenesis is temporarily suppressed; thus the prenatal wave subsides. A second, postnatal wave initiates immediately after parturition. This wave peaks on P0, then gradually declines through the first postnatal weeks. Formation of the DG ectal (suprapyramidal) limb slightly precedes that of the endal

(infrapyramidal) limb. Finally, a constitutive low level of neurogenesis is achieved that is maintained throughout adult life.[3-6]

Adult neurogenesis, however, was not characterized in these initial studies. More recent data show that adult-born cells appear to remain near the inner GCL, even well after division.[50] The rate of adult neurogenesis does vary somewhat with experience[7, 8], and it decreases with age.[9] Though the function of adult neurogenesis is not yet known, it is believed to generate up to 6% of total DG granule cell number per day[10], and the neurons generated functionally incorporate into the adult structure.[11] Thus adult neurogenesis contributes substantially to DG composition, whether by addition or replacement of cells.

These studies clearly undergird our understanding of DG development. However, DG cell layering by birthdate has never been quantified, has never been studied in the context of integrating developmental with adult neurogenesis, and has never been demonstrated within a single brain, in part due to technical limitations. In addition, the original 3H-T labeling of proliferating cells prohibited immunostaining for other cell markers[12], whereas modern techniques permit co-labeling (e.g., bromodeoxyuridine (BrdU)) and enhance visualization of cell morphology (e.g., retroviral delivery of fluorescent protein). Thus further study of layering within the DG seems both possible and appropriate. Integration of developmental and adult neurogenesis observations in a single study could provide a cohesive model for DG genesis.

But beyond layering: is the adult contribution of neurons to the DG interchangeable with the developmental contribution, or are these two populations

distinct? A number of existing studies have sought differences between developmentally born and adult-born neurons. The morphologic and physiologic course of neuronal maturation appear much the same in development as in adult.[13, 14] Electrophysiologically, there seems to be little if any difference between same-aged neurons generated during development vs. adulthood, in terms of membrane potential, current and long term potentiation (LTP).[15] However, there is clearly heterogeneity in molecular marker expression within the DG, both between development and adulthood, and between inner and outer layers of the adult DG.[16]. The location of progenitors contributing to the DG changes dramatically through development – from the lateral ventricle of the embryo, to the tertiary dentate matrix P3-10, to the subgranular zone (SGZ) in adult. Certainly the functional/behavioral consequences to knocking down neurogenesis have been vastly different at adult stages – with findings ranging from no effect[17] to memory deficits[18-20]– than during development – with findings ranging from similar memory deficits[21] and decreased neurogenesis throughout life[22], to vastly decreased brain and body weights and early postnatal death.[23] Thus whether cell fate and function are determined by birthdate is still unknown. However, it is clear that the circumstances under which DG neurogenesis occurs varies considerably with age.

The mouse is a useful system in which to study developmental vs. adult neurogenesis in the DG. Gestation and development are relatively condensed, allowing efficient study of the effects of manipulations during each of these periods in the adult animal. Rodents display spatial and temporal learning deficits after hippocampal manipulations similar to those seen in humans with hippocampal

injury.[24] Both DG morphogenesis and developmental and adult neurogenesis have been characterized extensively within this system. In particular, data concerning the knockdown of adult neurogenesis are currently being characterized in this system[20], and so a complementary characterization of a developmental knockdown would be useful.

The DG is intriguing to study in development vs. adulthood not only because it is a center for NG, but also because of its critical role in hippocampal function.[25] Within the broad spatial/temporal memory domain of the hippocampus, the adult DG appears particularly important for encoding of pattern separation and episodic memory, in the retention of spatial memory, and in metric processing.[24] It is the main input structure of sensory information to the hippocampus. It is believed to condense information coming into the hippocampus into sparse activity codes that are further processed by CA regions.[26] All these functions, as well as the DG structure subserving them, undergo delayed, extended development in both altricial rodents[27] and higher primates.[28] Interpretation of how new neurons incorporated function at any age can only be in the context of DG function in encoding, episodic memory and pattern separation, as this evolves developmentally.

Experiments investigating this issue have important implications for understanding human functioning. This is true because delayed development of the hippocampus is also characteristic of higher primates[28], and because neurogenesis has been demonstrated in the adult human DG.[29] The DG is also a promising candidate target structure involved in numerous neuropsychiatric disorders and sequelae. This follows partly from its key role as gatekeeper of information entering the

hippocampus, which is very vulnerable to traumatic, ischemic, and excitatory insults and their effects on memory. Increased neurogenesis and aberrant connections of the newly generated neurons have both been demonstrated in animal models of seizure[30], and as most epilepsy originates early in life, study of the role of developmental neurogenesis is especially important in this context.

Changes in neurogenesis are also implicated in depression[31-33] and post-traumatic stress disorder (PTSD).[34, 35], both of which can have possible etiologies – and can manifest – in childhood as well as adulthood. Decreased neurogenesis has been shown both in response to stressful experience and to stress hormones[31, 32, 36], and there is even suggestion that early stress [22, 37, 38] or early depletion of serotonin signaling[39] can result in decreased neurogenesis later in life. Such stress is often employed in generating animal models of depression.[40] Increasing serotonin in these models using selective serotonin reuptake inhibitors (SSRIs, as human patients do for depression) rescues both neurogenesis[41] and behavioral deficits[42], at a delayed time course similar to that seen in humans (too slow to be attributed directly to the drugs' direct effects on serotonergic signaling).[33, 42, 43]

Early life stress is known to put the human central nervous system (CNS) at risk for mood and anxiety disorders.[44] It has also been shown to produce hippocampal-mediated learning deficits in association with noted decreases in neurogenesis.[45] And both depression and PTSD have been correlated with decreased hippocampal volume. These findings together suggest that understanding the regulation and function of developmental neurogenesis is relevant to understanding human psychiatric/neurologic disease.

This study investigates several hypotheses related to the differences between postnatal and adult neurogenesis. It seeks:

1. To demonstrate and quantify birthdate-specific layering of cells within the dentate gyrus during embryogenesis, postnatal development and adulthood.
2. To examine the relative fates of embryonic-. postnatal-, and adult-born cells in the dentate gyrus, particularly which cells are dividing at each stage.
3. To assess the effects of knocking down a developmentally born population of cells, on DG anatomy, adult neurogenesis, and compensation/behavioral function, in order to compare these with existing data on the effects of reducing neurogenesis during adulthood.

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CHAPTER 2

# A DISTINCTIVE LAYERING PATTERN FOR DENTATE GRANULE CELLS GENERATED BY DEVELOPMENTAL AND ADULT NEUROGENESIS

**Abstract**

The dentate gyrus (DG) of the hippocampus appears to develop in an "outside-in" layering pattern. This phenomenon has never been quantified, however; nor has ongoing adult DG neurogenesis been integrated into this model. Here, we characterize the relative adult granule cell layer position of cells dividing in the embryonic, postnatal, and adult dentate, using BrdU and retroviral methodologies. Further, we identify both developmental and adult-born cohorts that contribute to the adult-dividing population, one month or more after initial labeling. Our data support an outside-in layering of the DG that continues through adulthood, and suggest a prominent contribution of early-dividing cells to the adult granule cell layer. We also find that a significant fraction of the adult subgranular dividing population has been dividing infrequently since early development. This information supports that cell cohorts dividing early in development contribute significantly to the structure and dividing population of the adult DG.

**Introduction**

The dentate gyrus (DG) of the hippocampus is one of the few mammalian brain structures that continue to generate new neurons through adulthood. Hence it is important to characterize the development of this structure throughout life and to determine what similarities and differences exist between cells formed at different life stages.

Early birthdating studies on the granule cell layer (GCL) of the rodent DG using tritiated thymidine established an outside-in developmental layering that

persists in the adult brain. Specifically, cells born during embryonic development line the outer GCL, and cells born postnatally remain closer to the hilus [1, 2].

These findings were further elaborated in the 1980s and 1990s, when the cell populations involved in DG morphogenesis were characterized[3-8]. At approximately E10 in mouse, cells lining the medial ventricles at the dentate notch, known as the primary dentate matrix, form the initial proliferative zone that will become the DG. Through a series of "dentate migrations," the proliferative zone moves from the ventricles to the hilus as the rodent approaches birth. The proliferative zone in the hilus from birth until approximately 3 weeks postnatal is known as the tertiary dentate matrix [3, 5, 6, 9]. About 85% of all granule cells are generated postnatally [2]. Gradually, the proliferative zone becomes restricted to a region known as the subgranular zone (SGZ), lying just beneath (hilar to) the GCL. Proliferation in the SGZ contributes to the addition of new neurons to the DG throughout the adult life of mammals. Studies of adult neurogenesis in isolation demonstrate that granule cells born in the adult are mostly positioned in the inner third of the GCL [10, 11].

All together, these studies suggest that the "outside-in" layering may also apply to adult neurogenesis in the DG. In other words, new neurons born in the adult may be positioned closer to the hilus compared to those born during embryonic and postnatal development. Such layering from embryogenesis through adulthood has, in fact, been shown in the case of the olfactory bulb, another structure in which neurons continue to be added throughout life [12]. However, no comprehensive study has tracked the location of neurons born from embryogenesis

through adulthood in the DG. To test this layering hypothesis, we labeled cohorts of granule cells born at different developmental stages in the same animals and compared their position in relation to each other.

Here, we labeled embryonic, postnatal, and adult cohorts of dividing cells, using 5'-bromo-2'-deoxyuridine (BrdU) or retrovirus, to elaborate our understanding of DG layering. BrdU labeling permits quantification of the number of cells from a birthdate cohort in any specific cell layer. Retroviral labeling allows lifelong, undiluted labeling of whole-cell morphology and visualization of birthdate-specific cohorts from more than one birthdate in the same brain. Both methods lend themselves to assessment of each cohort's cell phenotype in the adult brain if they colocalize with other molecular markers.

Taking advantage of this ability to colocalize, we sought, in addition, to characterize developmental vs. adult contributions to the pool of adult-dividing progenitors. The few studies that have most recently examined developmental contributions to the DG have focused on developmental contributions to the adult nondividing granule cell population (e.g., [13]). Recent work has characterized Sox2(+) multipotent and self-renewing neural stem cells in the adult DG.[14] Our work seeks to add to these findings by demonstrating that: 1) such multipotent, self-renewing cells infrequently divide from early development through adulthood; 2) they maintain their ability to divide and differentiate, or remain progenitors, over more than 50d; and 3) a large portion these adult stem cells are actively proliferating during embryogenesis and early postnatal life.

**Methods**

BrdU labeling

Female wild-type or Sox2-GFP transgenic mice in C57Bl/6 background were mated with Sox2-GFP C57Bl/6 transgenic males. The date of plug was considered E0.5, and the date of birth was considered P0. Male and female offspring from these pregnancies were injected at 3 time points: embryonic, postnatal, and adult. For embryonic injections, pregnant females received 50 mg/kg of 5'-bromo-2'-deoxyuridine (BrdU) intraperitoneally (i.p.) on E15.5. Only one injection was possible at this time point, due to known teratogenic effects of BrdU on embryos[15, 16]. For postnatal and adult injections, each mouse received 50 mg/kg BrdU twice daily, approximately 8h apart, on P5-7 (postnatal) or P35-37 (adult). Animals were sacrificed at P63 by lethal overdose of a ketamine/xylazine mixture followed by perfusion transcardially with 4% paraformaldehyde. This date of sacrifice was chosen to be one month after the adult (latest) BrdU injection timepoint, so labeled granule cells would have sufficient time to reach maturity [17].

Viral Vectors

Murine Moloney leukemia virus-based CAG-GFP and CAG-RFP vectors used in this study have been previously described [17, 18]. Concentrated retrovirus ($10^8$ pfu/ml) was prepared using HEK293T as packaging cells and was collected by ultracentrifugation [18-20]. Fluorescent protein will be expressed only in cells dividing at the time and location of retroviral injection. This is because retroviral

RNA can only achieve stable expression in cells that are dividing and can convert the RNA to DNA that incorporates into their genome.

Retroviral injections

In utero: Timed-pregnant C57Bl/6 female mice received surgery when embryos reached E15.5. Each female was anesthetized with 10% Nembutal administered intraperitoneally (i.p.). A longitudinal incision was made in the lower peritoneum, through which the intact uterus containing embryos was extracted, without disturbing its integrity or blood supply. The CAG-GFP retrovirus was mixed with 3% fast green dye 5:1 and was injected into one lateral ventricle of each embryo via a micro glass capillary tube inserted through the uterine wall. The diffusion of the dye into the other ventricle confirmed proper injection placement. The uterus was placed back into the peritoneal cavity and the mothers were sutured and allowed to fully recover before they were put back with the colony.

Postnatal and adult: Animals were anesthetized using 80 μg ketamine/8 μg xylazine at 8 μl/g i.p. Stereotaxic injections were performed to deliver the CAG-GFP or CAG-RFP virus to the DG in P5-P7 and P42 mice, as previously described in detail[17, 18]. P42 mice were housed with a running wheel starting 3 days before the injection. Both males and females were included in the analysis.

Immunohistochemistry

After transcardial perfusion, all mouse brains were postfixed with 4% PFA overnight at 4°C, then transferred to 30% sucrose and stored at 4°C until

sectioning. Brains utilized in BrdU experiments were hemisected immediately before sectioning. The right hemisphere was sectioned coronally and the left horizontally. Using a sliding microtome, all brains were cut through the extent of the hippocampus into 80-μm and 40-μm sections for postnatal and adult samples, respectively. Brains labeled with retrovirus were cut coronally in 40-μm thickness.

Sections were immunostained for BrdU (rat anti-BrdU Accurate 1:250), NeuN (mouse monoclonal, Chemicon, 1:100), and Ki67 (rabbit, Novacastra 1:500). Secondary antibodies used were donkey anti-rat Cy3 (Jackson, 1:250), donkey anti-mouse Cy5 (Jackson, 1:250), and donkey anti-rabbit FITC (Jackson, 1:250). After immunostaining, sections were washed 6 times in TBS; DAPI 1:1000 was included in the 2$^{nd}$ of these washes. Sections were mounted on double-subbed slides using DABCO mounting media. Immunostaining was visualized using the Bio-Rad R2100 confocal system with Nikon Eclipse TE300 or Olympus BX51 microscopes and a Zeiss Pascal Confocal microscope. Stereologic counts were performed using a MicroBrightField StereoInvestigator program optical fractionator (below).

Cell Quantification

Quantification of BrdU-labeled cells was performed stereologically. To determine the distribution of cells born at each time point within the DG, the GCL was partitioned into 3 "layers," using the StereoInvestigator program (Microbrightfield, Inc.). The outer borders of the GCL were outlined at 20x using DAPI (4',6-diamidino-2-phenylindole) stain at postnatal or NeuN stain at adult sacrifice time points. Cursor size was then set such that points marked by the

center of the cursor would be placed 20 μm in from the edge of the circular cursor (20-μm radius). The cursor edge was then repeatedly aligned with the edge of the GCL outline to delineate strips 20 μm into the GCL from the inner edge, and 20 μm in from the outer edge of the GCL. In adult animals, this procedure resulted in 3 approximately 20-μm "layers" (one inner, one middle, one outer) through the GCL. In some cases (especially at different ages and at the edges of the dentate), the thickness of the middle "layer" was less than 20 μm; inner and outer layer widths were constant. The numbers of BrdU-positive cells in each layer were quantified. Cells overlapping more than 1 layer were assigned to the layer in which a greater portion of their volume resided.

A single optical fractionator run was performed over the entire GCL for each section; a different marker was used to count cells in each layer. Stereologic counts were performed using grid and counting frame sizes to approximate 10 or more sampling sites per section and 2-5 cells per counting frame and to achieve a sufficiently low coefficient of error (CE Gundersen) between sections. Grid and counting frame sizes for each group were as follows, respectively: postnatal sac P8 (supplement only) grid = 75 $\mu m^2$, c.f. = 15 $\mu m^2$; embryonic sac P63 grid = 100 $\mu m^2$, c.f. = 25 $\mu m^2$; postnatal sac P63 grid = 100 $\mu m^2$, c.f. = 50 $\mu m^2$; adult sac P63 grid = 100 $\mu m^2$, c.f. = 100 $\mu m^2$. Counting frame depth for all 40-μm sections was 10 μm, with a 3-μm estimated guard zone on top and bottom.

Quantification of retrovirally labeled cells was performed by counting all cells in every $12^{th}$ coronal section throughout the septotemporal axis of the

hippocampus. For each section the GCL was divided in thirds and each retrovirally labeled neuron was assigned to the inner, middle or outer layer.

Colocalization was all performed using the Bio-Rad (Hercules, CA) Radiance 2100 laser scanning confocal system. Sequential 2 μm scanning was performed through the entire z-axis of every 12th DG-containing section with 40xoil immersion lens. Optical section thickness ranged from 16-26 μm. All Ki67(+) cells in each DG were identified using only that channel. Subsequently, each was evaluated in the other channels to determine whether it colocalized with BrdU. Evaluation was performed blind to BrdU treatment condition. Colocalization was defined as labeling in 2 channels that appeared to be in the same cell in multiple z planes. For Sox2(+) counts, all double-labeled Sox2(+)/BrdU(+) cells were counted using the confocal z-stack. The total number of BrdU(+) cells and of Sox2(+) cells was determined using the stereologic methodology described above (Sox2 grid = 150 $\mu m^2$, c.f. = 25 $\mu m^2$).

**Results**

*The GCL shows a distinct birthdate-based outside-in layering through adulthood*

To assess the contribution of perinatal and adult neurogenesis to different layers of the dentate GCL, we labeled dividing cells of the embryonic, postnatal and adult DG and studied the distribution of their progenies at later stages, when the cells had reached their mature position in the GCL. We selected BrdU and retroviral labeling techniques to undertake this study due to their distinctive strengths.

BrdU has two main advantages: its administration is non-invasive, and it labels all cells that divide around the time of injection, allowing an accurate estimation of the fate of the entire progenitor cell population [19-22]. However, it also may be diluted and become undetectable in neurons derived from progenitor cells undergoing multiple rounds of divisions [21-23]. Therefore it is unclear whether a decrease in BrdU(+) cells indicates cell death or dilution from repeated divisions. But for cells that are BrdU(+) at the time of evaluation, we can be confident that they have divided only a few times since the time of BrdU administration [21-23].

Conversely, retroviruses only label dividing cells near the site of intrahippocampal injection, and infectivity is highly variable. However, retroviruses are integrated into the host chromosome, thus rendering permanent, undiluted labeling of progenitor cells and all their progeny, regardless of the number of divisions [16, 17]. Retroviral labeling also permits visualization of the entire cell morphology, whereas BrdU immunohistochemistry only reveals the cell nucleus. We have therefore used both methods in the present study, so the results may be viewed in complement and directly contrasted.

BrdU

BrdU was injected in three groups of mice: embryonic day 15 (E15), postnatal day 5-7 (P5-7), and young adult (P35-37). All of these groups were assessed stereologically at P63 to evaluate the contribution of the labeled cells to the adult DG (Figure 1A).

Cells labeled in each successive group showed a distinctive outside-in chronological distribution along the adult GCL (Figure 1A). Cells born during embryogenesis tend to form the outermost layer of the GCL, closest to the molecular layer. Cells born P5-7 localize more towards the middle and inner GCL. Adult-born cells are restricted predominantly to the innermost layer, closest to the SGZ (Figure 1C; EA n=10, PA n=7; AA n=4). As previously documented, extensive proliferation of cells that remain in the hilus occurs postnatally (Figure 1A, middle image) [25, 26].

Quantitatively, embryonically dividing cells clearly make the largest numeric daily contribution to the adult DG, followed closely by postnatally dividing cells, with adult-born cells constituting only a small fraction (Figure 1B). The quantifications of BrdU cell number presented here are only semi-comparable between developmental stages given 1) the single embryonic vs. 3d postnatal and adult injections and 2) the variation in metabolism and placental/blood-brain permeability at these different developmental stages [27]. Nevertheless, these data suggest that the P63 DG contains mainly embryonic and postnatally derived cells. In the adult mouse, there are approximately 300,000 granule cells per unilateral DG [28, 29]. Nearly 60,000 E15-labeled cells per unilateral DG remain visible in the P63 DG, and almost 40,000 labeled by P5-7 injections. By contrast, adult-dividing cells (P35-37) constitute only approximately 3,000 cells per DG at the same time point, only one month after labeling. The number of BrdU(+) cells is significantly different between all groups ($p<0.05$, one-way ANOVA and follow-up Tukey tests).

Retrovirus

As a complementary method to assess DG layering, GFP-expressing retrovirus was injected into E15, P7 and P42 mice, and the distribution of GFP(+) neurons in the GCL was evaluated at P75 (10, 7 or 6 weeks after injection for respective groups) (Figure 2). Neurons born at E15 (Figure 2 A, B) were evenly distributed throughout the inner, middle and outer layers of the GCL, whereas neurons generated at P7 (Figure 2 C, D) and P42 (Figure 2 E, F) were localized predominantly toward the inner and middle GCL, with a marked decrease in the contribution to the outer layer. In particular, about 80% of adult-born GCs remained in the inner layer and the remaining cells were scattered within the middle GCL (Figure 2I, "adult"). Layering of embryonic and adult (Figure 2G) or postnatal and adult (Figure 2H) cells was also visualized within the same brain, using RFP- and GFP-expressing retroviruses injected into the same animal's dentate at different time points.

We infer from the above results a birthdate-based outside-in layering to the DG. However, the perceived inner layering of adult-born cells might be due to an insufficient time post-injection for cells to finish migrating in the GCL. Embryonic-labeled cells, for instance, had 68 days to migrate from labeled division to assessment of their location, whereas adult-labeled cells had less than half that time. Therefore, we evaluated whether adult-born cells would change to a more outer layering if given more time after division to migrate. We labeled neurons born at P42 and analyzed their distribution 7-15 months later (Figure 2I, "old").

We observed no differences in the distribution of granule cells at this later time compared with that of younger cells ("adult" vs. "old"), in agreement with the notion that adult-born neurons reach their position in the GCL within the first few weeks of their development [9, 10]. Hence, cell position in the DG depends on the developmental stage of the animal when a cell is born, and not on the cell's age.

*Cells dividing early and infrequently constitute much of the adult-dividing population*

Co-expression of dividing cell markers

A subpopulation of BrdU(+) cells in the GCL and SGZ from all injection time points was found to coexpress Ki67 (a marker of actively cycling, non-$G_0$ cells) and Sox2 (a progenitor cell marker) (Figure 3B, C). In contrast to some previous findings [23], this finding indicates that these cells divided in the embryo, in the neonate, or in the adult animal and are still actively dividing one month or more later.

In fact, cells that divided early in life make up a significant fraction of mitotic cells in the adult (Figure 3D, Table 1). One-way ANOVAs show that the number of BrdU and Ki67 co-labeled cells and the number of BrdU and Sox2 co-labeled cells are all significantly different ($p<0.05$) between injection timepoints. Post-hoc Tukey tests revealed significant differences between all groups. By contrast, the total number of Ki67(+) cells and Sox2(+) cells in the adult brain did not differ significantly between groups ($p>0.05$). The GCL volume of embryonic BrdU-injected animals did differ statistically from that of animals injected

postnatally or in adulthood, which may be attributable to the teratogenic effects of BrdU.[14, 15]

If 3.5% of the approximately 50,000 cells born on E15 are Sox2(+) on P63, then 1,750 embryonic-born cells constitute progenitors in the adult DG, almost 3% of the adult Sox2(+) population (estimated at 60,000 cells/unilateral DG, see Table 1). Postnatally dividing cells would constitute an even larger 5,000 cells, about 8% of the adult Sox2(+) population. In contrast, adult-dividing cells, some of which could in fact be a subset of those dividing on E15 or P5-7, constitute only around 0.2% of the adult Sox2(+) population.

These data likely underestimate the early contribution to the adult-dividing cell population, because BrdU can only be visualized in cells that have divided fewer than 9 times in vitro [30] or 4 times in vivo [23]; after this, BrdU is diluted beyond the detectable limit of immunohistochemistry. Thus our observation of BrdU label in any adult-dividing cells at all suggests that the remaining BrdU(+) cells have divided only a limited number of times between early development and adulthood. Such limited division supports the existence of infrequently dividing "stem" cells within the SGZ of the DG.

Co-localization of retrovirus

Retrovirus labels far fewer cells per DG than BrdU (highly variable infectivity; 200-800 cells labeled per DG per E15 injection). For this reason, double retrovirally labeled cells are rare when retrovirus is introduced at distant time points, as are retrovirus/Sox2 double-positive cells. However, we found

examples of each of these in P75 brains retrovirally labeled at E15 and P5, at E15 and P45, and at P5 and P45 (Figure 4).

These double retrovirally labeled cells demonstrate that cells in the DG divide again as much as 50d after an initial (labeled) prenatal or early postnatal division. Co-expression of Sox2, a progenitor cell marker, in early retrovirus-labeled cells supports long-surviving stem-like progenitors in the SGZ. In addition, these double-labeled cells demonstrate that such progenitors – dividing multiple times as much as 50d apart – are still capable of forming mature neurons integrated into the GCL 1 month later (Figure 4A, note the morphology and co-labeled dendrites of the retroviral double-labeled granule cells), as well as progenitor cells (Figure 4B, note morphology and Sox2 colocalization). Radial glial morphology was even observed in a postnatal retrovirally labeled cell co-expressing Sox2 in adulthood (Supplement 2). Such observations suggest stem-like abilities to differentiate and self-renew in these infrequently dividing progenitors.

**Discussion**

Data presented here confirm that a cell's birthdate affects its eventual status within the adult DG in several ways. First, consistent with earlier work, several methods demonstrate that cell birth date correlates with subsequent location within the GCL. Early-born cells layer to the outside (closer to molecular layer) compared with later-born cells (closer to hilus). Second, we show that early-proliferating, infrequently dividing cells constitute a considerable fraction of adult-dividing cells in the DG. These findings suggest several new ways to examine

whether developmental and adult neurogenesis are interchangeable or distinct processes, and hence whether increasing later adult neurogenesis can compensate fully for deficits in developmental neurogenesis.

We used two complementary methods to examine developmental and adult proliferation and layering. This proved advantageous, as each permitted us to examine different aspects of the fate of labeled cells. Using BrdU, we were able to take advantage of its quantitative, comprehensive labeling properties, as well as its known dilution after 4-9 cell cycles, which permitted us to see an infrequently but continually dividing population of cells. Using retrovirus, we were able to follow early-born cells without dilution of label in the adult and, using multiple fluorophores, we were able to see the adult morphologic fate and layering of more than one proliferating population in the same brain.

Both of these methods yielded strong support to previous anatomic findings [1, 2, 12, 31] that the GCL has an outside-in birthdate-based layering. The functional consequences of these anatomical differences are presently unclear. It is known that the medial entorhinal cortex projects preferentially to the outer molecular layer, whereas the lateral entorhinal cortex projects predominantly to the inner molecular layer [32-36]. It is possible that these segregated projection patterns extend to the GCL. If so, cells with different birthdates may preferentially receive different cortical inputs, although there is some existing evidence that such inputs can also converge on the same cells [32]. Birthdate-related topography raises the possibility of differences in cell function based on birthdate [37]. So far, only small differences have been shown between early- and adult-born cells, in

terms of electrophysiologic and cell morphologic properties [18]. Determining whether developmental- and adult-dividing populations differ in terms of their connectivity will be critical to determining whether these processes are interchangeable or distinct processes.

Our postnatal BrdU-labeled cell numbers in the adult DG considerably exceed the estimates of Muramatsu et al. using similar methodology [12], likely reflecting the fact that Muramatsu et al. assessed at 6 months whereas we assessed earlier, at 63d. Comparing our data to their results, considerable cell death and/or BrdU dilution seems to occur between 2 and 6 months, consistent with the findings of Dayer et al.[23] This reduction in BrdU certainly could result from label dilution in the high number of early postnatal-labeled cells that continue to divide through adulthood. Indeed, in our estimates, up to 12.5% of early postnatal-dividing cells continue to divide infrequently as progenitors through adulthood, and more may divide at a higher frequency (undetectable in adulthood by this methodology). In contrast, adult-dividing cells labeled with BrdU persist in the DG at relatively constant levels from about 4 wks to 11 months.[9, 23] This lack of significant BrdU dilution in adult-labeled cells is consistent with our findings of far fewer adult-dividing than postnatal-dividing progenitors.

In comparing results from BrdU and retrovirus experiments, it is evident that the percentage of labeled cells layered to the inside is considerably less after BrdU (Figure 1C) than after retroviral (Figure 2I) labeling in the embryonic- and postnatal-injected groups. We hypothesize that this difference is also due to BrdU dilution in cells continuing to divide in inner layers; such dilution does not occur in

retrovirus-labeled cells. Therefore, comparing BrdU to retroviral labeling gives us an even clearer idea of what becomes of E15-born cells. From undiluted retroviral measures, we determine that 30% of E15-dividing cells remain in the inner third of the GCL (includes SGZ). Ten percent of these divide few enough times that the BrdU label can still be seen in adulthood (Figure 1C): 3.5% as Sox2(+) progenitors (Table 1); the remaining 6.5% neurons or glia that differentiated after few enough divisions to not lose their BrdU label. The remaining 20% of inner-layering E15-rv-labeled cells must constitute a combination of proliferative cells whose label has been diluted beyond visibility, and differentiated cells formed from such invisible (unlabelled?) precursors.

This work presents a within-subjects demonstration that cells that divide during early development can continue to divide in the adult. Several different methods of birthdating and several markers of ongoing division support this conclusion. Importantly, the lack of BrdU dilution in early-labeled, adult-dividing cells (Figure 3) points to the existence of a population of infrequently dividing SGZ cells that continue to divide and differentiate through adulthood. These cells must have divided fewer than 9 times [30] between E15 and P63 – less often than once a week – and are still dividing in the adult. The number of such cells is not insignificant, as a single embryonic BrdU injection labels over 5% of the adult-dividing population (Table 1). These data certainly support the existence of a stem-like proliferative population that defies the previously proposed steady-state 12-25h cell cycle model for the DG [22, 38, 39], and although such cells also exist in the subventricular zones, their perpetual detectability in the DG – from all time

points of initial division – suggests the steady presence of a stem population in the DG rather than restricted progenitors regularly migrating in from the SVZ [40, 41].

We do not show that early birthdate instills any fate bias predisposing cells to be "stem-like." This conclusion is beyond the scope of the current study because 1) our methods cannot measure the fate of early-born cells with diluted BrdU, and 2) as a percentage of total cells birthdated, the fraction of labeled cells dividing is actually higher for the adult-dividing group (1.86%) than for the embryonic (0.171%) and postnatal (0.542%) groups (Table 1).

It is clear, however, that early-labeled cells constitute a greater proportion of the adult SGZ dividing population than adult-labeled cells. Cells labeled postnatally (P5-7) are over 4 times more likely to be dividing in the P63 adult 2 months later, than cells labeled in adulthood (P35-37) are to be dividing in the P63 adult 1 month later. Again, this suggests that developmental and adult neurogenesis are not fully interchangeable processes. Clearly, many more future DG adult progenitors are proliferating during early postnatal times than during adulthood. Does that mean that a developmental insult to proliferation will damage future dividing populations more extensively, or more chronically, than a similar insult to the adult? Will adult neurogenesis be fully able to compensate for the effects of such a developmental insult? Current data concerning these questions are conflicting [42-45;] and further exploration is needed.

Addressing the roles of cells dividing at different times in the DG enhances our understanding of the developing brain. In addition, this information will help determine whether early developmental neurogenesis figures distinctly in the form

and function of the adult DG. Determining the contributions of early developmental cohorts to the adult DG may help to reveal which properties of adult-born neurons make lifelong neurogenesis useful and evolutionarily conserved. It remains to be determined what functional role the lifelong addition of cells to the innermost layer of the DG might play in maintaining optimal DG function within the adult brain.

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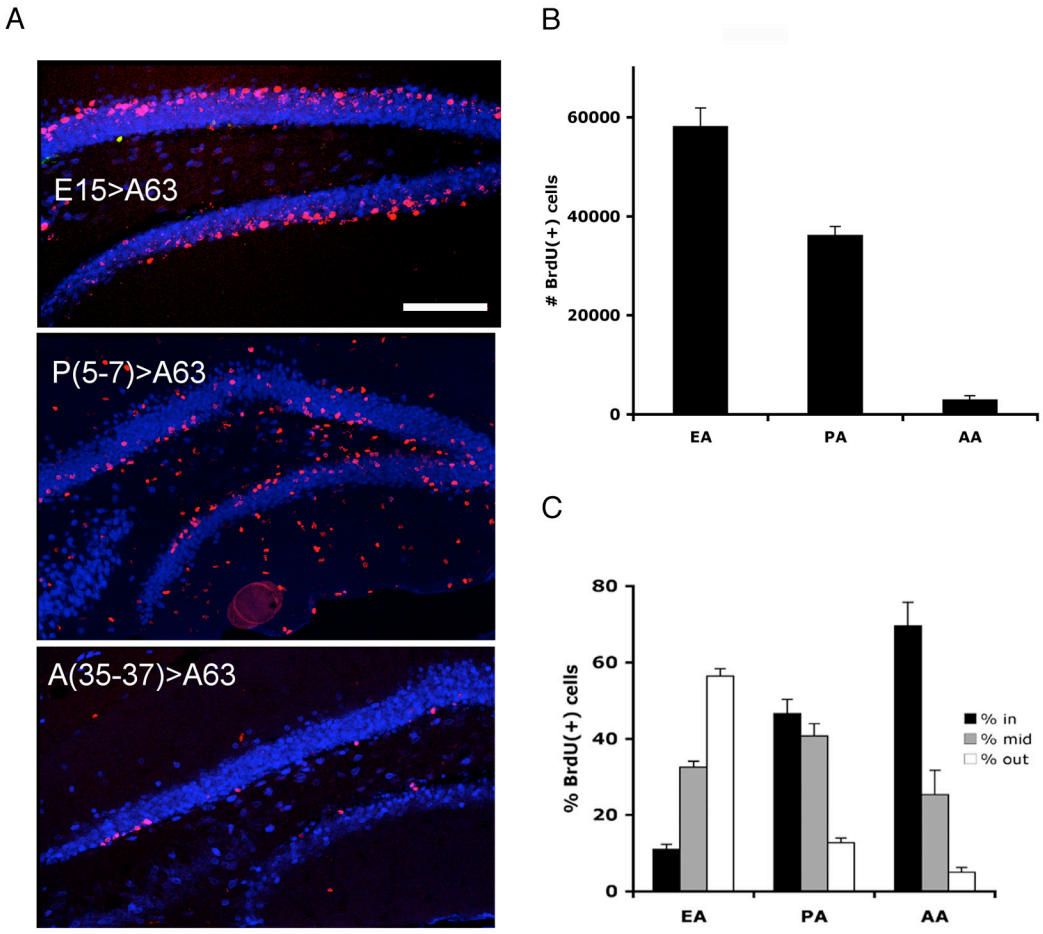



Figure 2.1

BrdU labeling demonstrates outside-in layering of cells from embryogenesis through adulthood in the adult mouse brain
A. Qualitative demonstration of birthdate-based outside-in DG layering. Confocal images (left) illustrate preferential localization of embryonic-born cells (top) to the outer GCL (near molecular layer), postnatal-born cells to middle to inner locations (mid), and adult-born cells to the innermost (hilar-most) GCL.
B. Total number of BrdU(+) cells per unilateral DG in P63 animals injected with BrdU at embryonic (E; 1 injection 50 mg/kg E15.5), postnatal (P), or adult (A) time points.
C. Percent of total BrdU(+) cells found in the inner, middle, and outer layers as a function of BrdU injection time point (same as in B). Red: BrdU; blue: NeuN.

Figure 2.2
Retroviral labeling of granule cells of the adult mouse reveals an outside-in layering based on birthdate. A-F, Overview of a transverse sections of the adult DG showing the distribution of GFP+ neurons (green) retrovirally labeled at E15 (A, B), P7 (C, D), or adult age (E, F). Images are merges of 16-20 confocal planes. The GCL was labeled by immunohisto-chemistry for the neuronal marker NeuN (blue). Scale bars: 100 mm (A,C,E) and 20 mm (B,D,F). G, Granule cells born during embryonic development (E15) labeled with a retrovirus expressing GFP (green) coexist in the same section with granule cells born during adulthood, retrovirally labeled with RFP (red). H, Double retroviral labeling of neurons born in the early postnatal (GFP) and adult hippocampus (RFP). Scale bars: 50 mm. Images are merges of 43 (G) and 60 (H) confocal planes taken from 13-week-old mice brains. I. Distribution of granule cells born at different ages, normalized to the total number of GFP+ neurons in the GCL. Data are mean ± SEM. N = 775 cells from 3 mice injected at E15 and counted 10 weeks later; 691 cells from 3 mice injected at P7 and counted 7 weeks later; 537 cells from 3 mice injected at P42 and counted 6 weeks later (adult); 227 cells from 3 mice injected at P42 and counted 7-15 months later (old).

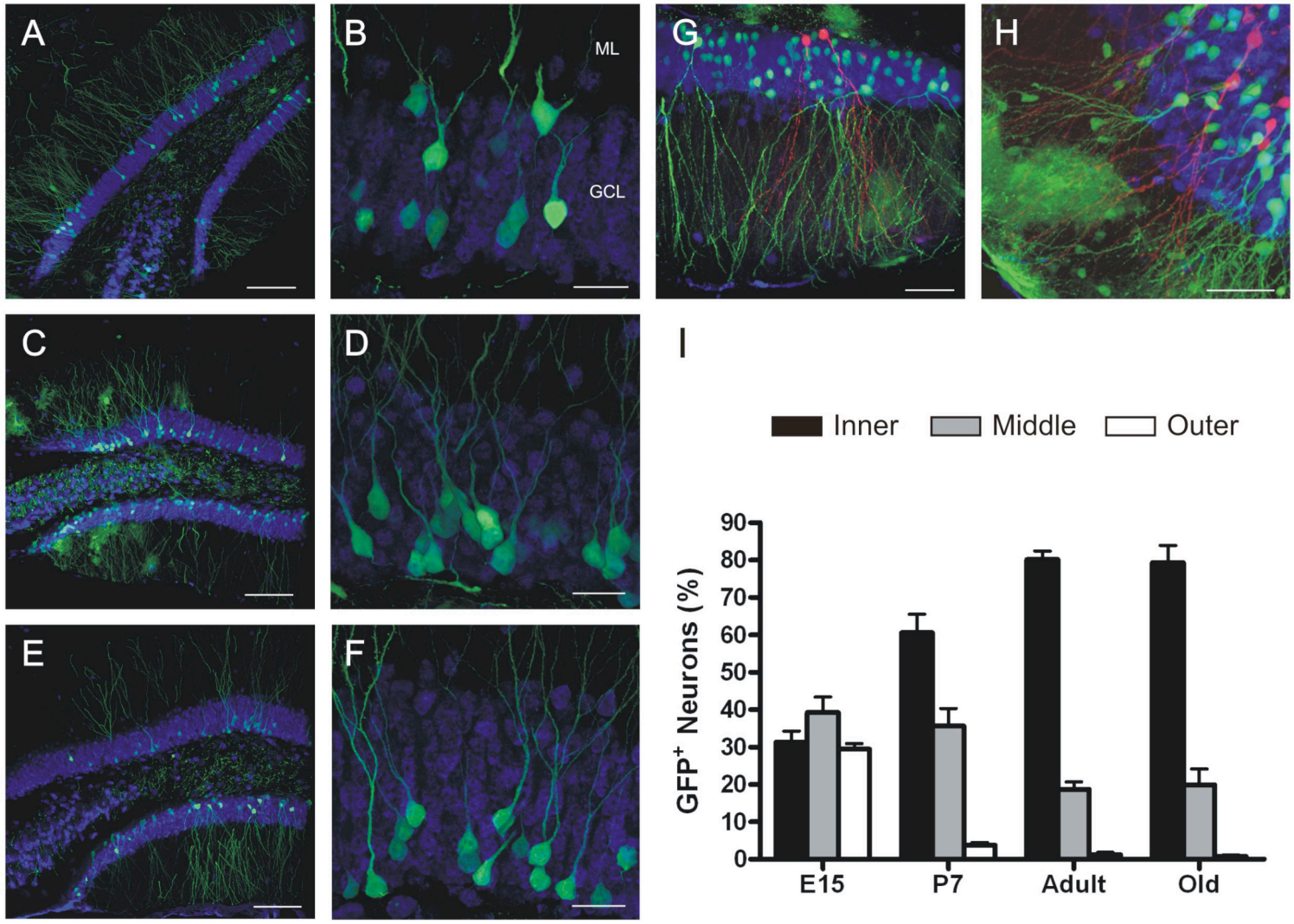

A
B
ML
GCL
G
H
C
D
I
Inner
Middle
Outer
E
F
GFP+ Neurons (%)
90
80
70
60
50
40
30
20
10
0
E15
P7
Adult
Old

Figure 2.3
BrdU labeling from embryonic and postnatal time points persists in dividing cells of the adult mouse DG
A. Schematic illustrating how the experiment demonstrates cells dividing in early life and adulthood. Cells labeled with BrdU (red) presumably underwent S phase of mitosis (indicated by curved arrow) at the time of BrdU injection - during embryogenesis (top), postnatal life (middle), or adulthood (bottom). Cells labeled with Ki67 or Sox2 (green) were actively cycling at the time of assessment (P63).
B. Embryonic BrdU is incorporated into comparatively few inner-GCL cells. However, these inner-located SGZ cells are the ones among the embryonically labeled cells that colocalize with markers of ongoing division in the adult.
C. Cells labeled with BrdU (red), presumably undergoing the S phase of mitosis during embryogenesis (left), postnatal life (middle), and adulthood (right), continue to divide in the adult animal 1 month or more after labeling, as evidenced by the co-labeling of individual cells in the SGZ with BrdU and the cell cycle marker Ki67 (green, top row), and the neural progenitor marker Sox2 (green, middle row) at a common adult time point (P63). Triple-labeled cells, presumably progenitors actively in cell cycle at the time of sacrifice, co-express BrdU (red), Sox2 (green), and Ki67 (blue). These channels can be viewed individually in Supplemental Figure 1.
D. A greater number of cells in the adult-dividing population divided (i.e., was BrdU-labeled) at early, than at adult, time points. The graphs represent data from Table 1 in columns BrdU/Ki67 (left) and BrdU/Sox2 (right), respectively. The fact that BrdU is not diluted from these still actively dividing cells suggests that they have divided only a few times between labeling and adult assessment, as would an infrequently dividing stem-like progenitor.

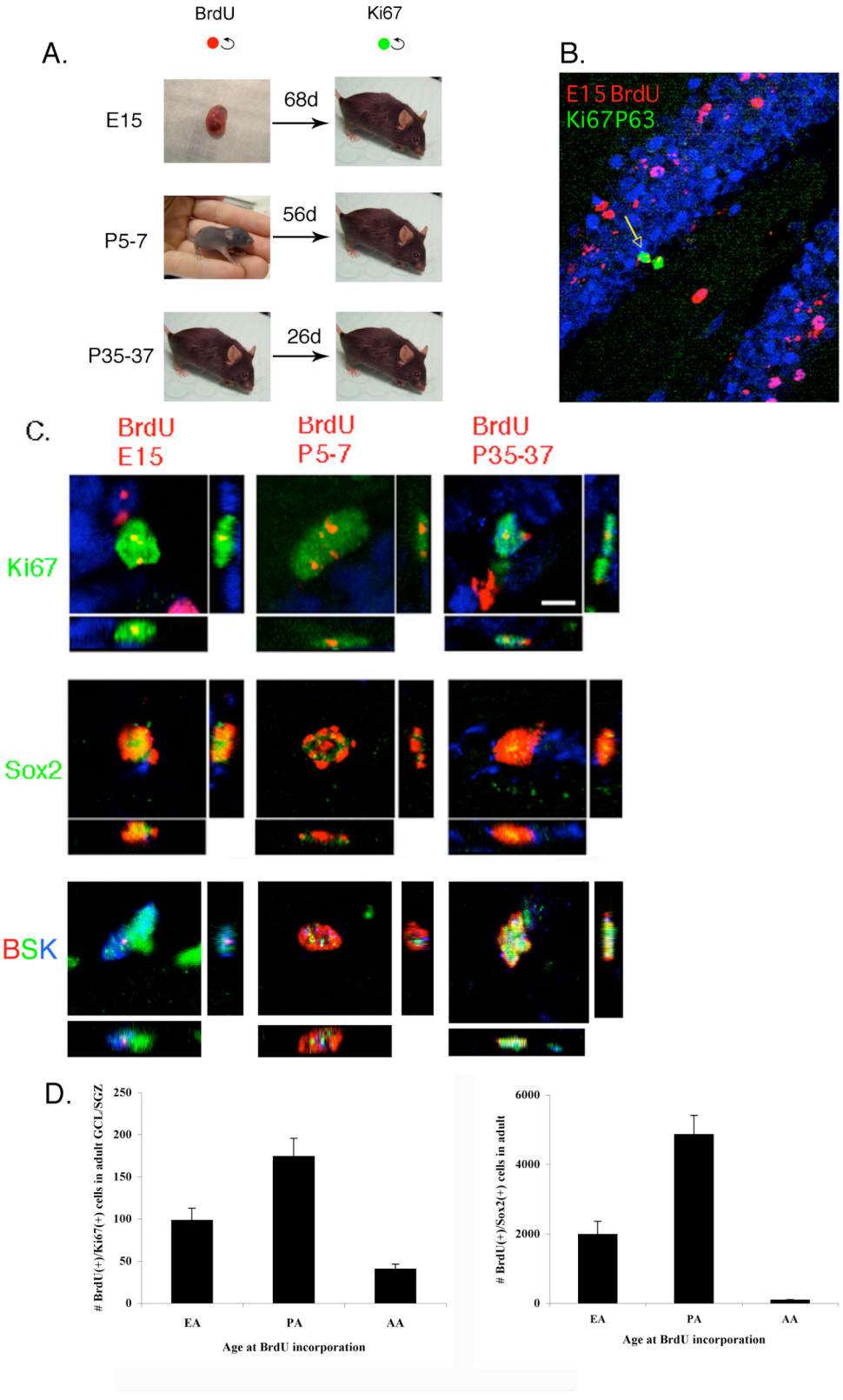
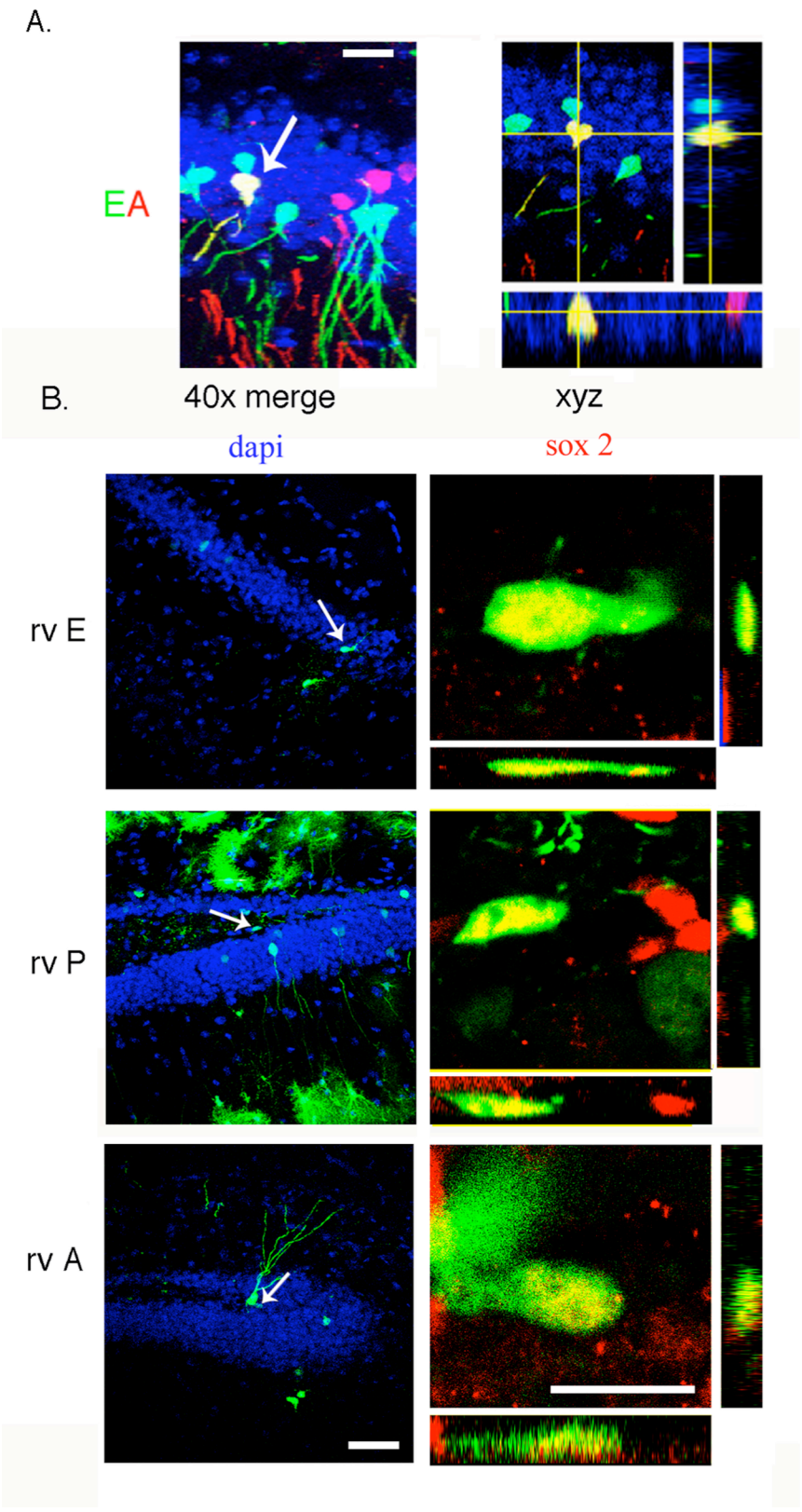
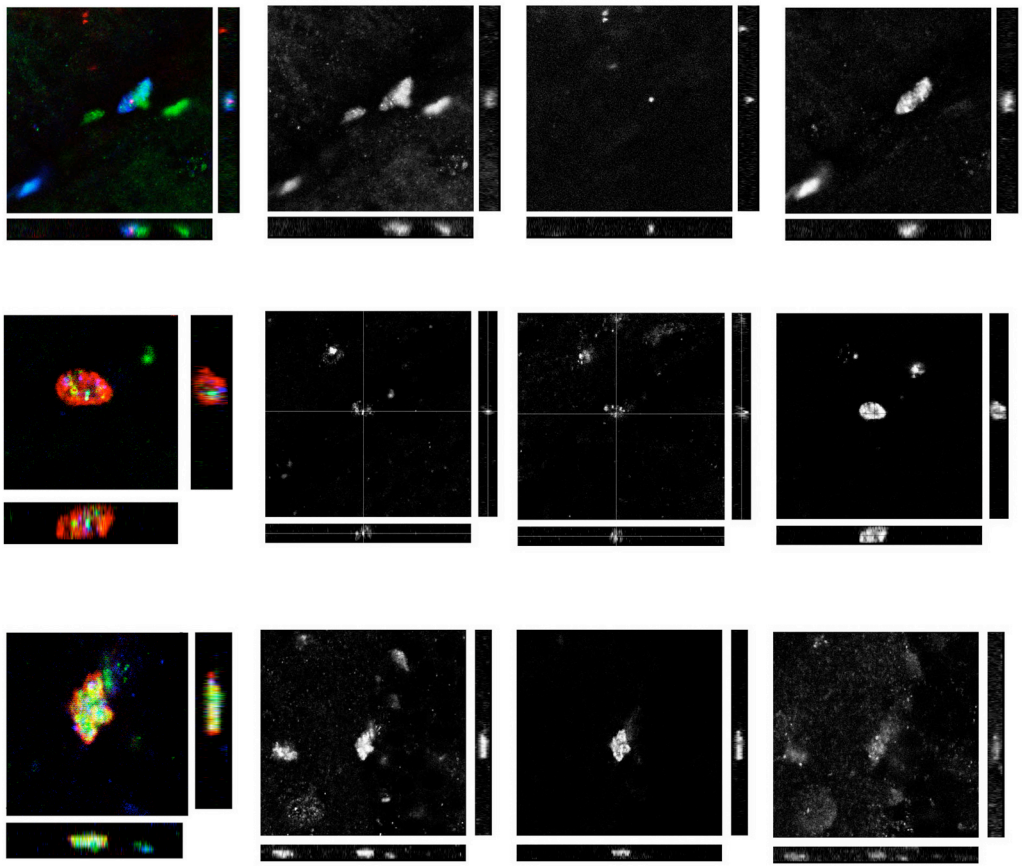
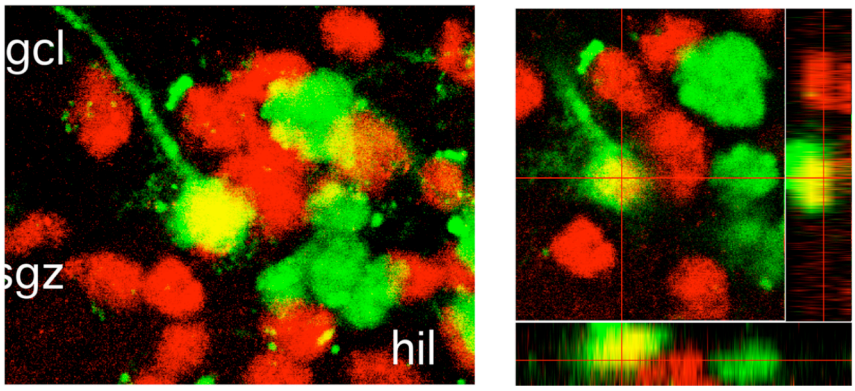

A.
BrdU
Ki67
E15
68d
P5-7
56d
P35-37
26d
B.
E15 BrdU
Ki67P63
C.
BrdU
E15
BrdU
P5-7
BrdU
P35-37
Ki67
Sox2
BSK
D.
# BrdU(+)/Ki67(+) cells in adult GCL/SGZ
250
200
150
100
50
0
EA
PA
AA
Age at BrdU incorporation
# BrdU(+)/Sox2(+) cells in adult
6000
4000
2000
0
EA
PA
AA
Age at BrdU incorporation

Figure 2.4
Retroviral colocalization in adult mouse DG illustrates chronically dividing cells
A. Retroviral double-labeling serves as further evidence that cells dividing early continue to divide more than a month after the initially labeled division. An embryonically dividing cell (labeled with GFP rv E15.5) divided again in the adult (labeled with RFP rv P45). Hence RFP and GFP colocalize in the depicted cell in an animal sacrificed at P75. Note colocalized fluorescent proteins in the dendrites of this morphologically mature neuron, suggesting that a cell dividing 50d after its initial embryonic division was capable of differentiating into a granule cell incorporated into the GCL. Examples of rv colocalization from all combinations of retroviral injection time points were identified (embryonic and postnatal, postnatal and adult, not shown), but the occurrence of retroviral double-labeling overall is very rare. Scale bar = 20 µm.
B. Retroviral colocalization with Sox2 underscores that early-born cells continue to divide in the adult. These double-labeled cells localize to the SGZ and exhibit both nonradial (depicted here) and radial (supplement 2) morphology. GFP rv was introduced at either E15.5 (top), P5 (mid) or P45 (bot). Left: scale bar 50 µm, GCL labeled using dapi (blue). Right: scale bar 10 µm, sox-2 labeled red (Cy5) on same section shown on left.


A.
EA
B.
40x merge
xyz
dapi
sox 2
rv E
rv P
rv A



Supplement 2.1
Adult proliferative markers colocalize with BrdU from all timepoints in DG development, as visualized in individual channels
Data from Figure 2.3C (Column 1) represented as individual channels collected from the confocal microscope. Column 2 is Sox2 (green); Column 3 is BrdU (red); Column 4 is Ki67 (blue).




Supplement 2.2
A postnatal-dividing cell expresses Sox2 and radial glial morphology in the adult DG
GFP-expressing retrovirus was injected into the DG on P5 (green). The animal was sacrificed at P75, stained immunohistochemically for Sox2 (red), and assessed for GFP(+) cell morphology. In this SGZ cell, GFP that indicates early postnatal division colocalizes with Sox2 and radial glial morphology, indicating ongoing adult division.

Table 2.1
Of adult proliferative cells, more are birthdated from embryonic and postnatal life than from adulthood

Data represent the mean (row 1) and standard error of the mean (SEM, row 2) of data collected for n=4 or 5 brains.
Column 1 indicates the total number of BrdU(+) cells stereologically estimated in the P63 unilateral GCL.
Column 2 indicates the number of Ki67(+) cells in the P63 unilateral GCL. This approximates the number of actively cycling cells in the adult brain and does not vary significantly between groups.
Column 3 indicates the number of cells co-labeled with BrdU and Ki67. Note that a considerable fraction of the adult-dividing cells (Ki67(+)) are labeled (BrdU(+)) during postnatal and embryonic development.
Column 4 indicates the stereologic estimate of the total number of Sox2(+) cells in the P63 unilateral DG. This approximates the adult DG progenitor population.
Column 5 indicates the number of cells co-labeled with BrdU and Sox2. Note that a large proportion of the adult neural progenitor cells (Sox2(+)) is labeled (BrdU(+)) during postnatal and embryonic development.
Column 6 indicates the stereologic estimate of the total GCL volume. The embryonically injected group (EA) has a significantly smaller volume in adulthood, perhaps due to the teratogenic effects of BrdU.
Column 7 indicates the percentage of the total BrdU-labeled population that is actively dividing in the adult brain (Ki67(+)), calculated as (Col 3)/(Col 1)*100 for each animal, and the value displayed is the mean of the individual percentages. Note that early-dividing cells constitute a greater number, though a smaller percentage, of the adult dividing population compared with adult-dividing cells, even after a longer opportunity for BrdU dilution.
Column 8 indicates the percentage of the total BrdU-labeled population that is part of the adult brain progenitor population (Sox2(+)),calculated as (Col 5)/(Col 1)*100 for each animal, and the value displayed is the mean of the individual percentages. Note that a greater percentage of the early-dividing population becomes adult progenitors.

| | BrdU | Ki67 | BrdU&Ki67 | Sox2 | BrdU&Sox2 | GCL volume | % BK/B | % BS/B |
|---|---|---|---|---|---|---|---|---|
| **EA** | $5.79 \times 10^4$ | 1699.2 | 98.4 | $7.43 \times 10^4$ | 1983 | $2.78 \times 10^8$ | 0.171 | 3.51 |
| EA SEM | $.673 \times 10^4$ | 110 | 14.5 | $.627 \times 10^4$ | 380 | $.104 \times 10^8$ | 0.0206 | 0.758 |
| **PA** | $3.71 \times 10^4$ | 2338.8 | 193.2 | $5.67 \times 10^4$ | 4860 | $3.08 \times 10^8$ | 0.542 | 12.5 |
| PA SEM | $.287 \times 10^4$ | 236 | 31.1 | $.702 \times 10^4$ | 553 | $.116 \times 10^8$ | 0.109 | 0.68 |
| **AA** | $.293 \times 10^4$ | 1692 | 40.5 | $6.85 \times 10^4$ | 90 | $3.15 \times 10^8$ | 1.86 | 3.44 |
| AA SEM | $.0939 \times 10^4$ | 219 | 5.92 | $.465 \times 10^4$ | 16.5 | $.130 \times 10^8$ | 0.751 | 0.689 |

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CHAPTER 3

# EARLY POSTNATAL DEPLETION OF NESTIN-EXPRESSING CELLS LEADS TO STRUCTURAL AND FUNCTIONAL DEFICITS IN THE ADULT DENTATE GYRUS

**Abstract**

An important part of characterizing the adult dentate gyrus (DG) is to determine the contribution of cell populations dividing early in development to its ultimate structure, function, and adult neurogenic capacity. In this study, we ablate an early postnatal-dividing cohort of DG cells, by injecting gancyclovir (GCV) into neonatal mice expressing herpes simplex virus thymidine kinase under the nestin promoter (NTK mice). NTK mice treated with GCV on postnatal days 5-9 (P5-9) show a 50% decrease in DG granule cell number and volume compared to wildtype littermates. 13 weeks later, these mice continue to exhibit decreased cell proliferation as adults. They also show hippocampal-dependent memory retention deficits in adulthood, but not deficits in olfactory, locomotor, or anxiety measures. These findings support the specific proposal that early postnatal neurogenesis uniquely contributes to adult hippocampal structure and function.

**Introduction**

The dentate gyrus (DG) of the hippocampus is a unique developmental structure in several ways. First, it is one of few brain structures to develop largely after birth. Eighty-five percent of rodent dentate granule cells are generated after parturition [1, 2]. The rodent DG also does not achieve its adult structure, with a fully defined granule cell layer (GCL) and subgranular proliferative zone (SGZ), until about 3 weeks of age [1]. Second, the DG SGZ is one of two brain regions exhibiting ongoing neurogenesis throughout adult life

[2-6]. This discovery has led to extensive study of adult DG neurogenesis and the DG progenitors that possess the lifelong ability to form new neurons.

Recent data has shown that a significant fraction of these chronically, infrequently dividing adult progenitors were previously proliferating in the early postnatal brain (Chapter 2). We also see that early postnatal-born cells layer distinctly, inside of embryonically generated and outside of adult generated cells within the GCL (Chapter 2). These observations lead us to ask: what is the role of early postnatal proliferation in the formation of the adult DG and its progenitors?

Existing evidence suggests that early postnatal DG cell proliferation may differ from proliferation during embryogenesis or adulthood. Although electrophysiologic properties of cells born at different stages of development appear similar [7, 8], the environments in which they mature at each stage are quite distinct [1, 9]. The site of DG cell proliferation changes from embryogenesis (a series of migrating germinal matrices) to postnatal life (a hilar proliferative zone called the tertiary dentate matrix) to adulthood (the SGZ) [10-12]. The amount of proliferation occurring at each stage and site differs dramatically [1, 6, 9, 13-15]. Cells created in these various proliferative sites also seem to have differing final positions within the adult DG. Cells born in the early DG layer to the outside of the GCL, implying different migratory properties from later-born cells, which layer progressively to the inside of the DG irrespective of migration time [13-16]. Earlier-born cells also seem to constitute a large proportion of the adult-dividing population [15]. This supports the

possibility that cells born at different phases may differ in their functions in the adult brain.

Analogy also suggests that early-born and late-born populations in the DG may be distinct. The other brain structure that receives new cells throughout life – the olfactory bulb – displays a birthdate-based layering similar to that in the DG, and in this structure, postnatal- and adult-born cells have different structural and functional properties [17].

One clear way to explore this question is to knock down early postnatal neurogenesis and assess the effects on the adult brain. Existing literature has pursued both postnatal and adult knockdowns of neurogenesis. In general, adult neurogenesis appears capable of full recovery after it is reduced. It is unclear, however, whether postnatal neurogenesis is able to recover. Behaviorally, adults have shown deficits during a period of reduced cell proliferation, while animals with early postnatally reduced proliferation show variable recovery of normal behaviors in adulthood. Specific results and methodologies of these studies are considered below; we consider "postnatal" animals to be 3 wks old or younger (pre-pubertal).

To knock down postnatal neurogenesis, past studies have used two methods, with conflicting results. The first is the injection of methylazoxymethanol (MAM), a DNA-methylating agent that kills proliferating cells. Different timing and duration of postnatal knockdown using MAM have yielded varying DG structural deficits. An early knockdown (MAM P0-1) produced rats with an 18% reduction in hippocampal weight on P60 [18]. As

adults, these rats also showed reduced body weight, ataxia, and increased locomotor activity [18]. However, in a more chronic model (MAM P3,5,7,9), decreases in DG volume and cell number were completely compensated between P16 and P90 [19]. Correspondingly, no behavioral deficits in water maze learning were observed in this group [19]. In both these studies, it must also be considered that effects seen after MAM administration are far from specific to decreasing early postnatal proiferation, both because MAM methylates RNA, and because it is a known teratogen, carcinogen and neurotoxin [20-24].

The second postnatal knockdown method that has been used is irradiation. Animals irradiated postnatally experienced chronic structural deficits into adulthood, including decreased hippocampal weight, neurogenesis, DG volume and cell number [25-27]. Behavioral tests showed accompanying abnormalities in adult learning, memory and anxiety [25-27]. The results of existing irradiation studies, therefore, conflict with the MAM studies already mentioned. And as with MAM, irradiation is far from a clean, specific knockdown method, as it too is teratogenic [28, 29], and produces long-lasting changes in the brain, including white matter necrosis [30, 31], long-term inhibition of neurogenesis, and inflammatory changes [27, 32]. Hence it remains unclear whether or not deficits in DG cell genesis during a defined early postnatal can be compensated later in life.

Results of adult neurogenesis knockdowns have also been mixed. Most report at least some ability of the adult DG to recover neurogenesis following adult-dividing cell ablation. By the time the animal reaches 6 weeks of age, one

study claims that the rat subventricular zone is able to fully recover its proliferation within 30d after irradiation [33]. Another study, however, reports that irradiating 8-wk-old mice produces a decrease in proliferation and Barnes maze learning deficits that persist 3 mo. later [34]. Functional deficits after reduction of adult neurogenesis have generally been subtle – evident only in specific hippocampal-dependent tasks [35, 36], and long-term memory retention [37, 38]. One adult hippocampal-specific irradiation model even shows improved working memory [39]. The use of transgenic models offers increased spatiotemporal specificity to these adult knockdowns, and a more complete picture of the function of adult neurogenesis.

The Nestin-Thymidine Kinase (NTK) mouse [40] was created to study adult neurogenesis, using the neural progenitor-specific promoter Nestin [41-43] to drive the constitutive expression of herpes simplex virus thymidine kinase. This enzyme converts the drug gancyclovir (GCV) to a lethal nucleotide analog that kills cells undergoing mitosis. Thus nestin-expressing progenitors of the DG that are dividing at the time of GCV injection should specifically be killed by this treatment in transgenic (NTK), but not wildtype (WT), littermates.

Here, we use the NTK transgenic model to reduce postnatal neurogenesis with greater temporal and progenitor-specificity. We administer GCV P5-9, then allow mice to develop undisturbed to adulthood and test them alongside similarly treated WT littermates, to assess the structural and behavioral effects of reducing postnatal proliferation.

GCV is a more selective method of dividing cell ablation than previous MAM or irradiation. GCV is not teratogenic in normal mice [44]. It should react specifically with cells expressing thymidine kinase AND dividing at the time of GCV treatment [45, 46]. The only expected systemic toxicity (outside the brain) would be to other dividing, nestin-expressing cells not in neurogenic areas. Immediately after treatment, it is clear that other affected locations include the gut [47] and hair follicles [48]. We chose a timing and duration of treatment at which animals survived and did not show differences in weight or fur appearance in adulthood.

We introduced GCV during postnatal days 5-9 to study the effect of selectively ablating DG progenitors postnatally. Although by P5 postnatal neurogenesis has subsided to about half its maximum level [13], we chose this timepoint because it is the time when nestin expression becomes restricted largely to DG and ventricular progenitors. Prior to this time, extensive cerebellar expression could produce confounding knockdown effects [49]. A large contribution to the rodent DG is still forming at this time: 50,000 granule cells are added per day between P5 and 8 in the rat [14]. This is also the period during which afferent projections from the entorhinal cortex are contacting the forming DG infrapyramidal blade [50]. Therefore we chose this critical early timepoint to assess the effects of knocking down early postnatal DG proliferation on adult DG structure, neurogenesis, and function.

**Methods**

Subjects

C57 background Nestin-thymidine kinase (NTK) mice were generated by Wei Deng [40]. For the present study, NTK transgenic females were bred to wild type C57 males, as NTK males are sterile. Offspring from these litters were used as experimental animals or as female breeders for experimental animals from subsequent generations.

For the core anatomic and behavioral assessments, male NTK and WT littermates were used. Ten NTK and 18 WT animals were tested for novel object recognition, rotarod, hidden cookie, and Morris water maze; only 7 NTK and 10 WT underwent the remainder of the tests. Anatomy from Figures 2 and 3 represents the animals used in behavioral testing. For the rescue experiment, the same animals were used in WT and NTK groups. In WT-rescue and NTK-rescue groups, n=3/group.

Drug administration

Experimental litters were injected with gancyclovir (GCV), 100mg/kg intraperitoneally (i.p.), by an investigator blind to genotype for 5 consecutive days, beginning on P5. Pups were removed for injection for less than 1 minute to minimize confounding effects of separation from the mother. On the $5^{th}$ day of injection (P9), each pup also received a single i.p. injection of 5'-chloro-2'-deoxyuridine (CldU, Sigma), 42.5 mg/kg [51], immediately following its final GCV injection to assess GCV effect on postnatal cell proliferation.

Pups were then left undisturbed to develop until 28 days of age, at which time they were tailed for genotyping, ear-tagged, and weaned to single-sex group housing. Male and female mice were both used in the experiments. All housing and procedures were performed in accordance with the Institute Animal Care and Use Committee (IACUC) regulations and approved protocols.

At least 6 hours before adult animals were sacrificed, 5'-iodo-2'-deoxyuridine (IdU, Fluka Biochemika) was administered, 57.5 mg/kg i.p. [51], to assess postnatal GCV effects on adult neurogenesis.

All drugs were dissolved in 0.9% saline and administered i.p. using a 25-gauge needle.

Behavioral testing

Behavioral testing began at 10 weeks of postnatal age. First, novel object recognition habituation, training and testing were performed over 3d. On d4, animals were tested on the rotarod, then for olfaction. Morris water maze training began on d5 and lasted 8 days. Water maze probe trials were performed 24h and 1wk after completion of training. Then mice were probed for open field activity, light-dark alternation, and prepulse inhibition, consecutively, over a 2wk period. Finally, 1 day of fear conditioning, one day of fear memory testing, and one day of elevated plus maze assessment were administered, concluding behavioral testing. Animals were sacrificed later that week. A total 10 NTK and 18 WT male littermates were tested in novel object recognition and water maze. Only 7 NTK and 10 WT males underwent the remainder of the testing.

Locomotion (rotarod)

Mice were placed, 4 at a time, onto a rotating rod with dividers creating a separate compartment for each mouse. The rod was set to accelerate gradually from 5 to 70 rotations per min over a 3-min time interval. The trial was ended when the mouse slid off the rotarod. The time it was able to remain on the rod was recorded. For each mouse, an average of 3 consecutive trials was taken.

Locomotor function was also assessed through measures used in other tasks, such as swim speed recorded during Morris water maze trials, and baseline locomotor activity, recorded during open field assessment and prior to fear conditioning.

Olfaction (hidden cookie task)

Mice were food deprived for approximately 7h before testing. Testing was performed under red light during the first hour of the regular dark cycle (6-7pm). Water was available to mice at all times.

A 0.5- to 1-cm piece of NutterButter (Nabisco) cookie was buried beneath 1-2 cm of bedding at one end of a clean standard cage filled with 4-6 cm total bedding and covered with a ventilator lid. Each mouse was placed into the center of the cage, and a stopwatch was started. The time from when the animal was placed in the cage to the time that the first bite of hidden cookie was taken was recorded. The trial was stopped at 3 min if the animal failed to find the cookie. Each animal was then returned to home cage with a small piece of cookie to

nibble until all mice had been tested. After that, mice were returned to their regular holding room, still in dark cycle, and their normal ad *libitum* dry food diet resumed.

Novel object recognition

For the novel object recognition task, mice were acclimated to the testing suite for 45 min prior to each introduction to the test chamber. They underwent 1 d of 10-min habituation to the test chamber. On day 2, they were placed in a standard holding cage for 30 s while the investigator placed 2 identical objects (cleaned w/ ethanol after each use) in the test chamber. Mice were then given 15 min in the test chamber to familiarize themselves with the objects. Groups of mice were counterbalanced for object type and location.

Testing for novel object recognition was performed both 30 min and 24 h after familiarization. Testing was performed identically to training, except one familiar object and one novel object were placed in the chamber. Mice were videotaped for 15 min in the test chamber, and their relative exploration of the familiar and novel objects was assessed by the investigator. Four mice were tested at a time, 1 per chamber counterbalanced across the 4 test chambers. Exploration of an object was scored when the animal's nose was oriented towards the object and the animal was either touching the object or close enough to whisk it.

Morris water maze

During postnatal week 11-12, animals began training in the Morris water maze (MWM) [52]. A 120cm diameter pool was filled with 25-28°C water made opaque by white tempera paint. At 0.5 inches beneath the water's surface in the northwest quadrant of the pool, a platform was placed that remained in the same location throughout training.

On each training day, mice were first acclimated to the behavioral testing room for at least 45 min. Each mouse was then individually trained to find the platform. Visual cues on the walls surrounding the pool, and the investigators in the room, served as navigational aids. Mice were placed into the pool starting in each quadrant other than the northwest. Each mouse was then permitted to swim until it climbed onto the hidden platform, or until each 40-s trial ended, at which time the mouse was placed onto the platform by the investigator. In either case, the mouse remained on the platform for 10 sec before being returned to the home cage. Three or four other mice were then trained starting in the same quadrant before the original mouse began training trial 2, starting from a new quadrant. During all trials, the time taken for the mouse to climb onto the platform and the swim path of the mouse were recorded using EthoVision software and a video camera above the pool tracking the mouse. Four training trials were performed per mouse on each of 8 consecutive training days. At the end of the trials, mice were dried with a soft towel and returned to their home cage.

After the completion of 8 d of training, the platform was removed for probe trials, in which the swim strategy of the trained mice was assessed to evaluate whether or not they remembered the location in which to search for the

platform. For each probe trial, the subject was released into the SE quadrant and permitted to swim freely for 60 sec, during which its swim path and speed were recorded. Each mouse was tested only once per day. Two total test trials occurred: one at 24h and one at 1 wk after completion of training.

Open field

Several days after completion of the last MWM probe trial, each mouse placed into the center of a 43cm square activity monitor cage, with 16 evenly spaced infrared beams crossing the area, to record its activity over 60 min. 8 chambers/8 mice were run at a time. Chambers were cleaned with ethanol between subjects.

Light-dark box

The same activity monitor boxes were used to assess light-dark alternation and preference. A black opaque Plexiglas box, covering 1/3 of the above open field chamber and with holes placed to allow laser beams to cross the floor, was placed in the rearmost part of each chamber. An opening in the front of the black box permitted the mouse to travel between light and dark chambers at will. Each mouse was placed into the dark chamber to start the trial. The mouse's path was tracked over the 10-min test period and analyzed with respect to number of crossings between and time spent in the light vs. dark zone.

Elevated plus maze

A standard elevated plus maze task was performed to assess anxiety, as previously described [53]. Briefly, each mouse was placed in the center of a maze, elevated 50cm from the ground, with two open (25 x 5 cm) and two closed (25 x 5 x 30 cm) perpendicularly crossing arms. The mouse was kept in a cylindrical paper cone in the center of the arms for 1 min. The cone was then removed, and the mouse was permitted to freely explore the maze for 3 min. Behavior was video recorded and hand-scored from the tapes using the same setup as in novel object recognition. Scoring was subsequently performed by hand using MatLab software. Data were scored to assess the number of entries into open and closed arms, and the total and percentage of time spent in open and closed arms. This scoring was performed twice, based on respective criteria of two feet, and all four feet, into the arm defined as an arm entry. The number of explorations during which each animal reached the end of the open arm with its nose was also scored.

Histology

After the completion of behavioral assessments, animals were sacrificed by i.p. overdose of ketamine/xylazine and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brains were dissected out, left overnight in 4% paraformaldehyde at 4°C, transferred to 30% sucrose and stored at 4°C until tissue processing (after brains sank in sucrose).

Brains were weighed using a Mettler AE100 balance immediately prior to sectioning. After whole brain weight was taken, olfactory bulb and cerebellum were removed with a razor blade, weighed separately, and saved in sucrose for embedding in OCT and cryostat sectioning at 20 μm. The main portion of the brain was sectioned coronally at 40 μm on a sliding microtome (AO Scientific Instruments). Sections were stored in cryoprotectant at -20°C.

Immunohistochemistry was performed on every 12$^{th}$ section. All antibody solutions were prepared in TBS 1% triton 3% donkey serum. Primary antibodies used: rat anti-BrdU (Accurate, 1:500) to detect CldU; mouse anti-BrdU (Becton-Dickinson, 1:100) to detect IdU; rabbit anti-Prox-1 (Jackson, 1:400); rabbit anti-Ki67 (Vector labs, 1:500). Secondary antibodies used: donkey anti-rat Cy3, donkey anti-mouse FITC, donkey anti-rabbit Cy5 (all Jackson, all 1:250).

Microscopy

All cell counts were performed using MicroBrightField StereoInvestigator software, coupled to a Nikon Eclipse TE 600 epifluorescence microscope. All colocalization and images for publication were captured using confocal microscopes Nikon Eclipse TE300 and Olympus BX51 using the Bio-Rad (Hercules, CA) Radiance 2100 laser system. Images were captured using a CCD camera (Spot RT; Diagnostic Instruments, Sterling Heights, MI).

**Results**

The Nestin-thymidine kinase (NTK) mouse was created and backcrossed into C57Bl6 background by Wei Deng, as a tool to study the effects of decreasing adult neurogenesis [37]. We hypothesized that this same model could be used to selectively determine the anatomic and behavioral effects of diminishing early postnatal Nestin(+) cell proliferation. In fact, we found that one effect of this early postnatal knockdown was to reduce adult neurogenesis. Therefore, the behavior results presented here represent the effects of reducing postnatal neurogenesis AND adult neurogenesis, rather than the effects of reducing postnatal neurogenesis in isolation with normal adult parameters. They also underscore the importance of early postnatal progenitor expansion in the formation of adult neurogenic populations (Chapter 2).

Anatomy

We first sought to confirm the efficacy of the NTK model in reducing postnatal neurogenesis. Postnatal NTK mice and WT littermates were injected with GCV i.p. on P5-9 (Figure 1A, n=5 per genotype). On P10 they were injected with BrdU to measure the amount of proliferation occurring after treatment, and sacrificed 6h later. This treatment resulted in an approximately 50% reduction in cell proliferation (Figure 1 B, C; $p=0.0359$), indicating that postnatal GCV treatment in the NTK mouse produces an effective knockdown of DG cell proliferation.

Next, we asked whether mice treated postnatally would survive to adulthood, with relatively normal gross neuroanatomy, and still have a detectable postnatal proliferation deficit. Mice were treated as in the first experiment, but this time they were sacrificed at 13 weeks of age (Figure 1D). The nucleotide analog used to measure postnatal proliferation was also changed from BrdU to CldU, so that adult proliferation could be distinguished from postnatal proliferation in the same animals by adult injection of IdU just prior to sacrifice (see Figure 2). Also, the time of CldU injection was moved back to the time of the last GCV injection on P9, in order to capture the extent of knockdown more effectively and avoid quantifying any rebound proliferation that might occur between cessation of GCV and introduction of CldU analog.

At 13 wks of age, overall body and brain weights did not differ statistically between WT (n=10 males) and NTK (n=12 males) (Supplement 1A, p=0.393). Since Nestin expression during early postnatal life also occurs in the olfactory bulb and cerebellum, we specifically weighed these structures and determined that they also did not differ statistically by genotype (Supplement 1B, olfactory bulb p=0.512, cerebellum p=0.944), confirming that nestin expression was largely restricted, and our knockdown was largely directed, to the DG by the dates of GCV injection. The number of postnatal-proliferating cells (CldU(+)) surviving in the adult was still significantly reduced – now by >50%, likely due to ongoing death and dilution (Figure 1 E, F, p=0.006). Thus the NTK mouse appears to be a suitable model for studying the adult effects of an early postnatal knockdown of DG cell proliferation.

Once this model was established, we again injected GCV P5-9 and CldU P9, allowed the mice to age to 13 wks, and this time injected IdU shortly before sacrifice (Figure 2A). A comparable reduction in CldU(+) cells was replicated (data not shown), so we went on to evaluate the anatomic effects of postnatal knockdown on the adult DG. Both the number of granule cells (Figure 2 B, C, $p=0.00819$) and the total DG volume (Figure 2B, D, $p=0.0152$) remained significantly reduced in adulthood in NTK animals. Hence it appears that the DG is unable to fully compensate the P5-9 deficit in cell genesis at a later timepoint.

Chronic deficits in cell proliferation were also evident in NTK animals, again indicating a lack of compensation for the postnatal knockdown (Figure 3). The number of IdU-labeled adult-dividing cells was significantly reduced in NTK-GCV animals compared to WT littermates (Figure 3A, B; $p=0.00666$). The number of progenitor cells, as measured by Sox2, was also reduced in NTK (Figure 3 C, D; $p=0.00365$), suggesting that, in addition to the GCL, the adult progenitor pool cannot fully recover from an insult to postnatal progenitor expansion. It appears that the Nestin(+) precursors dividing P5-9 give rise to a significant fraction of progenitors in the adult.

Behavior

From the $10^{th}$ week of age until shortly before sacrifice, NTK mice and WT littermates from the above groups underwent behavioral testing to explore whether any behavioral deficits accompanied the anatomic ones documented above.

Before assessing hippocampal-dependent functions, mice were tested on the rotarod and the hidden cookie task. These served to assess locomotor and olfactory deficits that might have resulted from depleting Nestin(+) cells in the cerebellum and olfactory bulb, respectively. As NTK and WT mice did not significantly differ in performance of either of these tasks (all $p>0.48$, Supplement 2A), we proceeded to examine memory and anxiety, assuming that both genotypes were not fundamentally impaired in basic olfactory or cerebellar functions.

To assess learning and memory, mice were tested in novel object recognition. Mice were familiarized with one object for 15 min. Twenty-four h later they were exposed to both this same object and a new, unfamiliar object that they then had 15 min to explore at will. WT mice were able to remember the familiar object at 24 h, as they spent significantly more time exploring the novel object (Figure 4A, t-test novel vs. familiar $p=0.000353$). NTK mice, by contrast, showed poorer memory, spending about the same amount of time with novel and familiar objects (Figure 4A, $p=0.609$). A two-way ANOVA showed a trend towards a group-by-object interaction ($p=0.0856$). This failure of NTK mice to explore the novel object as much as WT animals was not due to inactivity, as the total number of exploration events and the total time spent exploring the objects were not significantly different between groups ($p=0.415$ and 0.497, respectively; Supplement 2B).

These data suggested a functional deficit in memory, which we further characterized using the Morris water maze (MWM). Animals were trained for 8

d using 4 40-s training trials per day. Overall latency (Figure 4B, p=0.702) and distance (not shown, p=0.112) to platform did not differ significantly between groups, but in two-way repeated measures ANOVA showed a significant main effect of training day in both groups (for both latency and distance $p<0.0001$). However, looking only at the first training trial of the day, WT animals significantly improved their time to find the platform from d1 to d8 (t-test, p=0.00488), whereas NTK animals did not significantly improve (p=0.145). This was not due to motor deficits, as swim speed did not differ between groups (Supplement 2C). This pointed to a retention or consolidation, rather than an acquisition, deficit in the NTK group.

A retention deficit was confirmed by data from MWM 24-h and 1-wk probe trials (Figure 4 C, D). As soon as 24 h after completion of 8 d of training, NTK animals failed to spend more time searching in the target quadrant, whereas WT animals spent significantly more time searching there (p=0.0139, Figure 4C). This deficit remained at 1wk post-training, at which time several other measures of memory were also significantly or almost significantly impaired in NTK animals: NTK number of platform crossings was lower than WT (p=0.0429), and NTK latency to enter the target quadrant (p=0.0708) and to cross the platform area (p=0.0569) were longer on average than WT. These measures strengthen evidence for a memory retention deficit after postnatal proliferation knockdown.

As the hippocampus also figures critically in anxiety phenotypes, we tested these as well in WT vs. NTK littermates. No significant difference in any examined measure of open field exploration (Figure 5A, t-test for graph

p=0.250), light-dark alternation (Figure 5B, p= t-test for graph p=0.359), or elevated plus maze exploration was observed (Figure 5C, p=0.511) (all measures assessed $p>0.05$).

Postnatal knockdown rescue

Given the failure of NTK postnatal knockdown animals to spontaneously compensate DG structure and proliferation by adulthood, we sought to determine whether early intervention to stimulate neurogenesis could rescue DG structure and permit compensation. To explore this, we introduced 2 known strong neurogenic stimuli after the knockdown: a selective serotonin reuptake inhibitor, fluoxetine[54, 55], and wheel running [56, 57]. On P10, the day after postnatal knockdown and CldU labeling, NTK and WT littermates received i.p. 20 mg/kg fluoxetine injections daily for 14 d, after which they were weaned to cages with free access to running wheels.

Overall, the fluoxetine and running treatments appeared to have partially, but not fully, rescued DG structure and proliferation (Figure 6). One-way ANOVAs were performed between GCV-treated WT, NTK, WT-rescue, and NTK-rescue groups of animals, and significant differences between groups were found for all of the following parameters: number of Prox1(+) cells (p=0.0026), CldU(+) cells (p=0.0002), IdU(+) cells ($p<0.0001$), and Sox2(+) cells ($p<0.0001$). No significant difference was found between all groups for DG volume.

Post-hoc Tukey tests indicated that a significant ($p<0.05$) difference in DG volume was present between only WT and NTK groups, as previously reported. Therefore, at least partial volumetric recovery was possible in the rescue groups. This was nicely paralleled by a recovery in Prox1(+) GC number: the NTK group had significantly fewer cells than any of the other groups ($p<0.05$), but no other groups differed significantly in cell number.

Post-hoc tests also confirmed a recovery of the NTK-rescue group's dividing population. This recovery did not restore IdU adult proliferation to the levels of the WT-rescue group (Tukey NTK-rescue vs. WT-rescue $p<0.05$). However, it did restore them to levels significantly higher than the non-rescue NTK animals ($p<0.05$), and not significantly different from WT non-rescue animals ($p>0.05$). Sox2 data for the same animals further confirmed this recovery: the number of Sox2(+) cells in NTK rescue brains was significantly less than the WT rescue group (Tukey post-hoc $p<0.05$), but not significantly different from WT non-rescue values ($p>0.05$).

It appears, then, that early intervention with fluoxetine and running after postnatal knockdown of DG proliferation can at least partially rescue the adult DG structure and progenitor pool.

**Discussion**

In this study, we investigated whether decreasing postnatal dentate gyrus (DG) cell genesis – specifically the Nestin(+) progenitor population – had lasting effects on the adult animal. We found a failure of the DG structure and

progenitor pool to recover to normal levels by adulthood, and accompanying adult deficits in memory retention tasks.

This finding is of interest from several perspectives. First, it is clearly important to understand the chronic effects of insults to postnatal neurogenesis, as previous literature on the matter is conflicting (see Introduction). A vast array of events can cause an early neurogenic insult: maternal deprivation or early stress [58] or early life anti-mitotic treatments [18, 19, 26]. Interestingly, maternal separation at P9 can result in adult DG structural deficits, and memory deficits, quite similar to those seen here after GCV ablation of DG early postnatal proliferation [59]. Such early adverse events affect many brain parameters: inflammatory state, serotonergic transmission, and HPA axis activation, as well as cell genesis in all adult-neurogenic areas. Thus basic science needs to characterize how exclusively decreasing division of progenitors in the DG and SVZ alters the developing brain. This study suggests that early life insults specific to the neural stem cell population are sufficient to cause long-term diminution of the GCL and of the proliferative cell population in the adult. Further, such insults are sufficient to induce chronic memory-retention deficits.

It would be interesting to assess what downstream parameters these insults affect. For example, DG afferent connectivity from the entorhinal cortex is establishing in the early postnatal period [50]. The relative number and strength of synapses formed by surviving cells following such an insult might also be altered, and this could play a role in the functional deficits observed in the adult.

Second, this study of developmental neurogenesis complements previous studies of adult neurogenesis and its function. In many ways, developmental and adult neurogenesis have been shown to be similar processes: neurons born early in development and in adulthood undergo almost identical processes of morphologic and electrophysiologic maturation [5, 8], and they appear to have similar mature physiology in the adult brain [7]. However, there are differences in the adult locations within the GCL of neurons born in development vs. adulthood (Chapter 2) [13-16].

Data presented here suggest a possible difference between postnatal and adult neurogenesis in terms of the ability of the DG and its progenitors to recover after insult. In contrast to the significant reductions in DG volume and number of granule cells (Prox1(+)) we see in the DG after knocking down proliferation P5-9, no major DG structural deficits are noted after adult knockdowns [33, 37]. Perhaps this is not surprising, since only around 1% of DG cells are made in the adult [43], whereas over 8% per day are generated postnatally [14]. However, adult animals are able to recover their dividing population to its normal level within 2 wks [33] to 1 mo. [60] after GCV treatment. In contrast, postnatal-knockdown animals' proliferative populations remain approximately half the normal level, even 13 wks after GCV treatment.

This suggests that there is a critical period in the early postnatal DG during which the future progenitor population is capable of expanding to form the animal's adult progenitor reservoir. It also suggests that Nestin(+) cells in the

early postnatal brain are a necessary source and/or intermediary for this expanding pool.

Adult behavior, along with DG anatomy, was also altered following early postnatal knockdown of Nestin(+) proliferation using the NTK model. This agrees with other postnatal knockdown studies: in those that have shown adult anatomic deficits in the hippocampus, these were accompanied by behavioral deficits [25-28, 61]. NTK animals showed memory retention deficits as little as 24 h after learning. We also saw a deficit in novel object recognition 24 h after familiarization. However, we saw no alterations in measures of anxiety probed here. These deficits are more specific than those observed after irradiation or MAM postnatal knockdown, in which acquisition as well as retention deficits have been observed [61], and anxiety measures have been altered [62].

The causes of behavioral differences between postnatal and adult knockdowns will be more difficult to characterize. This is because behavior after adult knockdown is tested during the period of reduced proliferation, whereas behavior after early postnatal knockdown is assessed in the adult months after the antiproliferative treatment. Pups at the time of knockdown are incapable of performing tests that have been done on adult mice. This, in combination with the fact that the NTK postnatal knockdown also chronically reduces adult proliferation, makes postnatal and adult behavioral phenotypes difficult to compare.

It is possible that permanent structural deficits in the DG after NTK postnatal GCV – for instance decreased number of granule cells, or altered DG

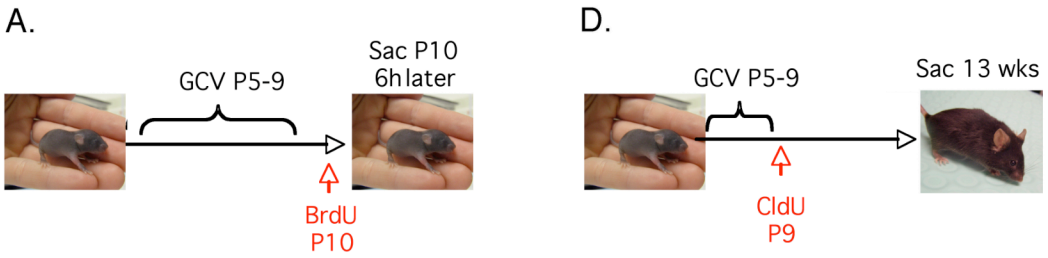
connectivity [18] – are responsible for memory deficits observed. It is also possible that chronic inability to generate new cells in the adult after postnatal knockdown has this effect, as memory deficits have also been shown in several adult neurogenesis knockdowns[35-38]. The most immediately parallel adult NTK data [37] are still preliminary, but appear to show that the adult animal can recover within 1 mo after a 14d treatment with GCV. The present data, viewed in concert, would suggest that a knockdown of early postnatal neurogenesis may cause more chronic and far-reaching effects than a similar or longer adult knockdown. Further comparison is important, to determine whether animals are less able to compensate postnatal than adult insults to Nestin(+) progenitor proliferation.

Rescue data presented here also support the idea that there might be a "critical period" in early postnatal life for progenitor recovery. NTK-rescue animals given fluoxetine after GCV starting P10 and access to running wheels beginning postnatal wk 3 were able to compensate their adult progenitor cell numbers to "normal" levels – at least commensurate with WT non-runners. This means that early in life, animals are still able to resupply long-term progenitors. Both treatments were administered to animals early enough in development that they were still undergoing higher rates of DG proliferation than adults, since 10% of granule cells form after P18 [9]. The treatments were administered between the 3 wks, at which time irradiation still causes chronic adult effects [27], and 6 wks, at which time full DG structural recovery is possible after knockdown [33]. It remains to be seen whether similar running and/or fluoxetine treatments would

be capable of restoring progenitor numbers after early postnatal depletion if they were administered only in adult animals. Do neurogenic interventions actually prolong or re-create the critical period environment? Or must they be made during a critical period in order to restore the progenitor pool?

Finally, this NTK knockdown could model insults to early postnatal human neurogenesis. Humans, like mice, undergo an extended DG GCL formation that continues through the first 11 months of life [63, 64]. The time course modeled here suggests what effects such insults as stress, hypothalamic-pituitary-adrenal axis overactivity, or brain chemotherapy might have on a child in the first years of life. It is known that early-life anti-cancer treatments frequently correlate with later learning and memory deficits [65]. Findings here suggest that such early-life insults to nestin(+) progenitors may result in later learning and memory deficits; this hypothesis merits further investigation in humans. Early, relatively benign interventions (e.g., running and SSRI administration) appear able to allow young animals to compensate for such insults, so that DG structure and cell proliferation is more normal in adulthood. It is tempting to consider what compensation such interventions might promise children for more normal adult lives after neurologically rough beginnings.

Figure 3.1
GCV P5-9 effectively knocks down postnatal DG proliferation, and this knockdown is detectable through adulthood
A. Experiment schematic: To test the efficacy of using the NTK model to knock down postnatal cell proliferation in the DG, mice were injected with GCV P5-9. The amount of cell division was measured by BrdU labeling on P10, and mice were sacrificed 6 h later for assessment.
B. Postnatal cell proliferation in the DG is decreased in NTK mice given GCV on P5-9. Mice were given their last dose of GCV on P9, then injected with BrdU and sacrificed 6h later on P10. Note that the mid-outer portions of the NTK DG also appear weakly stained, suggesting a gap in cell genesis. (Red – BrdU; blue – NeuN.) Scale bar = 50 μm.
C. P10 postnatal cell proliferation in the GCL is decreased by about 50% in NTK mice given GCV on P5-9 ($p=0.0359$).
D. Experiment schematic: To assess whether the NTK knockdown of postnatal cell proliferation in the DG is still detectable in adulthood, mice were again injected with GCV P5-9. The amount of postnatal cell division was measured by CldU labeling on P9. Mice then survived to 13 wks of age before being sacrificed for assessment.
E. Postnatal-proliferating cells surviving in the adult are decreased to an even greater extent in NTK mice given GCV P5-9, CldU on P9, and sacrificed at 13 wks of age. (Red – CldU, blue – Prox1.) Scale bar = 50 μm.
F. Postnatal-proliferating cells surviving in the adult is decreased by >50% in NTK mice given GCV P5-9, CldU on P9, and sacrificed at 13 wks of age ($p=0.0006$).


A.
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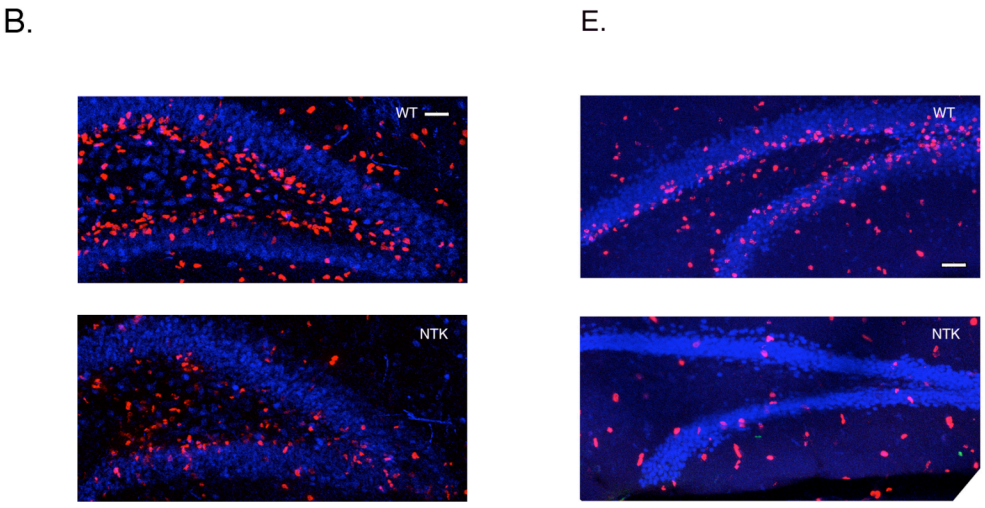
6h later
BrdU
P10
D.
GCV P5-9
Sac 13 wks
CldU
P9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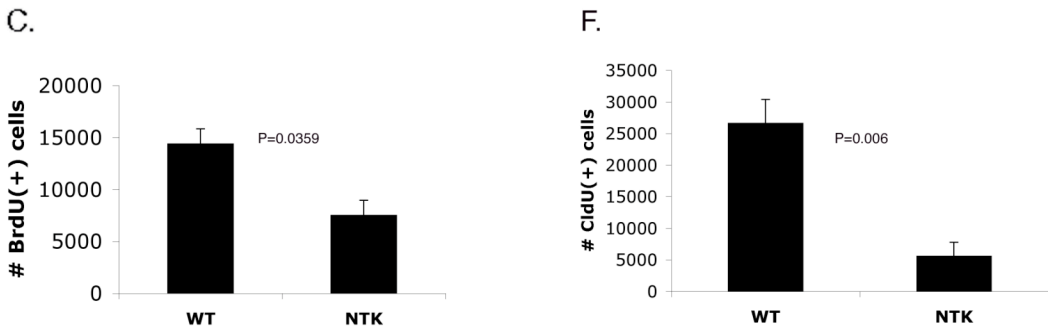
WT
NTK
E.
WT
NTK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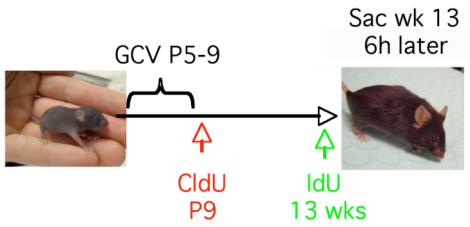
15000
10000
5000
0
P=0.0359
WT
NTK
F.
# CldU(+) cells
35000
30000
25000
20000
15000
10000
5000
0
P=0.006
WT
NTK

Figure 3.2
DG cell number and volume do not recover in adulthood after knockdown of proliferation P5-9
A. Experiment schematic: Mice were treated with GCV postnatally P5-9 to knock down cell proliferation in the DG. On the last day of knockdown, CldU was injected to measure the extent of knockdown achieved. Mice then matured to 13 wks of age without treatment. Six to 18 h before sacrifice, all mice were injected with IdU to measure adult DG proliferation.
B. Knockdown of postnatal DG proliferation appears to result in permanent deficits in the DG GCL, represented qualitatively in these matched coronal sections (more rostral matched sections stacked left; more caudal stacked right). Note reductions of the width of NTK suprapyramidal and even more so infrapyramidal layers relative to WT. Note also the small distance across the SGZ at the genu in NTK. (Stain – Prox1.) Scale bar = 50 μm.
C. The total number of granule cells in the GCL, labeled by Prox1, is chronically decreased after postnatal DG knockdown of proliferation. The graph represents stereologic estimates of the total number of GCs in the adult DG of WT or NTK mice.
D. Total GCL volume is chronically decreased after postnatal DG knockdown of proliferation. The graph represents stereologic estimates of total DG volume based on tracing the GCL outline based on Prox1 immunostaning of every 12th 40μm coronal section.

A.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6h later
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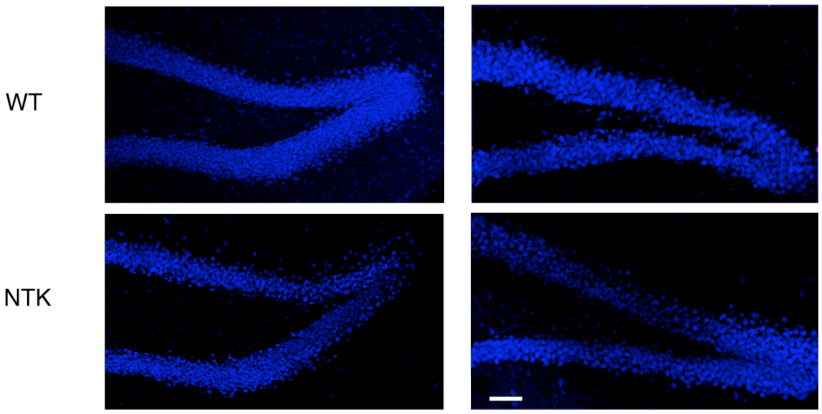
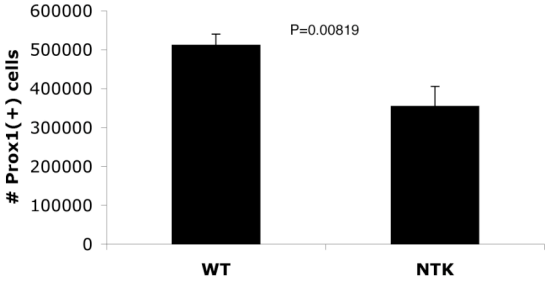
CldU
P9
IdU
13 wks

B.


WT
NTK

C.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400000
300000
200000
100000
0
# Prox1(+) cells
P=0.00819
WT
NTK

D.

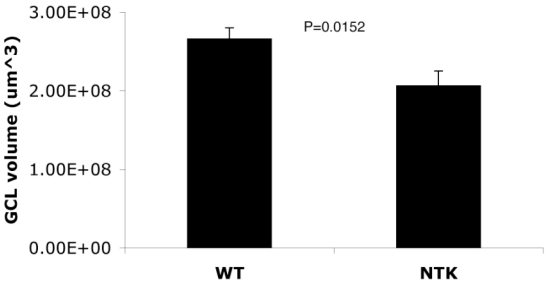
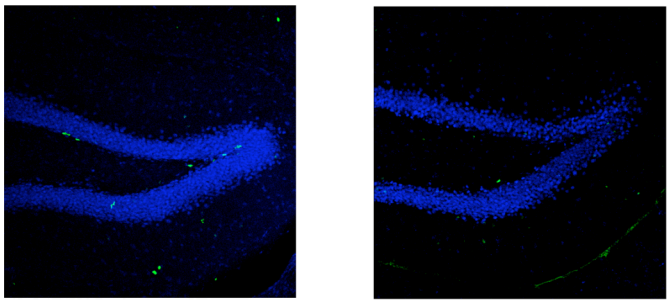
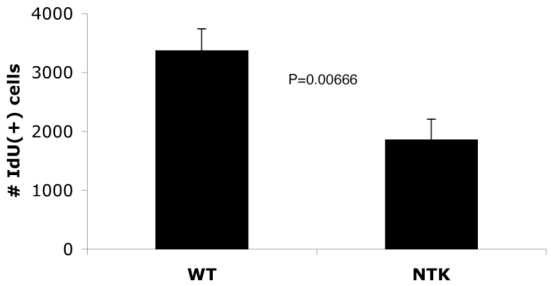

3.00E+08
2.00E+08
1.00E+08
0.00E+00
GCL volume (um^3)
P=0.0152
WT
NTK

Figure 3.3
The adult proliferative cell population does not recover after knockdown of proliferation P5-9
A. Adult cell proliferation, as measured by IdU label (green) injected shortly before sacrifice, is chronically decreased after postnatal DG knockdown of proliferation. (Blue – Prox1.) Scale bar = 50 µm.
B. Stereologic quantification of the decrease in IdU(+) adult-dividing cells.
C. The adult neural precursor population, as measured by Sox2(+) cells (green), is chronically diminished after postnatal DG knockdown of proliferation. (Blue – Prox1.) Scale bar = 50 µm.
D. Stereologic quantification of the decrease in Sox2(+) adult neural precursor cells.

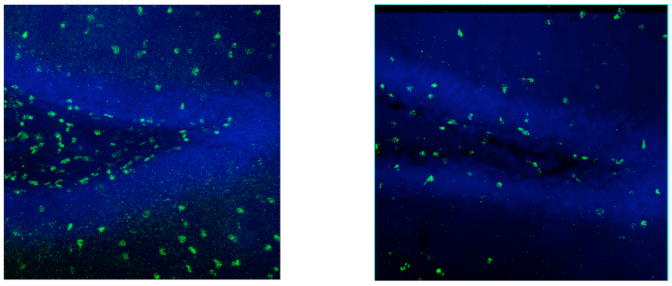
A.



B.


4000
3000
2000
1000
0
# IdU(+) cells
P=0.00666
WT
NTK

C.



D.

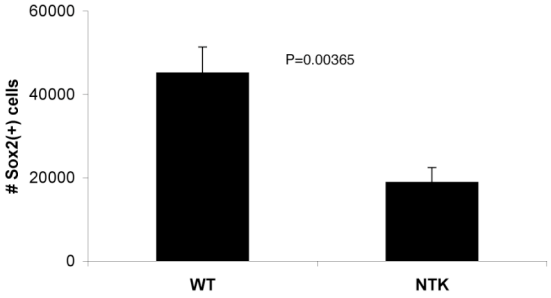
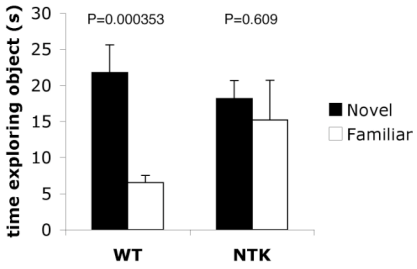
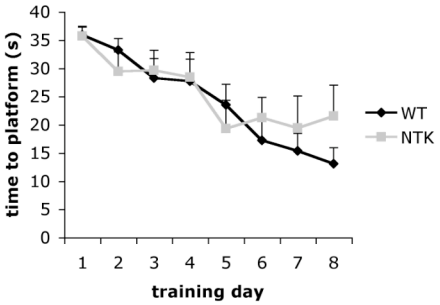

60000
40000
20000
0
# Sox2(+) cells
P=0.00365
WT
NTK

Figure 3.4
Adult animals show memory deficits after knockdown of proliferation P5-9
A. NTK animals fail to spend a significantly greater amount of time exploring a novel vs. a familiar object, 24h after exposure to the familiar object. WT littermates, by contrast, spend significantly more time exploring the novel object. This is without a difference in overall exploration time (see Supplement 2B).
B. NTK and WT animals exhibit similar learning curves during 8 d of training in the Morris water maze.
C. NTK mice are significantly impaired relative to WT mice in their memory for the location of the water maze platform as soon as 24 h after training. During the probe trial, WT mice spend more time searching in the target quadrant, while NTK show no quadrant preference.
D. NTK mice continue to show significant memory impairment relative to WT mice 1 wk after training. During the probe trial, WT mice spend more time searching in the target quadrant, while NTK show no quadrant preference.

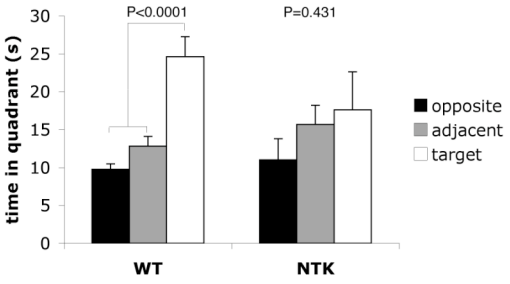
A.


P=0.000353
P=0.609
time exploring object (s)
30
25
20
15
10
5
0
Novel
Familiar
WT
NTK

B


time to platform (s)
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8
training day
WT
NTK

C.


P<0.0001
P=0.431
time in quadrant (s)
30
25
20
15
10
5
0
opposite
adjacent
target
WT
NTK

D.

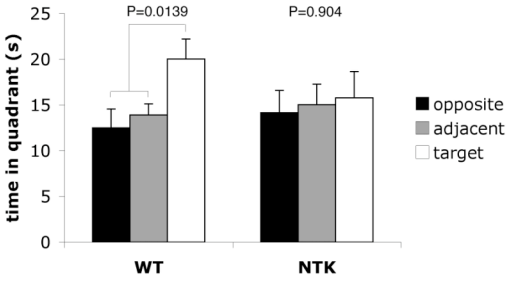
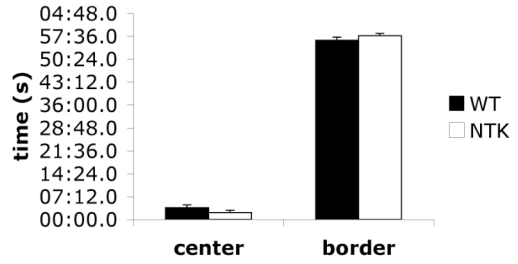
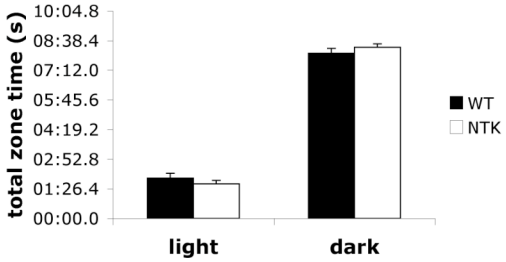

P=0.0139
P=0.904
time in quadrant (s)
25
20
15
10
5
0
opposite
adjacent
target
WT
NTK

Figure 3.5
Adult animals do not show altered anxiety after knockdown of proliferation P5-9
A. Adult WT and NTK animals spend the same amount of time in the center vs. border area of the open field over a 60-min test.
B. Adult WT and NTK animals spend similar durations in the light and dark areas during the 10-min light-dark box test.
C. Adult WT and NTK animals do not differ in the amount of time spent in open and closed arms of the elevated plus maze over a 3-min test.

A.


04:48.0
57:36.0
50:24.0
43:12.0
36:00.0
28:48.0
21:36.0
14:24.0
07:12.0
00:00.0
time (s)
WT
NTK
center
border

B.


10:04.8
08:38.4
07:12.0
05:45.6
04:19.2
02:52.8
01:26.4
00:00.0
total zone time (s)
WT
NTK
light
dark

C.

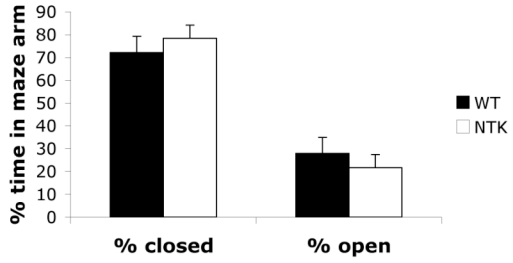
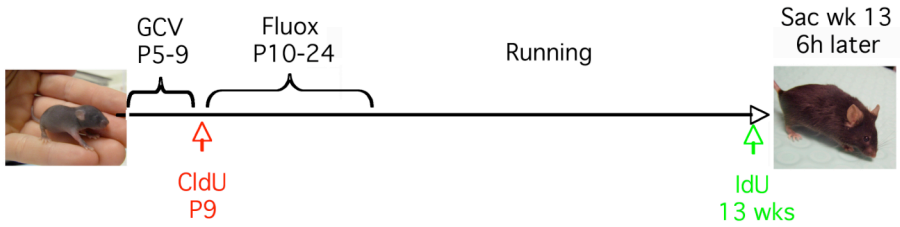
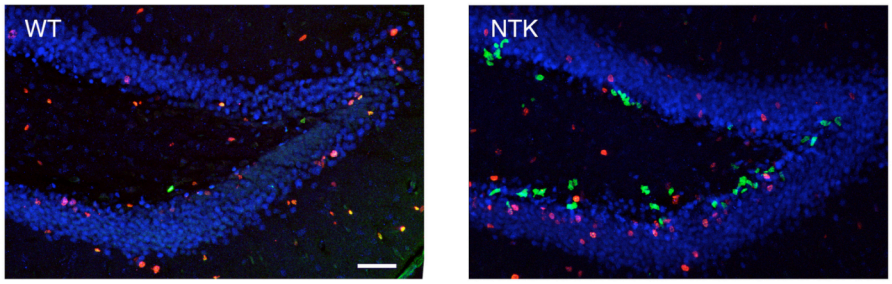

90
80
70
60
50
40
30
20
10
0
% time in maze arm
WT
NTK
% closed
% open

Figure 3.6
Fluoxetine and wheel running permit partial structural recovery of the adult DG after proliferation knockdown P5-9
A. In attempt to rescue the chronic DG deficits after postnatal knockdown, mice were treated with strong neurogenic stimuli immediately after postnatal knockdown. As with previous mice, GCV was administered P5-9 to knock down postnatal DG proliferation, followed by CldU to confirm the knockdown. In rescue mice, however, fluoxetine (20 mg/kg) was then injected daily for 14d immediately after GCV. For the rest of their lives, the mice were permitted free access to running wheels. Mice were then injected with IdU and sacrificed as in previous experiments.
B. Qualitative examination suggests at least a partial compensation in DG size of NTK-rescue (right) compared with non-rescue (left) knockdown animals, as well as a clear increase in proliferative cell populations. (Blue = Prox1, green = IdU, red = Sox2.) Scale bar = 50 μm.
C. Stereologic quantification reveals recovery of granule cell number (Prox1(+),) in the NTK rescue (NTK Tx) group. Only the NTK group had a significantly smaller cell number compared to all other groups ($p<0.05$).
D. Stereologic quantification reveals at least partial recovery of adult cell proliferation (IdU(+), left graph) and progenitor population (Sox2(+), right graph) in the NTK-rescue (NTK Tx) group. The NTK-rescue group never reaches the proliferation and progenitor pool size of the WT rescue group. However, after rescue NTK numbers are no longer significantly smaller than those of the WT group, and they are significantly larger than non-rescued NTK.

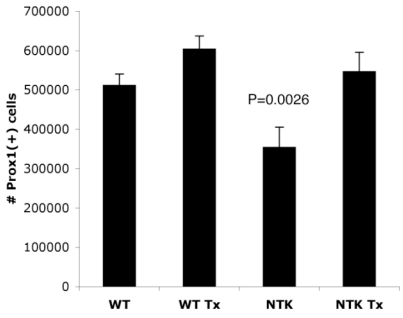
A.


GCV
P5-9
Fluox
P10-24
Running
Sac wk 13
6h later
CldU
P9
IdU
13 wks

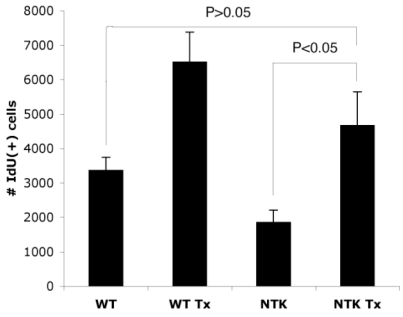
B.

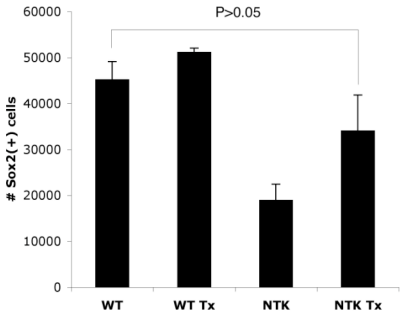

WT
NTK

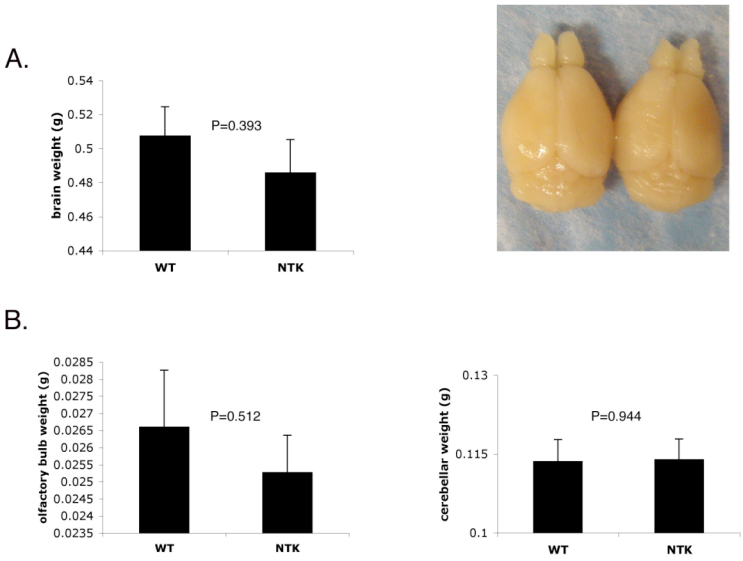
C.


# Prox1(+) cells
700000
600000
500000
400000
300000
200000
100000
0
P=0.0026
WT
WT Tx
NTK
NTK Tx

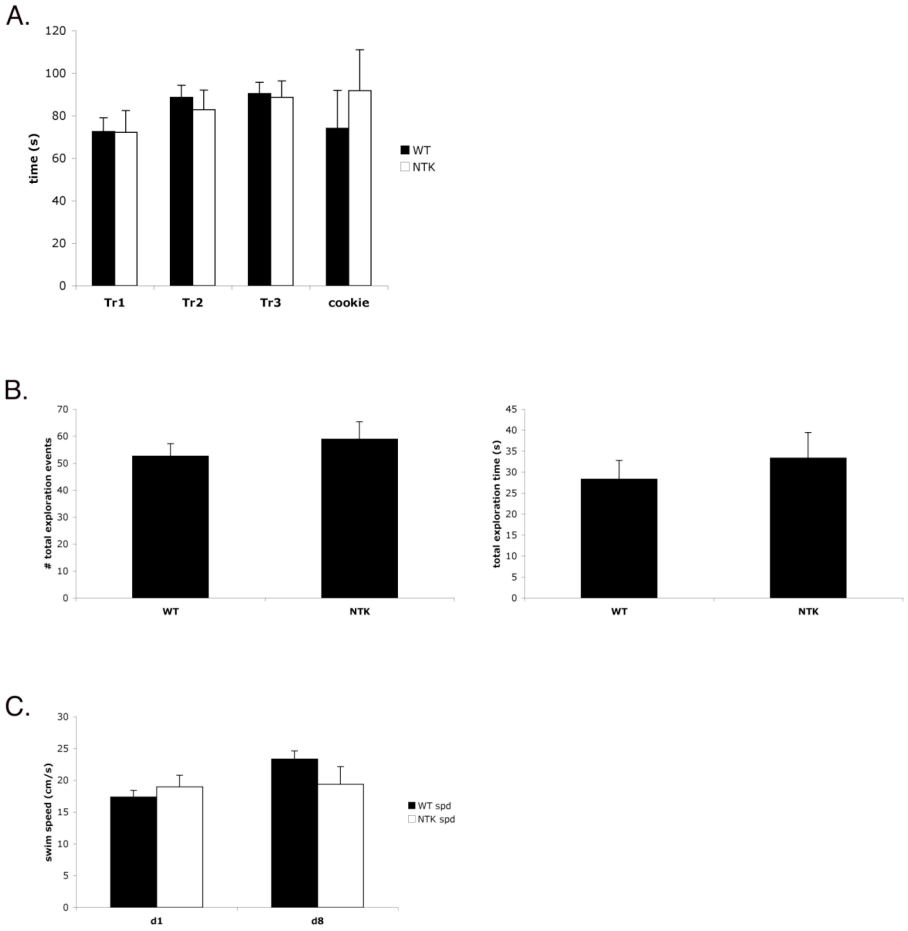
D.


# IdU(+) cells
8000
7000
6000
5000
4000
3000
2000
1000
0
P>0.05
P<0.05
WT
WT Tx
NTK
NTK Tx


# Sox2(+) cells
60000
50000
40000
30000
20000
10000
0
P>0.05
WT
WT Tx
NTK
NTK Tx




Supplement 3.1
Adult NTK and WT animals treated with GCV P5-9 do not show gross brain anatomic differences outside of adult neurogenic areas
A. Adult NTK and WT mice treated postnatally with GCV had similar whole brain weight (left) and brain external appearance (right).
B. Despite the fact that nestin is expressed in the olfactory bulb and cerebellum for a short period postnatally, WT and NTK groups had similar sizes (see right image of A) and weights of these structures after GCV P5-9.




Supplement 3.2
Adult NTK and WT animals treated with GCV P5-9 do not show significant differences in motor or olfactory abilities that would account for other behavioral deficits
A. Adult NTK and WT mice treated postnatally with GCV were tested to assess motor and olfactory function to ensure that changes in these were not responsible for any behavioral deficits classified as memory- or anxiety-related. “Tr1-3” indicate the SEM per group of each of 3 180-s trials on the accelerating rotarod; the number of seconds the animal was able to stay on the rotating rod was recorded. “Cookie” indicates the SEM per group of the number of seconds it took each animal to unearth and nibble a buried piece of cookie based solely on olfaction. On both tests, WT and NTK performance did not differ.
B. During novel object recognition testing, WT and NTK groups spent similar numbers of exploration events (left) and amounts of time (right) exploring the objects. This indicates that novel object recognition deficits observed were not due to neophobia, inactivity, or failure to explore generally.
C. Morris water maze deficits observed were not due to impaired ability to swim by the NTK animals, as evidenced by comparable swim speeds.

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CHAPTER 4

# CONCLUSION

The preceding chapters begin to respond to a recent call within the scientific community: "Now it is clear that more careful insight into not only adult neurogenesis but also [postnatal] neurogenesis is required to appreciate the function of the adult hippocampus. One of the next questions to be addressed is the differences and the relationships between postnatally born GCs and adult-born GCs in the adult hippocampus."[1]

Data presented here underscore that further knowledge of early DG development is critical – in order to understand its contribution to the progenitor pool and the function of the adult DG. Particularly as computational modeling is increasingly employed to investigate the function of the 1% of cells added to the DG during adulthood[2], we need to know any unique attributes of the approximately 35,000 cells per day contributed to the adult DG during early postnatal life.

In what ways do we know early-postnatal-born to differ from adult-born cells? At present, data addressing this question is sparse. Developmental and adult-born neurons appear to follow similar patterns of morphological[3] and electrophysiologic[4] maturation. Further, mature cells born during all stages of development display similar electrophysiologic properties in the adult DG[5]. However, subtle differences have been found even between these similar processes. For instance, adult-born cells have slightly delayed dendritic maturation – both in terms of length and complexity – compared with postnatal-born GCs[3]. It has also been shown that the DG molecular layer as a whole develops its mature synaptic properties around the end of the first/beginning of the second postnatal week[6]. This

means that developing early-postnatal-born cells encounter an entirely different electrophysiologic environment than developing adult-born cells.

The birthdate-based layering of the GCL, confirmed for the first time throughout life, within individuals in Chapter 2, suggests a few possible ways in which cells born early in development may be distinct. One such way is in their molecular marker expression in the adult DG. In situ hybridizations from our lab show preferential expression of certain molecules in the inner vs. outer portions of the GCL[7]. Thus early-dividing outer-layering cells may contribute more heavily to – or even possess the unique ability to add to – the cell population with "outer-layer" molecular properties. Certainly layer-specific differences in molecular marker expression could be determined by other factors than birthdate – for instance, by microenvironmental gradients between the adult SGZ and outer GCL. But such molecular differences may at least coincide with cell birthdate cohorts by layer. If so, these differences could indicate a bias towards different cell fate or function for embryonic- and early-postnatal-dividing cells. This hypothesis awaits further study.

Layering patterns by birthdate also raise the possibility that early-born GCs may differ from later-born cells in their afferent connectivity. Such differences would certainly be critical to modeling DG function. So far, electrophysiologic recordings from adjacent cells labeled at different birthdates find no difference in their responses to perforant pathway stimulation[8]. This, however, does not account for possible differences between cells at different layering positions with different birthdates, since the cells recorded were adjacent. It also does not account for the possibility of weighted afferent differences in overall GC cohorts by birthdate.

Little existing data addresses a possible difference in GC connectivity by birthdate. One retrograde tracing study has found a distinct efferent divergence of development-born but not adult-born axons to CA3[9], but this conclusion is based on extremely low numbers of cells. An afferent connectivity difference seems more promising, given the well documented layering of medial perforant path afferents to the outer DG molecular layer and the lateral perforant path to the inner molecular layer[10-14]. However, it remains to be shown whether dendritic connections within the molecular layer are topographic to cell location in the GCL. It is also unknown whether the extent or nature of afferent synaptogenesis is at all related to somatic position within the GCL.

In the other adult structure with lifelong, layering neurogenesis – the olfactory bulb – several functional distinctions have been found between development-born and adult-born populations[15]. Some of these parallel already known differences within the DG. For both structures, the location of the proliferating cell population moves through development and is distinct from its adult location[15-19]; this could certainly affect the phenotype and/or potentiality of cells born in such different niches and migrating to such different extents[20]. As demonstrated here, the DG exhibits a birthdate-based layering of granule cells, as does the olfactory bulb[15]. Both structures also display markedly larger contribution of early postnatal-born than adult-born cells to the adult structure[1, 15, 21].

Survival rates also differ between of early-born and adult-born cells in both the olfactory bulb and DG, with significant cell death occurring over a much longer period of time for cells born early in development[15, 22, 23]. In the DG, development-born

cells continue to decrease in number through 6 months of age[1, 22], whereas adult-born cells reach a stable number about 4 wks after their labeling that persists 11 months thereafter[22, 23]. It appears, then, that cell death and division (causing label dilution) have different dynamics in early-dividing and adult-dividing populations.

This last point raises an important difference that needs to be explored between DG cells dividing at different times: are early-dividing cells uniquely enabled to form the adult progenitor population in the DG? The numeric colocalization data from Chapter 2, indicating that a large percent of early-labeled cells colocalize with adult dividing cell markers, suggest that this is the case. This is supported by data from Chapter 3 that early postnatal insults to progenitor proliferation cause chronic deficits in adult progenitors. The observation in previous literature that BrdU dilution continues to occur through 6 mo. in early-labeled cells[1, 22], vs. only 1 mo. in adult-labeled[1, 21-23], also suggests that early postnatal-dividing cells constitute a more chronically, infrequently dividing population. It is not that earlier-born cells are predisposed in terms of fate to become dividers; the fraction of BrdU(+) cells labeled early that are adult-dividing is not greater than the fraction labeled in adult that are adult-dividing one month later (Table 2-1). But during early postnatal periods, vastly more of the future DG stem population appears to be dividing than ever will later in life.

A longitudinal fate-mapping study is needed to resolve the origin of the *in vivo* DG progenitor population. Such a study needs to address a question not raised here – whether adult neural stem cells reside within the DG from the time of birth[16, 17, 20] or migrate there from the lateral ventricles[24]. It should also confirm whether a

majority of the adult stem population is formed in early development (as suggested by Chapter 2), and to what extent it can be re-generated later in life (limits suggested by Chapter 3).

One of the most important implications of Chapters 2 and 3, taken together, is that early postnatal life is a critical period for the expansion of the adult DG progenitor pool. Nestin(+) cells that are dividing on P5-9 form a large fraction of the adult-dividing population. This is evidenced by retroviral and BrdU labeling data, which show that some of the cells dividing on P5-7 will go on to divide only a few times and remain part of the adult dividing population over 60 d later (Chapter 2). It is also evidenced by the loss of half the adult progenitor population when postnatal proliferation of Nestin(+) cells is halved (Chapter 3).

The importance of postnatal progenitor expansion to the adult progenitor pool raises an important question: is there a critical period during which this expansion must occur? Is there an age after which the total number of progenitors is set? After which cell proliferation is capped by some set limit of how much the existing progenitors can proliferate? Such a critical period is suggested by data that adult neurogenesis is capable of completely compensating a reduction in adult proliferation [25, 26], but grossly incapable of compensating an insult to postnatal proliferation (Chapter 3).

Several works suggest the existence of a DG critical period in the first weeks of postnatal development, though none to date has demonstrated it definitively. Other studies find, like Chapter 3, that the DG does not structurally compensate after early postnatal insults to the overlying meninges[27], blockade of serotonin signaling[28],

hypoxic-ischemic damage[29], epileptic seizures[30], or neonatal isolation[31]. The chronic effects of these manipulations in the adult range from dramatically decreased DG size, to inappropriate GC migration, to deficits in adult precursors and proliferation. Trophic effects on the DG may also be limited in their efficacy to the postnatal period. For instance, the enhancing effects of testosterone on female DG volume and behavioral performance in sexually dimorphic rats are restricted to the early postnatal brain[32]. After postnatal hypothyroidism causes chronic deficits in GCL volume and cell number[33], these can be rescued by restoring thyroid hormone by P25, but not by P90[34]. The consequence of these early postnatal changes to the adult brain is enormous, and underscores the importance of further defining a critical period for the DG.

The critical period could be determined by any of the many important changes occurring in the early postnatal DG. Rapid electrophysiologic maturation occurs in the DG molecular layer between P6 and 11[6]. Restricted expression of important functional molecules, such as the NR1-C1 receptor, also occurs in the DG between early postnatal life and adulthood[35]. The infrapyramidal blade continues to add cells [19, 20] and establishes its afferent connectivity via activity-dependent remodeling [13]. Could these, or the progressive restriction of cell proliferation to the SGZ by the third week of postnatal life[16, 17], help determine a critical period? Further specification of what, if any, critical period exists for the DG and of what defines that period are needed – for the understanding of both DG development and adult neurogenesis.

We know that many interventions are able to increase cell division in the adult DG[36-41]. Do these measures extend a critical period for progenitor expansion? Or do they simply allow cell division to increase within the constraints of the post-critical-period limits? Or do they extend post-critical period limits on progenitor expansion? It is evident that administering such measures during postnatal life is capable of at least partially restoring DG cell number and adult dividing population (Chapter 3). Would this same extent of rescue occur if measures to increase cell genesis were introduced later in life? Answering these questions is paramount to defining the parameters of any critical period existing between postnatal and adult neurogenesis. These answers will define on a practical level, what magnitudes of insults, at what times, might be addressed clinically using a subject's endogenous DG progenitors.

Finally, data presented in Chapter 3 suggest a clear functional role for early postnatal-dividing cells in the adult DG. It is important to note that behavioral deficits observed in adult animals have been observed only *during* the period immediately after treatment to reduce proliferation [25, 42-44], whereas here we demonstrate that deficits following early postnatal knockdown occur in the adult animal *months after* treatment, when proliferation is chronically reduced (Chapter 3) [45-47]. Without compensatory intervention, animals depleted of nestin(+) dividing cells P5-9 show memory deficits 24h or less after learning hippocampal-dependent tasks in adult life. These deficits are evident in both MWM and novel object recognition (Chapter 3). Thus somehow early postnatal-born cells and/or their irreplaceable progeny after depletion must be critical to DG memory retention or consolidation functions. This

suggests that there may be a critical period for functional, as well as structural, development of the DG.

As specific, transgenic means of depleting DG progenitors become more widespread, comparison of this NTK postnatal knockdown to other models will help identify specific features of the early postnatal-dividing population. This postnatal NTK model (Chapter 3) displays a combined decrease of postnatal AND adult neurogenesis. It was chosen because it reproduces the full clinical effects of an insult to proliferation in the early postnatal DG. From a basic science perspective, however, it will be important to isolate the effects of diminishing embryonic, postnatal, and adult DG proliferation. Thus it will be interesting to see whether the postnatal rescue model (Chapter 3), in which adult proliferation is restored to "normal" adult levels, will also show behavioral deficits. The immediate effects of reducing postnatal proliferation on behavior 1-3 weeks after the knockdown must also be determined. Such data would allow a more specific comparison of the function of newly developing cells with postnatal vs. adult birthdates, since existing behavioral data after adult progenitor depletions was collected during the period of reduced proliferation. The collection of postnatal behavioral data just immediately after depletion will require the development of improved learning and memory tests for 2-3wk-old pups.

Findings in Chapter 3 suggest several clear functional principles about early postnatal neurogenesis. First, insults to early developmental neurogenesis appear less than fully structurally and functionally recoverable. Practically, this suggests that some intervention will be necessary to restore normal adult function after an insult to postnatal proliferation. Second, behavioral deficits are not simply due to a

disproportionate reduction of adult neurogenesis relative to DG size. This is evident because adult proliferation and DG volume are reduced in proportion to one another after early postnatal knockdown, unlike in adult knockdown models. The fact that an amount of adult proliferation proportionate to DG size is not sufficient to restore normal behavior is important to future modeling of DG function.

A surprising finding of the NTK experiments (data not included here) was sex differences in the effects of postnatal neurogenesis knockdown. Experiments in Chapter 3 were initially run on both sexes of mice – all littermates from treated litters. This was because no sex differences in adult cell proliferation have been shown in normal C57Bl6 mice[49]. Here, we show that the NTK model is capable of reducing cell proliferation through adulthood in both sexes (combined male and female data, Figure 3-1; split by sex, the female data alone, like the male data alone, shows significant NTK knockdown). When behavioral testing was performed on male and female, NTK and WT mice, however, ONLY NTK males showed significant deficits in learning and memory. NTK females performed indistinguishably from WT female littermates. Time did not permit us to quantify CldU(+) cells in all female brains used for behavior experiments to check the magnitude of NTK female knockdown, which is why data from females were not included in this study. Thus we cannot rule out that NTK females simply did not have sufficient knockdown of proliferation to see a behavioral effect. However, since all littermates were treated blind to sex and genotype, it seems more likely that there is a selective ability in females to recover at least DG function, if not structure, after postnatal knockdown.

Others have documented developmental sex differences in the formation of the DG. The GCL is larger in males than females in terms of GC number and GCL volume, in some strains of adult mice[50], as well as in prepubescent rats[51]. C57Bl6 mice (used in this study), however, do not show this sexual dimorphism[52]. Nonetheless, in C57Bl6 mice, male GCs born P0 tend to layer more towards the molecular layer than female GCs born the same day[1]. P7-born GCs, by contrast, layer more towards the hilus in males compared to females, though the total GCL density remains the same in both groups from both these times[1].

In rodents that do have sexual dimorphism to the DG, it appears that this dimorphism correlates with functional deficits on MWM and radial arm maze, which can be rescued by testosterone given to females during early neonatal life[32]. This rescue is evident prior to puberty, indicating that sex steroids can have an early and profound effect on GCL structure and later function[32, 53].

Despite the apparent advantages of the male DG in intact sexually dimorphic animals, females seem to have the advantage in recovery after insults to the DG. Following DG deafferentiation, female rats recovered MWM performance to the level of sham-lesioned animals, while males did not[54]. Females have greater neurogenesis deficits in adulthood after prenatal maternal stress than males[55]. However, after postnatal maternal stress, males but not females showed a reduction in DG cell genesis weeks to months later[56]. Taken together with Chapter 3, these findings suggest that the female brain may have an increased capacity for recovery from postnatal DG neurogenesis insults compared to the male. The cause of this preferential ability to recover, as well as its time course, must be further defined.

The implications of a DG critical period for rescuing adult structure and function have immense implications for humans. Humans also undergo extended DG development in postnatal life[57], and have conserved adult neurogenesis[58]. And humans undergo a wide variety of early life insults to neurogenesis, of which we have yet to fully understand the long-term biologic consequences.

The most specific insult to early DG cell division is treatment of early life brain cancers. Following antimitotic treatments to the brain (chemotherapy and/or irradiation) to eradicate these cancers, many children show memory deficits similar to those observed in the early postnatal NTK model[59]. They also show decreased hippocampal volumes for years after irradiation, even when irradiation is directed to another location (posterior fossa)[60]. As these authors note, "further exploration of the relationships between radiation therapy, memory dysfunction, and hippocampal pathology in [the pediatric] population is warranted."[60]

Epilepsy also appears to decrease cell genesis in the early postnatal GCL [61-63]. Deficits seen after postnatal epilepsy include thinning of the DG and abnormal migration, as well as decreased cell number[62, 63], as seen in NTK mice. We did not test the NTK model for epileptic susceptibility, but no spontaneous seizures were observed in these mice.

Finally, early life stress predisposes humans to a variety of psychiatric disorders, including posttraumatic stress disorder (PTSD) and depression[64-66]. PTSD is correlated with a decrease in adult hippocampal volume[67], suggesting that deficient hippocampal neurogenesis could play a role in pathology, and early intervention to rescue neurogenesis might help rescue function. Measures that rescue

neurogenesis, such as antidepressants[68], also reduce depressive and PTSD symptoms[67, 68]. These same measures appeared to restore adult DG structure after postnatal NTK knockdown (Chapter 3). It is important to replicate these results, and to explore whether such treatments can rescue DG structure and depressive behaviors after childhood stress.

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