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A Functional Role of Mammalian Adult Hippocampal Neurogenesis in the Normal and Diseased Brain

A Dissertation submitted in partial satisfaction of the Requirements for the Degree of Doctor of Philosophy in Neurosciences by Claire Dudley Clelland

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2009
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AD: Alzheimer’s Disease, an age-related neurodegenerative disease

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, a specific agonist for the AMPA receptor

Ascl1: Achaete-scute complex homolg 1, a member of the bHLH family of transcription factors

BDNF: Brain-derived neurotrophic factor

BrdU: 5-bromo-2-deoxyuridine, a synthetic thymidine analogue commonly used for the detection of in vivo proliferating cells

CA1: Cornu Ammonis 1 subregion of the hippocampal formation

CA3: Cornu Ammonis 3 subregion of the hippocampal formation

CANTAB: Computerized neuropsychological tests from Cambridge Cognition

CBP: CREB binding protein, a transcriptional co-activator

CCL5: Chemokine (C-C) Motif Ligand 5, also known as RANTES, a protein chemokine involved in inflammation

CNS: Central Nervous System

CNTF: Ciliary Neurotrophic Factor, a polypeptide hormone and central nervous system nerve growth factor

DAPI: 4’-6-diamidino-2-phenylindole; intercalates between nucleotide bases to stain cell nuclei

DCX: Double cortin, a protein expressed in migrating immature neurons

DG: Dentate Gyrus, subregion of the hippocampal formation and site of ongoing
neurogenesis throughout adulthood

**DGC**: Dentate Granule Cell, the primary neuronal cell type of the dentate gyrus

**dnWnt**: Dominant Negative Wnt, a dominant negative protein used to disrupt Wnt signaling

**EE**: Environmental Enrichment, a form of external environmental stimulation

**EGF**: Epidermal Growth Factor

**GABA**: γ-Aminobutyric acid, a neurotransmitter

**GC**: Granule Cell

**GCL**: Granule Cell Layer, a subregion of the dentate gyrus or olfactory bulb

**GLAST**: Glutamate Aspartate Transporter, also known as EEAT1, a high affinity glutamate transporter expressed in glia

**GLT**: One of two major glutamate transporters in the central nervous system

**GFAP**: Glial Fibrillary Acid Protein, an intermediate filament protein that is found in glial cells such as astrocyte as well as radial glial cells

**GFP**: Green Fluorescent Protein, commonly used to label proteins or cells

**GFPcon**: GFP Control, a lentiviral vector expressing GFP or any mouse or group of mice in which such a vector has been injected

**HAP1**: Huntington Associated Protein 1

**HD**: Huntington’s disease, an autosomal dominant neurodegenerative disease

**Hdh**: the mouse HD gene homologue

**Hes1**: Hairy and enhancer of split 1

**Hip14**: Huntington Interacting Protein 14
**bHLH**: Basic Helix-Loop-Helix, a protein structural motif that characterized a family of transcription factors

**Htt**: Huntingtin protein

**IGF**: Insulin-like growth factor

**IL**: Interleukin, a group of cytokines

**IR**: Irradiation, a treatment involving exposure to X-rays, or any mouse or group of mice who have received an X-ray treatment

**ER**: Endoplasmic Reticulum

**Id2**: Inhibitor of DNA binding 2

**KA**: Kainic Acid, a toxin used to induce lesions in living tissue

**Ki67**: A protein expressed during cell cycle and used as a marker for proliferating cells

**KO**: Knockout

**LPS**: Lipopolysaccharides, a lipoglycan endotoxin that can act to elicit a strong immune response

**LTP**: Long-term potentiation

**MAM**: methylazoxymethanol acetate, a toxin used to block proliferation and neurogenesis

**Mash1**: Mammalian Achaete-scute Homolog 1, a bHLH transcription factor mammalian homologue of achaete-scute gene family

**NE**: Non-Enriched, referring to a control condition of the experimental condition EE

**NeuN**: Neuronal Nuclei, a neuron-specific nuclear antigen, a specific marker for neuronal protein
NGF: Nerve Growth Factor

NKCC1: A sodium-potassium-chloride cotransporter

NMDA: N-methyl-D-aspartic acid, an amino acid derivative agonist of NMDA receptors

NPC: Neural Precursor Cell

NR: NMDA receptor

NRSE: Neuron-Rsetrictive Silencer Element, also known as RE1, A conserved DNA response element found in genes that code for neuronal proteins

NRSF: Neuron-Restrictive Silencer Factor, also known as REST, a Kruppel-type zinc finger transcription factor that binds the NRSE and is thought to act as a transcriptional repressor

NSC: Neural Stem Cell

OB: Olfactory Bulb

PD: Parkinson’s Disease, an age-related neurodegenerative disease

PFA: paraformaldehyde

PSA-NCAM: Polysialic Neural Cell Adhesion Molecule, a protein expressed in immature neurons

PSD: Post Synaptic Density

QA: Quinolinic acid, a toxin used to induce lesions in living tissue

R6: The transgenic mouse line expressing exon 1 of the mutant human Huntingtin gene

R6/1: A transgenic mouse line expressing exon 1 of the mutant human Huntingtin gene with greater than 115 CAG repeats
**R6/2:** A transgenic mouse line expressing exon 1 of the mutant human Huntingtin gene with greater than 150 CAG repeats

**RANTES:** a protein chemokine involved in inflammation, also known as CCL5

**REST:** RE1-Silencing Transcription factor, also known as NRSF, a Kruppel-type zinc finger transcription factor that binds the NRSE and is thought to act as a transcriptional repressor

**RILP:** Rab Interacting Lysosomal Protein

**S100B:** A calcium binding protein commonly found in glial cells such as mature astrocytes

**SC:** Stem Cell

**SGZ:** Sub Granular Zone, subregion of the dentate gyrus in the hippocampal formation

**SH:** Src Homology

**SHH:** Sonic Hedgehog Homolog, one of three proteins in the mammalian hedgehog family and a ligand of the hedgehog signaling pathway

**Sox2:** the transcription factor Sry-related homeobox (HMG-box) 2, used as a marker of neural stem cells and embryonic stem cells.

**Sp1:** a transcription factor involved in early development

**SUMO:** Small ubiquitin-related modifiers

**SVZ:** Subventricular Zone; region lining the lateral walls of the lateral ventricles and site of ongoing neurogenesis throughout adulthood

**TNF:** Tumor Necrosis Factors, a groups of cytokines that can cause cell death

**TrKA:** Neurotrophic Tyrosine Kinase A
UTR: Untranslated Region, either of two sections on each side of a coding sequence on a strand of mRNA.

VEGF: Vascular Endothelial Growth Factor, a subfamily of a platelet-derived growth factor family and a signaling protein involved in angiogenesis.

Wg: Wingless, A gene encoding for a secreted signaling protein of the Wnt family originally identified in *Drosophila melanogaster*.

Wnt: A large family of signaling proteins embryonic and adult neurogenesis, among other roles.

WT: Wild Type

YAC: yeast artificial chromosome; a technology used to create transgenic mice expressing the large HD causing gene.
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ABSTRACT OF THE DISSERTATION

A Functional Role of Mammalian Adult Hippocampal Neurogenesis in the Normal and Diseased Brain

by

Claire Dudley Clelland

Doctor of Philosophy in Neurosciences

University of California, San Diego, 2009

Professor Fred H. Gage, Chair
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This work explores the functional role of adult hippocampal neurogenesis in the context of dentate gyrus (DG) and hippocampal function as well as in the context of long term dysfunction characteristic of the degenerative brain. To probe the role of adult hippocampal neurogenesis in pattern separation function, two behavioral tasks have been developed and applied to models in which neurogenesis has been artificially ablated or reduced, increased by environmental stimulation, and impaired long term in the diseased brain. The work presented in this thesis provides evidence for a functional role of adult hippocampal neurogenesis in spatial discrimination consistent with a role in spatial pattern separation, a function that does not appear to be modulated by environmental enrichment. Despite the well known correlations between increased rate of neurogenesis and performance on learning and memory tasks, modulating the
number of mature dentate granule cells, without increasing the pool of immature neurons stably over time, does not appear to affect spatial discrimination behavior. However, long term deficits in adult neurogenesis, as examined in a rodent model of the neurodegenerative disease Huntington’s, may contribute to deficits in spatial discrimination observed in R6 mice expressing the mutant human huntingtin transgene. Further investigation into the mechanism(s) underlying the role of adult neurogenesis in spatial pattern separation would both provide insight into the biological process of neurogenesis in the adult brain and contribute to our understanding of cognition in the context of the normal and diseased brain.
CHAPTER 1

INTRODUCTION
PART I: Adult Hippocampal Neurogenesis

1. Adult Neurogenesis

1.1 Neurogenesis Background

Ramón y Cajal famously conjectured in the early 20th century that the adult brain was fixed permanently at birth: “In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated” (314). The first suggestion to the contrary, that the adult brain retained the ability to generate new neurons postnatally, was reported by Altman more than 50 years later (7, 9). It has now been well established that neurogenesis continues throughout adulthood in two regions of the adult mammalian brain, the subgranular zone of the hippocampus and the subventricular zone of the telencephalon (8-10, 106, 118, 185, 206, 234, 235, 238, 246, 371). New neurons born in the adult DG and SVZ-olfactory bulb system are derived from putative multipotent neural stem cells that reside near the ventricular zone. The existence of adult NSCs is evidenced by the demonstrations that cells of the SGZ and SVZ have the potential to differentiate into all lineages of the adult central nervous system (119, 178, 206, 229, 292).

NSCs in the SVZ and hippocampal SGZ develop into electrically mature and fully-integrated neurons (55, 171, 383, 399). Neural precursor cells born in the SVZ of the lateral ventricle migrate tangentially through the rostral migratory stream, guided by astrocytes (77, 236, 415) to the olfactory bulb where they become fully differentiated and electrically competent (see review (230)). In the SGZ of the hippocampus, progenitor cells predominately become neurons and functionally integrate into the DG (54, 171, 399). Adult hippocampal neurogenesis may play a
potentially critical role in hippocampal dependent learning and memory (137, 348, 397, 399), and deficits in neurogenesis (214, 308) may contribute to memory impairments observed in some neurodegenerative diseases, such as HD (Lione, Carter et al. 1999; Cybulsk-Klosowicz, Mazarakis et al. 2004; Grote, Bull et al. 2005; Mazarakis, Cybulsk-Klosowicz et al. 2005; Morton, Hunt et al. 2005; Van Raamsdonk, Pearson et al. 2005; Morton, Skillings et al. 2006; Nguyen, Kobbe et al. 2006; Pang, Stam et al. 2006).

1.2 Development of Newborn Neurons in the Adult Hippocampus

Dentate granule cells (DGCs) are generated beginning at E14 in the rodent brain, but 80% of DGCs derive postnatally. In the adult brain, newborn hippocampal neurons are born into a DG that is densely packed by 1 million GCs in a space of approximately 0.5mm$^3$ in the adult mouse (2, 153, 397). In the adult rat, cells proliferate at an estimated rate of 9,000 new cells per day, a rate that equals 6% of the total GC number per month (53). However, about half of NPCs die within the first four weeks of birth (193, 194, 306, 398). Cells that survive this initial high death rate period remain relatively stable for several months to a year post mitosis, the longest period studied thus far (194). Proliferating cells in the DG primarily generate neurons at baseline; i.e. approximately 75% of proliferating cells generate neurons and less than 10% differentiate into astrocytes (207, 397, 398, 437).

The ongoing birth of new neurons expands the population of GCs and increases the volume of the DG over time. Recent analysis has shown that unlike the SVZ-OB system in which continuous neurogenesis replaces existing neurons,
constitutive neurogenesis in the DG increases the volume of the DG (168, 227, 282). Using a long-term genetic label to mark NSCs and some NPCs in 8 week old (adult) mice, Imayoshi and colleagues showed that labeled GCs increase the total population of murine DGCs by approximately 15% at 6 months after induction of the label, an increase in volume that is stable over the 6 months the mice were further studied. Furthermore, ablating neurogenesis using irradiation or antimitotic drugs prevented this increase in DG volume (168). Similarly, using genetic fate mapping to label Dcx-positive NPCs, newborn neurons were estimated to contribute approximately 8% to the total number of NeuN-positive DG neurons (282). These data are consistent with reports that overall GC number does not change with age despite the well documented age-related decline in neurogenesis (31, 246).

1.3 Cell lineage and morphological development of newborn adult hippocampal neurons

The development of newborn neurons in the adult hippocampus resembles embryonic and early postnatal development of neurons (437), both morphologically and via the molecules and pathways that regulate this development. NSCs and amplifying progenitor cells give rise to postmitotic neuroblasts, immature neurons, and ultimately mature and integrated DGCs (54, 171, 399). Most newborn neurons expand into the inner side of the GCL (168, 207, 282) and predominantly remain on the upper blade of the DG (168, 282).

It has been suggested that the origin of newborn neurons resides in a subset of slow dividing, predominantly quiescent astrocyte-like SCs (3, 91, 194, 268, 341, 342).
These radial-glial-like SCs are positive for the astrocyte markers nestin, GFAP, vimentin and Sox2, and comprise two-thirds of nestin-positive cells in the SGZ. They are easily identified by their triangular soma and prominent radial processes extending across the width of the GCL and terminating in elaborate dendritic arbors in the molecular layer (104, 194). Radial-glial-like SCs are mostly quiescent, dividing infrequently, and may give rise to amplifying progenitor cells. Cell divisions in radial-glial-like stem cells account for only 5% of divisions by nestin-positive cells (194) and 2-5% of this population of cells appear to be active at baseline (comprising 23% of the total Ki67-positive dividing cells in the DG) (104, 370). The rate of cell division of radial-glial-like SCs is unresponsive to running and environmental enrichment, factors that can modulate the overall rate of neurogenesis in the DG (194).

Symmetric cell division has not been observed in nestin/GFAP-positive radial-glial-like SCs and fate mapping verification that these cells do indeed give rise to neurons has not been conclusively shown. However, a subset of Sox2-positive cells morphologically distinct from the nestin-positive radial-glial like population has been shown to have the SC property of multipotency and appear to self-renew in vivo (370). Lineage analysis reveals that these non-radial Sox2-positive cells form both neurons and astrocytes in vivo. Approximately 10% of these cells maintain Sox-2 expression at least 1 month after cell division and the pool of Sox2-positive SCs does not change after running though more Sox-2 positive cells are generated, suggesting that Sox-2-positive cells have self-renewal capacity (370). Furthermore, targeting single-cells in vivo by low titer expression of a Sox2 driven Cre/GFP expressing retrovirus revealed GFP expression in a GFAP- and Sox2-positive cell, suggesting that Sox2-positive
non-radial cells (presumably infected with the virus) may have the capacity to ‘de-
differentiate’ into radial glial-like cells (370). These data suggests that non-radial
Sox2-positive cells comprise a SC population within the DG, but whether these cells
are distinct from a population of nestin-positive radial glial-like SCs, or represent a
stage in a linear lineage from SCs with radial morphology to differentiated cells, or
whether non-radial Sox2-positive cells are the only SC population within the adult DG
remains to clarified.

Non-radial Sox2-positive, GFAP-, vimentin-, and Dcx-negative are highly
proliferative and represent nearly a quarter of dividing Ki67-positive cells within the
DG (194, 370). The rate of mitotic activity in these cells has been estimated at
between 5% (by co-localization with the mitotic marker Ki67) (370) and 20-25% (by
incorporating BrdU or expressing Ki67) (194). The variance in this range may be due
to the time points examined; rapid proliferation drops off dramatically less than 3 days
after the initial mitotic event, decreasing the proportion of transit amplifying cells that
are in cell cycle from over 20% at 3 days post division to less than 2% at 7 days post
division (198, 437). Progenitors amplify to increase the population of NPCs by up to 5
fold (104, 194, 437).

Proliferating Sox2-positive cells predominantly give rise to postmitotic
neuroblasts. Neuroblasts express Dcx, PSA-NCAM, Prox1, and β-III-tubulin, but not
nestin, Sox2, or NeuN. Dcx-positive neuroblasts migrate a short distance into the GCL
on the scaffold provided by radial glial-like SCs and extend rudimentary 10-20µm
processes. Almost all of these cells are postmitotic, in that they do not newly
incorporate BrdU, and are characterized by larger, 10-15 µm somatic bodies (104,
In contrast, less than 1% of neuroblasts carry short 1-5 µm horizontal processes and maintain mitotic activity, exhibited by BrdU incorporation (104), which may represent a distinct population within the neuroblast developmental stage.

As neuroblasts mature, their somas increase in diameter to greater than 15 µm, their horizontal processes elongate to roughly 40 µm, and they begin to extend processes obliquely into the GCL. Development progresses for an additional 2-3 weeks as immature Dcx-positive neurons continue to grow, begin to express the mature neuronal markers NeuN and calbindin, and begin to resemble mature granule cells (104, 194). This process of neurogenesis culminates in the formation and survival of mature granule cells that express neuronal markers NeuN, Prox1, Calbindin, and β-III-tubulin, but no longer express Dcx and PSA-NCAM. Mature granule cells have large soma (>20 µm), well developed apical dendrites, and axons that form a mossy fiber (104, 194). An analysis of how cell lineage is affected in HD is described in Chapter 4, and formed part of my thesis work.

1.4 Axonal and dendritic development of newborn adult hippocampal neurons

Axonal projections from newborn adult hippocampal neurons reach their appropriate targets by 10-11 days post mitosis (155, 249, 383, 437). Adult born hippocampal neurons establish functional synapses with CA3 pyramidal cells, mossy cells, and hilar interneurons (releasing glutamate as their primary neurotransmitter) within three weeks of their birth (184, 249, 383).

Dendritic growth and arborization in newborn cells provides important insight into the functional development and relevance of newborn neurons. In the OB, it
appears that dendritic patterning is predetermined (191), but whether a similar mechanism exists in the DG is unclear. By 48 hours post mitosis, the apical dendrites of Dcx-positive newborn hippocampal neurons, though somewhat thinner than those of mature GCs, first reach the molecular layer; by 96 hours, the dendrites of newborn neurons have ramified (343). Newborn adult hippocampal neurons receive synaptic input from the perforant path during the first 2-3 weeks post mitosis (107, 123, 437). By 30 days post cell division, newborn granule cells have long processes extending into the molecular layer and an arborized dendritic tree (207). Dendrites of newborn neurons grow at an estimated rate of 15µm per day for three initial days and double in length at 4 and 5 days post mitosis, a period of time that coincides with retraction of the basal dendrite (343). Dendritic arborization and synapse formation appear to be developmentally regulated, and GABAergic signaling may play a significant role in this process (123, 124).

1.5 Spine development of newborn adult hippocampal neurons

Synaptogenesis of newborn neurons in adult mouse hippocampus recapitulates that of earlier postnatal spine growth. Newborn neurons from fully functional synapses with mature CA3 pyramidal neurons and hilar interneurons and their dendritic spines resemble those of mature granule cells (383, 437). Interestingly, the initiation of spine development is slightly delayed in the adult mouse hippocampus as compared to postnatal counterparts (437). Like mature granule cells, newborn neurons have axosomatic, axodendritic, and axospinous synapses and do exhibit filopodia or small spines in initial phases of spine development similar to that observed during earlier
developmental periods (383, 384). Using a disabled retrovirus expressing GFP to label dividing cells, Zhao et al. quantified and compared the maturation of nascent spines on hippocampal neurons born in both the 7-10 week old adult brain and postnatal (P10) mouse brain. Initiation of spine formation occurred 4 days earlier in the postnatal brain compared to the adult brain (12 days post injection (dpi) in postnatal born neurons versus 16 dpi in adult born neurons). Dendritic complexity has been shown to be delayed by a similar amount of time. Spine density increased at peak rates between 3 and 4 weeks from $0.43 \pm 0.09$ spines/$\mu$m at 21 dpi to $1.95 \pm 0.08$ spines/$\mu$m at 28 dpi. Newborn neurons remained highly labile as spine density continued to increase, albeit at a slower rate, until 8 weeks post cell division when a spine density plateau of $2.61 \pm 0.07$ spines/$\mu$m was reached. Spine motility, however, continued during the entire period of spine development studied (up to 56 dpi) (437). Spine density is maintained at roughly 2.3 spines/$\mu$m even in one year old mice, the latest time point studied to date (266). Despite the well documented decline in neurogenesis that occurs with age (by approximately 40-fold for 10 month old mice), newborn neurons in the aged rodent brain reach a similar spine density (266), demonstrating the capacity for dynamic plasticity even in aged mice.

Newborn neurons initially make contact with preexisting synapses as most spines form within 200nm of preexisting boutons (384). Newborn neurons remain highly mutable for several months as spine rearrangement continues at high rates (384, 437) and mossy fiber bouton growth in CA3 continues to increase for several weeks after neuron genesis, eventually reaching the same size as mature GC’s preexisting
boutons (383). However, the size of mossy fiber boutons in the hilus (which are smaller than those of the CA3) did not change during maturation (383).

1.6 Enhanced excitability of newborn neurons

Neurons born into the adult hippocampus form fully functional synapses, display active synaptic transmission, and integrate into host circuitry (171, 209, 358, 383, 399, 437). Functional connectivity between mature GCs is virtually identical, regardless of whether neurons were born in the postnatal and embryonic brain, suggesting that neurons born in adulthood eventually contribute to a homogenous functional group of GCs (209). However, during the period of their development and maturation, newborn neurons have distinct electrical properties from those of mature granule cells and may contribute uniquely to processing of the DG.

Newborn neurons are ‘excitable’ and display elevated resting membrane potential and enhanced LTP (339, 356, 410, 438). Newborn neurons have a lower threshold for firing action potentials than mature granule cells, and due to the expression of low-threshold calcium channels, newborn neurons generate low threshold calcium spikes under physiological conditions. In contrast, mature granule cells generate similar spikes only in the presence of potassium channel blockers (339, 356). Newborn neurons display a lower threshold for induction of LTP as early as 2 weeks after birth (124, 339, 438). In addition to a decrease in threshold, enhanced LTP in newborn neurons is expressed by an increase in amplitude/magnitude of LTP in cells up to 6 weeks of age (124, 339), a property which is absent in neurons younger
than 4 weeks of age and lost in mature granule cells that are 4 months or older in age (124, 438).

In the early stages of development, the excitability of newborn neurons is largely regulated by response to GABAergic synaptic events. While there appear to be no difference in GABAergic inputs or inhibitory connectivity to GCs that are born in the adult or developing animal once they are fully mature, newborn neurons are electrically distinct from mature neurons in their response to GABA signaling (123, 210). Input to newborn neurons is initially exclusively GABAergic and tonic activation of nonsynaptic GABA\(_A\) receptors in immature/proliferating cells regulates differentiation and synaptic integration (107, 123, 124, 289, 385). GABA may act in concert with BDNF signaling as the NPC response to GABA\(_A\) receptor stimulation is absent in mice with depleted central BDNF (60). Young, 2-4 week old neurons are not inhibited by GABA as mature DG cells are, but rather are excited, due to the depolarized reversal potential of chloride and the absence of GABA\(_B\) receptor mediated potassium responses (12, 289). Newborn neurons exhibit slow rise and decay phases of response to GABAergic events, and along with depolarizing reversal potentials, these immature characteristics suggests that adult born neurons progress through similar stages as neurons in the developing brain (124).

GABAergic dependent depolarization in young neurons appears to play a role in the activity dependent regulation of newborn cells and provides a mechanism for “excitation neurogenesis coupling” (86, 124). Reversing the response of immature neurons to GABA by genetically manipulating the chloride importer NKCC1, thereby hyperpolarizing rather than depolarizing newborn neurons \textit{in vivo}, results in reduced
dendritic arborization and delayed synapse formation of both GABAergic and glutamatergic synapses (123). Similarly, reversing the chloride gradient impairs neurogenesis in the developing CNS (318). Furthermore, GABA-induced depolarization of NPCs promotes neural development by driving pro-neural gene expression, including NeuroD (385).

1.7 Activity dependent regulation of adult hippocampal neurogenesis

The ability of the NPCs to respond to network activity by promoting proliferation and survival of NPCs and neural differentiation has been termed “excitation neurogenesis coupling” (86). GABAergic depolarization of newborn cells favors neural production through the expression of pro-neural NeuroD and inhibition of pro-astrocytic Hes1 and Id2 genes. The interface of coupling between excitatory signals and regulation of neurogenesis occurs primarily through NMDA receptors expressed by NPCs (86, 379).

The proliferation and survival of newborn neurons, both in vitro and in vivo, is dependent upon input activity in a cell specific manner (379, 405). LTP enhances survival of 1-2 week old neurons in the DG (45). The NMDA receptor 2B is expressed in newborn adult hippocampal neurons early in their development and activation of NR2B regulates several developmental steps of adult neurogenesis, including neuronal survival and enhanced LTP, similar to that observed in the developing CNS (124). Blockage of NMDA receptors in vivo increases NPC proliferation, but has no effect on fate choice (52, 139, 379). Similarly, mice expressing low levels of the NR1 subunit show increases in proliferating cells in the
DG (48). Using a disabled retrovirus expressing Cre/GFP in dividing cells only in a floxed NR1 mouse, Tashiro and colleagues demonstrated that the critical period for activity-dependent regulation of cell survival corresponds to the period of peak synapse formation, between 2 to 3 weeks post mitosis. In addition, the administration of an NMDA receptor antagonist during this critical period increased the survival of NR1KO neurons by almost 50% suggesting survival of newborn neurons is competitive and depends upon activation of NMDA receptors (379).

Finally, glutamate is a positive regulator of neurogenesis and functional metabotropic glutamate receptor subtypes are found in both NSC and NPCs. Activation of metabotropic glutamate receptors regulates proliferation, differentiation, and survival of NPCs and self-renewal of NSCs. Knock-out of the mGlu5 receptor or antagonists to mGlu3,4 receptors decreases the number of NPCs in the DG and SVZ (for review, see (257)).

While the enhanced excitability of newborn neurons clearly plays a role in the maturation and integration of newborn, immature neurons, the significance of this property within the context of the function of newborn neurons remains unclear. It is logical that lower thresholds for both firing and induction of LTP, in addition to decreased inhibition (unpublished data from our lab), would allow immature neurons to provide a unique computational dynamic to processing within the DG and hippocampus. Indeed, Aimone et al. has suggested that it is by virtue of distinct physiological properties that immature neurons contribute uniquely to memory formation within the DG (4).
2. Function of adult hippocampal neurogenesis

2.1 The hippocampal formation: anatomy and connectivity

Adult hippocampal neurogenesis occurs in a highly specialized archicortical region, both anatomically and functionally. The functional contribution of newborn neurons is necessarily dependent upon the physiological context in which new neurons are born; neurogenesis must modulate or contribute to the function of hippocampus as a whole, and the DG specifically. Therefore, the anatomy and functional output of the hippocampal formation and its subregions will be briefly examined.

The hippocampal formation is thought to encode and consolidate memory (340, 363), play a role in memory retrieval (125, 298), and code information about space (102, 244, 245, 267, 272). Superficial layers II and III of the entorhinal cortex (the interface between the hippocampus and input regions from the cortex) feeds directly into the DG via the perforant path (reviewed by (419)). Granule cells are sparsely connected to CA3 excitatory pyramidal cells via mossy fibers (11), and given that mossy fiber synapses are large and close to the soma, provide a powerful input into CA3 (181, 325). It should be noted that single spikes within the DG fail to elicit neuronal activity in the CA3; rather, an increased spike rate in the DG is both sufficient to discharge CA3 pyramidal cells and increases the probability of firing within CA3 (156). Granule cells are also interconnected through hilar excitatory interneurons and receive recurrent inhibition through hilar inhibitory interneurons. Through sparse coding, the DG is thought to be responsible for pattern separation (181, 196, 250, 285, 324). At the cellular level, pattern separation by the DG occurs by virtue of the fact that granule cells have small place fields (260) exhibit low (sparse),
but highly reliable firing rates (181), and must overcome inhibitory interneuron input, and thus there is a low probability that a similar subset of DG cells will send input to the same CA3 neurons (285, 325).

The major trisynaptic loop of the hippocampus involves projections from the DG to CA3, CA3 to CA1, and CA1 in turn projects to deep layers V and VI of the entorhinal cortex through the subiculum (for review see (325)). Substantial recurrent connectivity, particularly that of CA3, has been hypothesized to participate in episodic and working memory (141, 197, 253, 388, 416). Place cells—CA3 and CA1 pyramidal neurons and DG granule cells—encode information about current and intended spatial location, and, along with grid cells and head-direction cells, are suggested to participate in formation of a cognitive map, remapping to novel stimuli/environments, and memory consolidation (5, 102, 115, 284).

2.2 The hippocampal formation: role of subregions DG, CA3, and CA1 in learning and memory

Hippocampal lesions or disruption of the function of the hippocampus impairs spatial working memory (22, 57, 217, 248, 418). In addition, hippocampal lesions appear to specifically impede recollection, but not familiarity (333). Macaques and humans with damage to the hippocampus or fornix are impaired at object-place memory tasks (46, 74, 297, 352), such as Paired Associates Learning of words in humans (74).

While rats with DG lesions (103, 255, 406), CA3 lesions (130, 151, 169), and CA1 lesions (83, 84) exhibit spatial working memory deficits similar to complete
hippocampal lesions, closer examination reveals that subregions of the hippocampus contribute differentially to learning and memory. CA3 appears to process spatiotemporal memory while the CA1 subregions participate in temporal processing (130, 164). Lesions or inactivation of CA3 leads to deficits in spatial working memory (130, 151, 169, 197). Specifically, CA3 and the trisynaptic pathway (DG-CA3-CA1) are thought to be important for rapid and one trial spatial and contextual learning (novelty detection) and to tune CA1 place fields (275, 276). CA3 is also thought to mediate pattern completion and learning of associations where space or sequence is a component (130, 134, 196, 222, 325). While pattern separation refers to a process of encoding, pattern completion is the process of retrieval of a whole representation/memory from an incomplete part. In contrast, CA1 participates in consolidating memory through establishing associations between the hippocampus and neocortex as well as processing temporal information (83, 84, 132, 164, 325).

Lesion and inactivation studies of the DG subregion result in impaired spatial working memory (103, 255, 281, 406). Several studies in rodents and humans have shown that the DG specifically functions in the formation of distinct representations of similar or interfering inputs (be it spatial, object-related, or episodic), a process termed pattern separation (20, 131, 132, 135, 164, 165, 196, 218, 221, 222, 254, 325, 425). Pattern separation is typically tested by comparing behavioral or physiologic response to conditions in which inputs are more similar to those in which inputs are dissimilar. In behavioral studies, a (novel) goal-related object/space serves as the input that must be separated from a familiar object/space, such as arms of a radial arm maze, (218). Similarly, pattern separation is often tested by comparing cellular response to
similarities/dissimilarities between environments in which an exploration task is performed, such as by manipulating the features of a testing box or room (218, 221, 222, 376). Lesions of the DG, and not lesions of CA3 or CA1, produce deficits when the similarities between spaces or objects to be discerned are maximal or testing environments are very similar, but DG lesioned rodents are not impaired when (spatial) interference is low (131, 132, 135, 164, 165, 218). Strictly speaking and with deference to its earliest conception, pattern separation is a process of encoding (250), and therefore the DG’s role in pattern separation is certainly required for initial memory formation. Whether the DG is important for memory reactivation or reconsolidation is unclear. It has been suggested that memory retrieval may bypass the DG altogether (196, 221, 222, 388). Once a spatial pattern separation has lead to the encoding of distinct spatial representations, presumably CA3 place cells are able to maintain (for at least brief periods of time) these ‘memories’ and selectively reactive them depending on experience (221, 222, 285, 376).

2.3 Function of adult hippocampal neurogenesis

Adult hippocampal neurogenesis has been implicated in learning and memory (137, 348, 397, 399) and may be necessary for the functional integrity of the circuits into which new cells are born (77, 348). Neurogenesis is thought to play an important role in the normal function of the DG in spatial learning and memory (88, 99, 435, 438). It has been noted that differences in basal neurogenesis of different mouse strains correlate with spatial learning and memory performance (192), suggesting that neurogenesis may positively contribute to learning and memory; however, the function
of adult hippocampal neurogenesis remains unclear. It has been suggested that at their earliest states of maturity, when newborn neurons are hyperexcitable, neurogenesis plays a role in the formation of temporal associations and pattern completion (4). However, the role of neurogenesis in pattern separation and pattern completion has not yet been investigated and forms the basis of my studies in Chapter 2.

Newborn neurons in adult hippocampus are functional (171, 399, 437) and integrate into hippocampal circuitry (171, 188, 313). Newborn neurons contribute to synaptic plasticity (LTP) in the DG (335, 356, 438), but the contribution of newborn neurons to changes in activity dependent responsiveness of CA3 neurons remains unclear. CA3 pyramidal neurons do respond differentially to granule cell activity (156, 213), and given the distinct electrophysiological properties of immature neurons, newborn neurons may have the capacity to contribute uniquely to encoding within the hippocampus. A critical period for response of newborn neurons to environmental cues occurs between 2-3 weeks post mitosis (348, 378) and cells trained during this critical period to respond to particular tasks or environments are preferentially activated by re-exposure to the same task/environment even after they have fully matured (188, 378). Thus, newborn neurons may contribute functionally to DG processing differentially depending on their maturity state and electrophysiological properties.

Approaches to investigating the function of adult hippocampal neurogenesis have followed a common design: knock-down adult born neurons in the intact adult hippocampus in mice or rats and then behaviorally challenge the animals in paradigms designed to test learning and memory. Approaches differ by the techniques used to
knock-down neurogenesis, the effectiveness and selectivity of various ablation techniques, the interval between treatment and behavioral testing, and the behavioral tests employed. The studies examining the function of adult hippocampal neurogenesis are summarized in Table 1 and discussed here in more detail.

Early studies on the function of neurogenesis often examined the behavioral effects of reducing adult neurogenesis using the systemic antimitotic agent methylazoxymethanol acetate (MAM). Spatial navigation in the Morris water maze and contextual fear conditioning were unimpaired following MAM treatment in rats, but the formation of trace memories was impaired (348, 349). However, the extent to which off-target effects, such as general perturbation of protein synthesis and signaling, confound results in MAM experiments has encouraged researchers to find more specific methods to ablate neurogenesis and the use of MAM treatment to address questions regarding the function of newborn neurons has generally fallen out of favor.

A common and widely used technique used for the long-term elimination of newborn neurons has been focal X-irradiation treatment of the hippocampus while sparing the eyes, ears, snout, and forebrain (including the SVZ). Irradiation techniques vary, but those used recently in mice have generally involved several low-dose (typically 5Gr) treatments spaced several days apart. A single 10-Gr dose or less decreases proliferating cells in the SGZ by up to 96% and induces a dose-dependent increase in apoptosis of NPCs that returns to baseline by 48 hours after treatment (30, 259, 302). Inflammation and other irradiation-induced responses return to baseline
after a period of recovery (258, 259). Irradiation does not appear to affect the proliferation of astrocytes or oligodendrocytes (30, 259, 262).

The behavioral outcomes of ablating neurogenesis using irradiation or genetic techniques have been mixed. Irradiation of rodents has been shown to disrupt both spatial memory (355) and object recognition (200). Both whole brain and focal irradiation of rats had no effect on performance in both T-maze match- and non-match- to place tasks, and did not impair long-term retention of a no-alternation rule, nor ability to reverse, in the T-maze (157). Genetic ablation of newborn neurons has resulted in impairments in the Morris water maze (99, 435) and spatial memory retention in the Barnes maze (168). However, following similar irradiation treatments, others have not found impairments in spatial learning and memory in either the Morris water maze (335, 420) or the Y-maze (335). The results of knocking down neurogenesis on contextual fear conditioning are equally contradictory. Irradiation and genetic ablation treatments have been shown to impair contextual memory in some instances (157, 168, 203, 335, 420), but have no effect in others (99, 435). In one case, ablation of neurogenesis using both irradiation and genetic ablation appeared to improve spatial working memory in a radial arm maze task when mice were forced to make choices between very similar (and repetitive) arm presentations (336).

The demand for techniques that selectively ablate adult neurogenesis within the DG without impacting the integrity of the mature DGCs, the hippocampus, and surrounding cortex (and can be demonstrated to do so unequivocally) is high. Our group has been working to develop new tools to selectively ablate neurogenesis in the adult DG while sparing mature DGCs and other brain regions. Lentiviral expression of
dominant negative Wnt, a protein that plays a role in the proliferation and neuronal maturation in the adult hippocampus (228) similar to its role in development, is one such example. Selectively reducing the numbers of newborn adult neurons locally in the DG using lentiviral expression of dominant negative Wnt resulted in object recognition impairments and spatial memory deficits on the Morris water maze that were dependent on the percentage of viral coverage of the DG (170). In addition to focal X-irradiation, I have also used lentiviral expression of dominant negative Wnt in my investigation of the functional role of adult hippocampal neurogenesis, which will be discussed in detail in Chapter 2.
<table>
<thead>
<tr>
<th>Ablation Method</th>
<th>Extent of Ablation (%) decrease BrdU+ cells</th>
<th>Species</th>
<th>Behavioral Task</th>
<th>Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAM</strong></td>
<td></td>
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<tr>
<td>Dose</td>
<td>Dose duration</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0-15 mg/kg</td>
<td>1 x daily, 14d</td>
<td>~80%</td>
<td>Rat (Sprague-Dawley)</td>
<td>Trace eye blink conditioning</td>
<td>(348)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Delayed eye blink conditioning</td>
<td>N.D.</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(no difference)</td>
<td></td>
</tr>
<tr>
<td>7 mg/kg</td>
<td>1 x daily, 14d</td>
<td>~75%</td>
<td>Rat (Sprague-Dawley)</td>
<td>Morris water maze</td>
<td>(349)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trace fear conditioning</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(no difference)</td>
<td></td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>1x daily, 14d</td>
<td>~50%</td>
<td>Mouse (C57BL/6 NCrljBgi)</td>
<td>Contextual fear conditioning</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(203)</td>
<td></td>
</tr>
<tr>
<td><strong>Irradiation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Dose</td>
<td>Recovery period</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>2 x 10Gr (d1,2)</td>
<td>1 month</td>
<td>~90%</td>
<td>Rat (Long Evans)</td>
<td>Morris water maze</td>
<td>(355)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- (long term retention only)</td>
<td></td>
</tr>
<tr>
<td>2 x 7Gr (d1, d2)</td>
<td>2 weeks or 8 weeks</td>
<td>36-68%</td>
<td>Rat (Long Evans)</td>
<td>T-maze (match- and non-match to place and reversal)</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>~36%</td>
<td></td>
<td>Contextual fear conditioning</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(short and long term retention)</td>
<td>-</td>
</tr>
<tr>
<td>3 x 5Gr (d1,5,8)</td>
<td>3 months</td>
<td>~75%</td>
<td>Mouse (129SvEv)</td>
<td>Radial arm maze</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(336)</td>
</tr>
<tr>
<td>3 x 5Gr (d1,5,8)</td>
<td>3 months</td>
<td>~85%</td>
<td>Mouse (129SvEv)</td>
<td>Contextual fear conditioning</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(335)</td>
</tr>
</tbody>
</table>

**Table 1.1:** Summary of ablation methods used to investigate the function of adult hippocampal neurogenesis and associated behavioral performance
Table 1.1: Continued

<table>
<thead>
<tr>
<th>Ablation Method</th>
<th>Extent of Ablation</th>
<th>Species</th>
<th>Behavioral Task</th>
<th>Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x 10Gr, 15Gr or 20Gr</td>
<td>3 months</td>
<td>~75%</td>
<td>Mouse (C57BL/6 NCrIjBgi)</td>
<td>Contextual fear conditioning</td>
<td>(203)</td>
</tr>
<tr>
<td>3 x 5 Gr (d1,3,5)</td>
<td>3 months</td>
<td>~90%</td>
<td>Mouse (129SvEv)</td>
<td>Noveltly-suppressed feeding</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

**Transgenics/Targeted Ablation**

<table>
<thead>
<tr>
<th>Ablation Method</th>
<th>Extent of Ablation</th>
<th>Species</th>
<th>Behavioral Task</th>
<th>Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP-TK (line 7.1)</td>
<td>~85%</td>
<td>Mouse (C57Bl6-BALBe mixed)</td>
<td>Contextual fear conditioning</td>
<td>-</td>
<td>(335)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cued fear memory</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>GFAP-TK (line 7.1)</td>
<td>~75%</td>
<td>Mouse (C57Bl6-BALBe mixed)</td>
<td>Radial arm maze</td>
<td>+</td>
<td>(336)</td>
</tr>
<tr>
<td>CreER-TM</td>
<td>~25% (estimated from previous experiments)</td>
<td>Mouse (C57Bl/6J)</td>
<td>Barnes maze, contextual fear conditioning</td>
<td>-</td>
<td>(168)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cued fear conditioning</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Tlx&lt;sup&gt;f/f&lt;/sup&gt; x Nestin-Cre</td>
<td>~66%</td>
<td>Mouse (backcrossing to C57Bl/6J)</td>
<td>Contextual fear conditioning, cued fear conditioning</td>
<td>N.D.</td>
<td>(435)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Morris water maze</td>
<td>~ -</td>
<td>(short term retention only)</td>
</tr>
<tr>
<td>Lentiviral expression dnWnt</td>
<td>~75% (Dcx+ cells)</td>
<td>Rat (Sprague Dawley)</td>
<td>Morris water maze</td>
<td>- (long term retention only)</td>
<td>(170)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Object recognition</td>
<td>- (short term retention only)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Novelty-suppressed feeding</td>
<td>N.D.</td>
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</tbody>
</table>
One explanation of the mixed findings following knock-down of adult hippocampal neurogenesis is that while neurogenesis may contribute to hippocampal-dependent learning and memory, its contribution may be specific and thus commonly used behavioral paradigms do not adequately tease out functional deficits. A second explanation is that mature granule cells within the DG or other hippocampal subregions can compensate for the absence of neurogenesis during learning and/or memory reactivation. It appears that with repeated trials and additional training, mice with lesions to or inactivation of the DG and/or CA3 can still learn spatial information incrementally, presumably mediated by CA1 (103, 218, 275, 276). While DG cells are initially active during a task that requires spatial working memory, CA3 and CA1 become active after repetitive training (310). If normal memory can be formed through compensatory mechanisms of the CA3 or CA1 following lesion of the DG, then these subregions may compensate for more subtle manipulations such as ablating neurogenesis. Accounting for all of the mechanism by which animals learn is difficult, and specified and well-controlled tasks are demanded in order to make sense of learning and memory dependent behaviors. Furthermore, many of the experiments designed to investigate the function of neurogenesis to date may not be sensitive enough (i.e. not difficult enough) to bring out deficits caused by eliminating neurogenesis. To investigate the role of neurogenesis in hippocampal processing, we must design unambiguous tasks that address specifically hypothesized functions of newborn cells. Finally, off-target effects of ablation techniques may cause damage to DG, other regions of the hippocampus, or other brain regions. More selective techniques to ablate adult born neurons at specific time points are warranted. At
present, different experiments using non-overlapping techniques, and presumably non-overlapping patterns of off-target effects, provide the best support for the functional contribution of adult hippocampus neurogenesis.

3. Modulation of adult hippocampal neurogenesis, and associated behavioral consequences, by environmental enrichment in WT mice

The contribution of newborn immature neurons to hippocampal function and memory is of interest in cases where memory is impaired, such as in neurodegenerative diseases like Alzheimer’s and Huntington’s, particularly regarding whether manipulation of endogenous stem cells and/or neurogenesis has the potential to impact or rescue deficits in memory and cognitive function. The rate of adult neurogenesis is dynamically regulated by a variety of physiologic and pathologic factors (17, 177, 295, 397). Among those neurogenesis-modifying stimuli studied thus far, environmental enrichment is particularly attractive in the context of neurodegenerative disease given the magnitude of the effect on the survival of newborn neurons, the behavioral improvements seen following periods of environmental enrichment, and the fact that it is a non-invasive treatment.

Exposure to an environment with stimulating and changeable features, typically toys, tubes, nesting material, and houses, often for prolonged periods of four weeks or longer, has striking effects on the survival and differentiation rates of adult generated hippocampal neurons (45, 110, 195, 398). The rate of survival of BrdU-positive cells in the DG after four weeks exposure to an enriched environment is similar to that seen after a four week exposure to a running wheel (398). However,
evidence indicates that the mechanisms modulating these effects are distinct: while running increases the proliferation of NPCs, environmental enrichment increases both the survival and percentage of proliferating cells that become neurons (286). Environmental enrichment also plays a role in neuronal viability and has been shown to maintain synapses (216) and may contribute to growth of the GCL (195). A component of environmental enrichment, learning, has also been shown to increase the survival of adult NPCs (98, 137, 190, 398).

Environmental enrichment has a positive effect on learning and memory of spatial or object-dependent information (45, 110, 195) and may prevent cognitive declines associated with normal aging (216). Even short exposure to an enriched environment of one week may be sufficient to improve spatial learning and memory (173). While improvements in hippocampal-dependent cognitive functions following environmental enrichment correlate with increases in neurogenesis (45, 195, 216), at least one report has suggested that the beneficial effects of environmental enrichment on spatial learning and affective behavior are not dependent on neurogenesis. Enriched irradiated mice (with decreased neurogenesis) demonstrated both equivalent spatial learning and memory to sham enriched mice, and behavioral performance was better than both irradiated and sham non-enriched mice (258). However, in this study, the Morris water maze task used was not itself sensitive to neurogenesis, as irradiated sham mice showed no deficit in acquisition and probe trials, leaving open the possibility that gains in learning and memory following enrichment are dependent on neurogenesis and will only be observed following the employment of neurogenesis-sensitive tasks.
PART II: Studying adult hippocampal neurogenesis in the context of neurodegenerative disease: relevance to Huntington’s disease (HD)

Recently, pathologic conditions characteristic of a number of neurodegenerative diseases such as Huntington’s, Parkinson’s and Alzheimer’s diseases, have been linked to changes in adult neurogenesis (78, 162, 214, 215, 308, 322, 377). Abnormalities in adult neurogenesis in the context of neurodegenerative disease may contribute to net cell loss or circuit disruption (15). The ability to tease apart dysfunction caused by abnormal neurogenesis from other pathologies in the degenerating brain is particularly difficult, especially considering the potentially subtle normal functional contribution of adult neurogenesis to processing within neurogenic regions coupled with widespread pathology observed in many neurodegenerative diseases. In addition, it is not clear whether abnormalities in adult neurogenesis in the context of neurodegeneration contribute to disease progression and/or cognitive dysfunction or whether neurogenesis is merely a sensitive, secondary read-out for other degenerative changes. Understanding abnormalities in adult neurogenesis in the context of the neurodegenerative environment may inform our understanding disease pathology as well as the function of neurogenesis under normal conditions. In addition, neurogenesis has been shown to respond to non-invasive physiologic stimuli as well as pharmacologic agents, and represents a viable and accessible therapeutic target.

The question of the functional significance of changes in the plasticity of the adult brain, and particularly that of adult neurogenesis, in the context of neurodegenerative disease warrants investigation. However, approaching this problem
requires care. Firstly, we must have an informed understanding of the function of the
system in question under normal, wild-type conditions as well as robust and consistent
tests to investigate proposed functions (see Chapter 2). Secondly, we must have
adequate models, animal or otherwise, through which we can probe our questions.

Huntington’s disease (HD) is an attractive model to study the effects of
neurodegeneration considering that the disease is caused by a single known gene,
unlike most other neurodegenerative disorders (e.g., Parkinson’s disease). Despite the
fact that more in depth analysis of HD pathology, its underlying causes, and effects on
cognition and function reveals that the mutant Htt protein and the disease itself are
substantially more complex than the seemingly genetic simplicity of this disease
would initially suggest, the identification of the gene underlying HD has nonetheless
permitted the creation of several animal models of HD, forming a window into the
disease. Furthermore, expression of the mutant Huntingtin transgene in rodents has led
to stable and consistent modeling of neurologic pathologies and behavioral deficits
and several of the transgenic HD models, including the earliest transgenic model, the
R6 line, have been well characterized.

Abnormalities in adult neurogenesis as exhibited by both HD patients (78-80)
and transgenic HD mice (26, 128, 129, 214, 215, 308, 380) may have clinical
relevance for patients afflicted with HD. While striatal pathology contributes to some
motor and cognitive defects of HD patients (13), non-striatal pathology, especially that
of the hippocampus, may mediate aspects of affective and cognitive deficits in HD
(78, 301, 327). Investigating the functional significance of abnormalities in adult
hippocampal neurogenesis in the context of HD and underlying causes may contribute
to our general understanding of whether abnormal plasticity in the adult diseased brain contributes to disease pathogenesis and/or progression, as well as inform our understanding of the function of newborn cells in the adult brain.

4. Huntington’s Disease Background

4.1 Clinical and genetic overview

Huntington’s disease (HD), for which there are currently no disease modifying treatments, is a genetically dominant neurodegenerative disease characterized by movement abnormalities, cognitive impairments, dementia, and affective disturbances (154). The disease typically presents in mid-life, with progression lasting for approximately 20 years and eventually resulting in death (114). Pathological CAG expansions within exon 1 of the huntingtin (Htt) gene on the short arm of human chromosome 4 results in the progressive degeneration of the basal ganglia (caudate nucleus and putamen), cerebral cortex, brainstem, spinal cord, thalamus, and hypothalamus (1). The hallmark of HD is the progressive loss of medium-spiny GABAergic neurons in the striatum (58, 85, 305, 403, 404), but degeneration of cortical and hippocampal neurons (85, 327, 360, 403) contributes to both disease progression and cognitive deficits observed in HD, and probably occurs in parallel with striatal loss from disease onset.

In 1872 Dr. George Huntington gave the first medical account of the hereditary disease that would later become his eponym. Over a century later the HD-causing gene was cloned (1). The human Htt gene is located at IT15 and is comprised of 67 exons totaling 180 kb coding for a 350 kDa protein that is ubiquitously expressed.
Greater than 35 trinucleotide CAG repeats, beginning 17 codons downstream from the ATG initiator of exon 1, leads to pathological expansion of glutamine residues within the Htt protein. Exon 1 is less than 1 kb in length. Two primary messenger transcripts are produced from *Htt*, comprising either 13.7 or 10.3 kb, and the human Htt protein is composed of 3144 amino acids (1).

HD is caused by 36 or more CAG repeats in exon 1 of the *Htt* gene. There is an inverse relationship between the age of disease onset and the number of CAG repeats; repeats of 40-50 are typical in adult onset HD cases, and repeats greater than 50 result in the more severe juvenile onset form of HD (1, 389). Non-pathological *Htt* typically contains 17-20 repeats, and repeats within the range of 27-35, while below the clinical threshold for diagnosis, can expand into the disease range of greater than 36 repeats over successive generations (1, 167). Repeats in the range of 36-41 may result in incomplete penetrance (256, 312). A decrease in the age of onset and an increase in the severity of the disease over successive generations is a result of anticipation through meiotic instability which is greater in spermatogenesis than oogenesis (315, 389). Somatic instability has also been observed HD, and may be related to disease progression within individuals (186).

4.2 The Huntingtin protein is required for CNS formation and neuronal viability

Htt is required for CNS formation (278, 414, 432) and cellular viability of adult neurons and testis (94). Htt is necessary for embryonic development; deletion of the Htt homologue in mice is embryonically lethal between day 7.5 and 10.5, a period critical for neurogenesis and CNS formation (278, 414, 432). However, expression of
mutant Htt at a level comparable to expression levels of the Htt murine homologue can rescue lethality of the null mutant (414). Deletion of a single WT allele results in no pathological features (100). CAG repeats themselves appear to be dispensable at least during embryonic stages (69).

The Htt protein is ubiquitously expressed in the brain during all stages of development in rodents and is expressed in humans at midgestation (19-21 weeks). Between P7 and P15 in the rodent, a period of considerable postnatal neurogenesis, Htt expression is increased by 60% to levels that remain stable into adulthood (38). The induction of Htt expression in neuronal subtypes follows the pattern of neurogenesis and neuronal maturation during development: Htt is first initiated in the basal forebrain and brain stem from E15-17, the cortex from P0-1, and the striatum from P7 (38).

4.3 Localization of WT and mutant Huntingtin and functional relevance

WT Htt is expressed at high levels in the cytoplasm, with low levels of expression found in the nucleus (189). Both WT and mutant Htt can be cleaved by caspases to generate N-terminal fragments which localize mainly to the nucleus (117, 140, 251). Mutant N-terminal fragments appear to be more toxic than the full-length mutant (117, 140) and nuclear localization of mutant Htt enhances toxicity in both cultured cells and in vivo (332, 338). Furthermore, the Htt mutation interferes with its nuclear translocation, causing an accumulation of mutant Htt in the nucleus (73, 426). N-terminal fragments of mutant Htt are sufficient to produce both intranuclear
inclusions (291, 338) as well as other characteristics of HD pathology in mice and primates (82, 291, 338).

One of the hallmarks of HD pathology is the formation of inclusions containing mutant Htt in the nucleus and cytoplasm of neurons (90). In the human cortex, the density of inclusions correlates with CAG repeat length (90) and the formation of nuclear inclusions in HD transgenic mice (R6) precedes neurological symptoms (82). Aggregate formation correlates with cellular toxicity and disease progression in mammalian culture systems, and neurons and glia of transgenic mice and primates (148, 237, 337, 407, 424, 429). Whether inclusion formation is deleterious to cell survival (148) or neuroprotective (16, 329) is the subject of ongoing debate.

The Htt protein is associated with the plasma membrane, endocytic pathways, autophagic vesicles, endosomal compartments, the ER, Golgi, mitochondria, microtubules, DNA, and nuclear components of transcriptional regulation (18, 33, 111, 189, 288, 321, 428). Mutant Htt has been implicated in disrupting axonal transport, endocytosis, protein trafficking, and synaptic transmission, and plays a role in dysregulation of transcription, and neurotransmitter and apoptotic systems (see Introduction, Part III for a more in-depth review).

4.4: A review of the function of the Huntingtin protein and dysfunction caused by mutant Huntingtin (and possible/theoretical relevance to abnormalities in adult hippocampal neurogenesis)

4.4.1 The Huntingtin protein
Although the HD-causing gene was identified well over a decade ago (1) the function(s) of normal Htt and biochemical processes by which mutant Htt causes HD have not been fully elucidated. The large size of the Htt protein makes isolation and biochemical analysis of the protein difficult and Htt has little or no homology with other known proteins. Furthermore, given that Htt is expressed throughout the cell and in most tissues, and has over 200 interacting partners, the ability to deductively hypothesize about the precise functional abnormalities of mutant Htt that underlie disease pathogenesis and progression in HD is limited (167). While aggregation of mutant Htt appears to be cell autonomous (144), the persistent presence of the mutant Htt protein and pathological cell-cell interactions are necessary for the development of significant neurodegeneration and functional deficits (144, 145, 427). Thus, while HD appears to be a relative simple model of neurodegeneration given that a single known gene is at its root, its underlying cellular pathologies and the consequences of mutant Htt are complex. Understanding the function of WT Htt and the loss and gain of function(s) induced by mutant Htt remains key to understanding: (1) pathogenesis in HD, (2) underlying causes of abnormalities in adult neurogenesis in the context of the degenerative environment, and (3) treating HD patients.

Examination of the structure of Htt reveals insights into its possible functions. Upstream of polyQ repeats and a proline-rich domain in exon 1, the first 17 N-terminal amino acids of Htt provide a substrate for SUMOylation and ubiquitination (182), post-translational modifications that can differentially affect localization, nuclear export, and half-life in the WT and mutant proteins. Htt is thought to be a superhelical solenoid with a diameter of ~200 Angstroms and the presence of ten
HEAT repeats distributed throughout the length of the protein favor this interpretation (225, 374). Like other proteins with high HEAT content, the alpha helical structure of Htt suggests Htt and its interactors may be involved in scaffolding, intracellular transport, microtubule dynamics, and chromosome segregation (225, 374). In addition, Htt contains a conserved nuclear export signal at the C-terminus (426), and a nuclear export signal has also been suggested for the 17 amino acids of the N-terminus (73, 426). In addition to SUMOylation and ubiquitination, Htt is also post-translationally palmitoylated by Hip14, a modification that is essential for its trafficking and distribution, particularly at the cellular membrane (163, 429).

4.4.2 Axonal Transport and Htt interaction with the dynein/dynactin complex

Htt facilitation of bidirectional long and short term transport along microtubules directly and through interaction with Hap1 has been shown in mammalian cells, Drosophila, and mouse models (59, 122, 146, 226, 290, 326, 390, 428). Htt interacts directly with dynein/dynactin microtubule motor complex in retrograde transport in vivo and in vitro and through its interaction with Hap1, Htt participates in kinesin-dependent anterograde transport to regulate lysosome/endosome trafficking (59, 122, 146, 226, 290, 390). Phosphorylation of WT Htt by Akt is thought to act as a switch favoring anterograde over retrograde vesicle transport in neurons by recruiting kinesin 1 to the dynactin complex; retrograde transport is promoted by the detachment of kinesin 1 following Htt dephosphorylation (71). Furthermore, the IGF-1/Akt pathway favors anterograde transport in a Htt-dependent manner and may compensate for transport defects caused by mutant Htt (71, 431). Expression of mutant Htt or loss of Hap1 decreases association with
dynactin and kinesin light chains, and impairs neurite outgrowth through, at least in part, an impairment in internalization, trafficking, and stabilization of the NGF receptor TrkA (326). Furthermore, mutant Htt inhibits vesicle, mitochondrial, and receptor transport (e.g., of the EGF receptor) along neuronal projections, and impairs actin polymerization via its interaction with profilin (47, 122, 146, 226, 290, 390).

4.4.3 Endocytosis, and vesicle and protein trafficking

In addition to participating in axonal transport, Htt also interacts with cellular organelles integral to protein processing and trafficking, including the Golgi apparatus and the endoplasmic reticulum. Disrupting WT Htt or the mouse homologue in vitro via RNAi disrupts the Golgi apparatus and induces aberrant organization of the endoplasmic reticulum (59, 287). In addition, abnormalities in cholesterol biosynthesis and trafficking result in an intracellular accumulation of cholesterol in striatal neurons of transgenic mice (391, 393, 394). Cholesterol transcriptional dysregulation has been observed in human HD striatal and cortical tissue (394).

Htt participates in both clathrin mediated and non-clathrin mediated vesicle trafficking and recycling (353, 369, 372, 391), which likely contributes to abnormalities in trafficking of synaptic transmission machinery observed in HD patients and mice (116), and deficits in synaptic communication and plasticity observed in transgenic HD mice (127, 202, 241, 274). Htt is expressed both presynaptically (116, 296, 411) and as part of the postsynaptic density, where it indirectly complexes with NMDA and kainate receptors through the SH3 domain of PSD-95 (see (109) for review).
The Htt interacting protein Hip 1 modulates presynaptic plasticity, and endocytosis and intracellular transport of synaptic and signaling molecules including synaptobrevin (265, 296). Hip 1 is normally enriched in mouse and human brain, particularly in the hippocampus (411). Hip 1 has a reduced binding preference for mutant Htt (265). Delta-Notch signaling plays a key role in regulating genes important for NPC survival and differentiation, and transduction of the Notch signal depends upon endocytosis of the cleaved intracellular portion of the transmembrane receptor and translocation to the nucleus. Through its endocytic and transport functions, Hip 1 plays a key role in Notch mediated neurogenesis (265). Notch signaling is crucial to the regulation of both embryonic and adult neurogenesis. High notch expression maintains NSC populations in the SGZ, induces proliferation of NPCs, influences cell fate determination, and mediates dendritic complexity of newborn neurons in vivo (41, 261), while low Notch expression induces proliferating NPCs to withdraw from the cell cycle, migrate, and differentiate (41, 161, 198).

The mutant Htt dysregulation of another important class of receptors (NMDA receptors) is thought to play a key role in mediating neuronal vulnerability and death and NMDA receptor mediated excitotoxicity has been a pervasive hypothesis of the underlying cause of neurodegeneration in HD (109). An imbalance in subunit expression in HD increases NMDA evoked currents and calcium transients to levels toxic to cells (6, 108, 224, 433, 434). Most of the work regarding the contribution of activity-dependent toxicity to HD progression has focused on medium spiny neurons of the striatum, cells particularly vulnerable in HD. Diseased medium spiny neurons are characterized by increased insertion of NR2B subunits into NMDA receptors, a
depletion in NR2A subunits, and probable decrease in NR1 subunits (6, 32, 108, 224). WT Htt negatively regulates the trafficking of glutamate receptors as well as their function (372, 373) and mutant Htt appears to dysregulate receptor subunit expression by accelerating the forward trafficking of NR2B subunits or increasing their available pool (32, 108). A similar imbalance in NR2B subunit expression and an increase in NMDA receptor binding has also been reported in the hippocampus (32, 224, 239). Neurotransmitter dysregulation, including an increase in glutamate transmission, reported in HD transgenic rodents, likely exacerbates receptor-mediated excitotoxicity (28, 240, 319).

4.4.4 Autophagy

Normal catabolic turnover and recycling of proteins and other cellular components by autophagic processes is essential to cellular health. Loss of autophagy in murine neurons leads to neurodegeneration, motor dysfunction, inclusions, and the appearance of hind-limb clamping, hallmarks of HD as expressed by transgenic mice, even in the absence of other disease causing agents (152, 205). Htt and its mouse homologue Hdh are critical to autophagic control (69, 432).

4.4.5 Apoptosis

Loss of Hdh results in high levels of apoptotic cell death in the ectoderm and embryonic lethality (432). Overexpression of WT Htt protects against injury induced apoptosis (159, 320). Htt may also regulate the cell cycle, and Hdh lacking its 7 polyglutamines has been shown to cause cell cycle arrest in fibroblasts in vitro (69). The effect of Htt on apoptotic cell death may result from the interaction of Htt with HIP 1, an endocytic protein that influences the transport and function of clathrin.
endocytosed AMPA and NMDA receptors. HIP 1 induces apoptosis through caspase cascades (65).

4.4.6 Transcription regulation by WT Htt and dysregulation by mutant Htt

WT Htt is involved with transcriptional regulation by acting on transcriptional machinery, transcription factors, and influencing mRNA expression (33, 76, 89, 97, 311, 374, 426, 428). Transcriptional dysregulation may result from a loss of function or a deleterious gain-of-function of the mutant Htt protein (167). Abnormal interaction between mutant Htt and DNA, in a polyglutamine dependent manner, alters mRNA expression (33). In addition, abnormal regulation of transcription factors, including Sp1, and alteration of the histone code appear to be important components of pathologic progression in transgenic rodent and cellular models of HD (201, 240, 369, 440, 442). Mutant Htt interaction with the CREB and its transcriptional co-activator CBP, leading to CBP sequestration in Htt aggregates and cellular toxicity (180, 187, 283, 366). Mutant Htt also impairs the histone acetylation function of CBP which leads to transcriptional repression (283, 366).

In addition to acting as a transcriptional activator (180, 442), WT and mutant Htt can act as transcriptional repressors (97, 189) by sequestering transcriptional machinery such as RNA polymerase II or impairing transcriptional regulation and processing (240, 334). The well characterized role of mutant Htt in transcriptional dysregulation of BDNF has been discussed. In addition, depletion of Htt or expression of mutant Htt compromises RNA mediated gene silencing in vitro via interaction with Argonaute and associated P bodies (334). Transcriptional dysregulation by mutant Htt may be particularly disruptive given that Htt appears to regulate transcripts for
proteins with a myriad of functions including secreted proteins important in receptor binding and hormone signaling, and the integrity of the extracellular matrix, e.g., murine SFRP-1 which is involved in Wnt signaling (369).

4.4.7 A model explaining the interaction of CAG repeat length on late disease onset in HD

An open question in the study of all age-related neurodegenerative diseases concerns the biology governing the late onset manifestation. This is of particular interest in HD given that the mutant gene is present from conception and development appears grossly normal in HD, though some abnormalities may precede clinical diagnosis of the disease. One interesting model explaining the age-related onset of HD proposes that the age of onset and disease progression in HD is determined by the rate of CAG expansion in somatic cells (186). This model fits nicely with data correlating increased CAG repeat number with a decrease in age of onset (1, 389) and the observation that HD patients homozygous for the mutant gene do not have an earlier age of onset, but may exhibit faster disease progression (277, 364). Kaplan and colleagues hypothesize that HD manifests when somatic repeat expansion reaches a pathological threshold in a sufficient number of cells and that the rate of disease progression is determined by the number of cells reaching the pathological threshold (predicted to be 115 repeats) at any period of time. Variability in repeat length is initially negligible since all cells inherit the same allele size. As cells age, instability, resulting from slippage or mishybridization of the two DNA strands during cell division or repair due to high complementarities of CAG repeat sequences, expands CAG repeat number differentially and stochastically in somatic cells. The process is
self-perpetuating and self-accelerating in that the probability of mishybridization increases with an increase in repeat length. Age of onset thus results from a critical number of cells reaching the disease threshold, those patients inheriting higher repeats will reach this threshold faster than those with lower repeat number. Rate of disease progression is determined by the cellular distribution of repeats: a fast progression rate results from increased numbers of cells reaching pathologic thresholds. Homozygosity does not confer an additional decrease in age of onset because repeat length is the critical factor in onset; rate of progression is faster in homozygous patients because the repeat distribution is narrower. Patients with juvenile onset (resulting from CAG repeat lengths greater than 55 repeats), have both decreased age of onset due to accelerated somatic instability, and faster disease progression due to increased likelihood that more cells will reach the pathological threshold faster (186). The model also predicts that for transgenic and knock-in mice to exhibit disease symptoms during their short lifespan, they must be born with disease alleles close to the pathological threshold, a prediction that is consistent with the transgenic models currently in use (247).

5. R6 transgenic mouse model of HD: neuronal and behavioral dysfunction

5.1 Generation of the R6 transgenic mouse line

The generation of the first HD transgenic mouse line, R6, has facilitated examination of neuropathological changes in the adult murine CNS, reflecting changes that also occur in human HD patients (56, 247). The R6 line is particularly useful in the study of HD due to its accelerated disease phenotype. The presence of rotorod
motor deficits are apparent in R6/1 mice after 15 weeks of age (252, 294) and in the R6/2 by as early as 5-6 weeks of age (56); overt motor symptoms, including hindlimb clasping, can occur as early as 4 months of age (247) and 8 weeks of age (56) in R6/1 and R6/2 mice, respectively. The R6 transgenic line overexpresses a 1.9 kb fragment of the human Htt gene including approximately 1 kb of 5’ UTR, promoter regions, exon 1 of the human mutant Htt protein and 262 bp of intron 1 on a CBAxC57Bl/6 background (247). The R6/1 line is comprised of a single gene insertion and 116 CAG repeats in the coding region of exon 1. The R6/2 line functions as a single copy integrant, though it was created by a triple insertion and subsequent deletion at two of the three insertion sites. The R6/2 transgene contains 144 or more repeats and codes for a mutant Htt protein predicted to be 23 kD (247). It is important to note that in mouse transgenic models of HD, overexpression of the mutant human Htt gene occurs alongside normal expression of the mouse homologue Hdh. Hdh is a 3120 amino acid protein containing 7 CAG repeats interrupted at the third triplet with CAA (24). It is also important to note that the observed increase in CAG repeat length as a colony ages (due to anticipation), a statistic that is not reported, may be important to interpreting findings based on studies involving animals from different colonies and may make comparative studies difficult.

5.2 Neuropathology in the R6 mouse model

Neuropathology in the R6 model is consistent with that of early stages of HD. Both R6/1 and R6/2 mice exhibit progressive motor (56, 232, 247) and cognitive deterioration (127, 232, 274, 361). Brain volume is reduced overall by up to 19% in
later disease stages in the R6/2, consistent with the YAC128 HD transgenic model (400), but formation of the CNS appears to be grossly normal, including formation of the DG and hippocampus (247). However, cortical and pyramidal neurons in both R6/1 and R6/2 transgenic HD mice show decreased spine density and a reduction in spine shaft diameter, decreases in dendritic field size, and reduced dendritic arborization (202, 361). Cytoplasmic and ubiquitinated nuclear inclusions of mutant Htt (82, 223) in the R6/2 line are similar to neuronal intranuclear inclusions observed in postmortem human HD tissue (90), and are found in high concentrations within the hippocampus of transgenic mice (270). The development of neuropil aggregates and nuclear inclusions in R6/2 cortical, striatal, and hippocampal neurons correlates with the development of neurological symptoms and increases dramatically with age and disease progression (223).

While aggregation of mutant Htt appears to be cell-autonomous (144), the persistent presence of the mutant Htt protein and pathological cell-cell interactions are necessary for the development of significant neurodegeneration and functional deficits (144, 145, 427). The expression of mutant Htt in cortical pyramidal neurons alone is insufficient to induce neurodegeneration and motor deficits; however, cell-cell interactions between cortical interneurons expressing mutant Htt and diseased striatal neurons is sufficient to cause neuropathology and induce motor deficits (145). In addition, surgical deafferentation of corticostriatal pathways and chemical deafferentation of nigrostriatal pathways in the R6/2 transgenic mouse ameliorate neuronal atrophy, lower glutamate levels (thereby potentially decreasing excitotoxicity), and decrease Htt aggregates in the striatum, as well as improve motor
function and extend survival (365). Using chimeric models to increase the longevity of diseased neurons, it has been shown that the growth of nuclear inclusions, striatal degeneration, and neuronal death appears to depend upon the presence of a diseased environment (317).

6. Abnormalities in adult neurogenesis in HD

6.1 Abnormalities in neurogenesis in HD

Degenerative diseases and other pathologic conditions, including Huntington’s, Parkinson’s, and Alzheimer’s diseases, depression, ischemic brain injury, epilepsy, and stress have been linked to abnormalities in adult hippocampal neurogenesis (138, 162, 199, 215, 308, 322, 367, 368, 375, 377, 421). Abnormalities in neurogenesis in the context of neurodegenerative disease, such as HD, may contribute to net cell loss or circuit dysfunction (15).

Abnormalities in adult neurogenesis as exhibited by transgenic HD mice (26, 128, 129, 214, 215, 308, 380) and abnormalities in proliferation in the SVZ in HD patients (78-80) may have clinical relevance for patients afflicted with HD. While striatal pathology contributes to some motor and cognitive defects of HD patients (13), non-striatal pathology, especially that of the hippocampus, may mediate aspects of affective and cognitive deficits in HD (78, 301, 327).

6.1.1 Abnormalities in the neurogenic region SVZ in HD patients

While the effect of HD on adult human hippocampal neurogenesis has not been examined, cell proliferation in the adult human HD SVZ has been previously investigated. Increased cell proliferation in the human HD SVZ and SVZ thickness
correlate with pathological grade and CAG repeat length (78). It is unclear whether
this increase in proliferation is a protective response by the CNS and represents a
replacement of damaged cells in the striatum, or whether this upregulation in
proliferation contributes to pathology in the striatum. Most of the cells born in the
diseased SVZ exhibit phenotypes of glial cells (progenitors or mature GFAP-positive
cells) with fewer neurons (78, 80). Furthermore, this effect is region specific—there is
increased proliferation in the central and ventral regions of the SVZ, while striatal
atrophy occurs mostly in the dorsal region (79). The potential positioning of newborn
neurons in close proximity to the degenerating striatum, in addition to the possibility
that neurogenesis may by unregulated in this region in response to damage in HD,
raises the possibility that the brain may have at least a partial capacity to compensate
for neurodegeneration (70). Indeed, in a rat lesion model of HD, SVZ progenitor cells
have been shown to migrate from the site of their birth toward the lesion site (380).
However, conflicting results have been observed in SVZ-derived neurogenesis in HD
transgenic mice. While one group has seen an upregulation of neurogenesis in the
SVZ of R6/2 mice (26), our group has demonstrated normal levels of neurogenesis in
the SVZ of R6/2 mice (307, 308) and others have similarly found comparable levels of
baseline SVZ neurogenesis between WT mice and HD transgenic mice in the
N171Q82 HD rodent model (96). A better understanding of the mechanisms
underlying adult neurogenesis specifically within the context of HD would contribute
to our ability to harness the therapeutic potential of these cells (70).

6.1.2 Abnormalities in adult hippocampal neurogenesis in HD transgenic mice
NPC proliferation, survival, and morphology are abnormal in the DG of mice transgenic for the human HD gene (128, 129, 214, 215, 308), an effect that can be partially restored by environmental enrichment (129, 214, 215) (see summary below). NPC proliferation and survival are progressively reduced in the DG of R6/1 and R6/2 mice (128, 129, 214, 215, 308), especially late in disease progression (128, 214). Newborn, immature neurons have fewer, smaller, and irregularly shaped soma, shorter and less branched neurites, and migrate shorter distances into the GCL (215, 308). Fate determination of precursor cells appears unchanged (215). Htt expression coincides temporally and spatially with neurogenesis (38) and at least one report suggests that mutant Htt may alter neurogenesis through abnormalities in Hip1/Notch signal transduction (265).

Neurogenesis appears functionally unresponsive to induction by lesion in the R6/2 model. Neurogenesis in R6/2 mice is resistant to upregulation induced by QA and KA lesions (308), but upregulation of SVZ neurogenesis does occur following stroke (Phillips et al., 2007, unpublished data). Cytokines that are shown to prevent excitotoxicity and increase neurogenesis in WT animals have no apparent effect in the R6/2 DG (128). In one study, R6/2 mice with access to activity wheels did not exhibit improvements in neurogenic deficits (204), but, given the severity of motor deficits in HD mice, controlling for time spent running is difficult.
### Table 1.2 Summary of abnormalities in the adult neurogenesis in R6/2 and R6/1 HD transgenic mice

<table>
<thead>
<tr>
<th>Cell/Process of Interest</th>
<th>R6/2</th>
<th>R6/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferating Cells (BrdU+)</td>
<td>Decreased BrdU+ and Ki67+ cells as early as 3.5 wks age (decreased by half by 8 wks age) (129)</td>
<td>(1) Decreased number of BrdU+ cells at 10 wks age (2) No significant difference in %BrdU/NeuN (214, 215)</td>
</tr>
<tr>
<td>Immature Neurons (Dcx+)</td>
<td>(1) Dcx+ cells: decreased number, branching, and migration into GCL (129, 215, 308) (2) NPCs fail to upregulate in response to QA, KA seizures (308)</td>
<td>Similar abnormalities in morphology as seen in R6/2 (215)</td>
</tr>
<tr>
<td>NPCs In vitro</td>
<td>Proliferation rate and differentiation fate of R6/2 dentate-derived NPC is comparable to WT-derived cells (308)</td>
<td>Has not been examined</td>
</tr>
<tr>
<td>Effect Environmental Enrichment</td>
<td>Has not been examined</td>
<td>EE from 4wks age: (1) delays motor onset disease (396) (2) Partially rescues deficits in the number of BrdU+ cells at 22wks age (214)</td>
</tr>
</tbody>
</table>

### 6.1.3 Rescuing deficits in neurogenesis in HD rodent models improves cognitive functioning

Strikingly, the behavioral impact of rescuing or restoring adult neurogenesis in HD rodent models has shown promise for the therapeutic potential of endogenous NPCs. Induction of neurogenesis through trophic factors or antidepressant treatment has alleviated some motor and cognitive deficits in HD rodent models. In an HD mouse model, treatment with FGF-2, a neuroprotective growth factor, increased cell proliferation by 150% in the SVZ (compared to 30% in WT littermate controls) with a
concomitant increase in immature Dcx-positive neurons and a decrease in aggregations formed by mutant Htt protein. These cells migrated to the striatum (a primary site of pathology) and formed projections. Improved motor performance on a test for motor coordination (rotorod) and extended lifespan were also observed (176). Antidepressants have been shown to have a positive effect on neurogenesis and cognitive function in HD transgenic mice (96, 142, 303), possibly through neuroprotection induced by an upregulation in BDNF (96, 303). Antidepressants increase the pool of NPCs, accelerate maturation of newborn cells, promote neuronal differentiation, enhance neurogenesis-dependent LTP, and improve behavioral outcomes in tests measuring affect in WT mice (104, 408, 413). In addition, some behavioral improvements observed following antidepressant treatments in WT mice have been blocked by focal irradiation (330), suggesting that antidepressant treatment may in part depend upon plasticity within neurogenic regions. Interestingly, cognitive and hippocampal neurogenic deficits in transgenic R6/1 HD mice can be rescued by chronic administration of the antidepressant fluoxetine without impacting motor deficits characteristic of HD (96, 142, 303), suggesting that in addition to striatal pathology, adult hippocampal neurogenesis may play a role in the affective symptoms of HD. In addition, the forced induction of neostriatal neurogenesis also appears to slow disease progression and alleviate motor and cognitive impairments in a mouse model of HD (64).

6.2 Environmental enrichment partially restores neurogenic deficits and ameliorates cognitive and memory decline in HD transgenic mice
The potential for brain self-repair through manipulation of endogenous stem cells and/or neurogenesis is of particular interest in HD given promising results that rescuing deficits in neurogenesis in HD transgenic rodent models are associated with improvements in memory and cognitive function (142, 176, 303). Both physiologic stimuli (environmental enrichment) and antidepressant treatments have been shown to ameliorate deficits in neurogenesis and delay disease onset in HD mice (96, 142, 160, 214, 215, 303, 362, 396).

It has been shown that the onset of impaired spatial cognition (127, 232, 274, 361) and hippocampal dysfunction (274) observed in R6 mice may be delayed by environmental manipulations, such as long-term exposure to an enriched environment (160, 214, 215, 362, 396). Environmental enrichment or physical activity, which modulate the proliferation and survival of newborn neurons, improves motor and cognitive deficits in transgenic HD mouse and rat models (160, 294, 362, 392, 396) and enrichment has been shown to delay disease onset and slow disease progression in HD mice (160, 362, 396). In the R6/1 mouse, continuous environmental enrichment from 4 weeks of age rescued the total number of proliferating BrdU-positive cells in the DG at 7 weeks of age and DG neurogenesis was still responsive in 25 week old mice (214, 215). The benefit of environmental enrichment to ameliorating abnormalities in neurogenesis is specific to the DG, as no effect is seen in the SVZ of R6/1 or WT mice following enrichment (215). However, it remains to be clearly delineated whether a partial restoration of neurogenesis resultant from environmental enrichment directly contributes to behavioral changes in HD mice.
The functional and therapeutic relevance of adult hippocampal neurogenesis in the context of HD remains unclear. An obvious consideration in examining the significance of abnormalities in adult hippocampal neurogenesis in neurodegenerative diseases is the significance of adult hippocampal neurogenesis to normal functioning of the hippocampus (see chapter 2). However, hippocampus-dependent pathology and resulting cognitive deficits have not been a component of the classic definition of HD. This stems not from excluding, by empirical evidence, the contribution of hippocampal dysfunction and pathology, but largely due to ignoring the possibility that degeneration and functional abnormalities within the hippocampus may play a role in disease progression. Whether cognitive dysfunction observed in HD is dependent on hippocampal degeneration or other abnormalities has not been the subject of any focused study to date. Indeed, relatively little work in the field has focused on non-striatal-derived cognitive impairments in relation to HD. However, some evidence from cognitive studies involving HD patients or HD transgenic mice, regardless of the hypotheses motivating these studies, may offer insight into the significance of memory and cognitive impairments to HD disease pathology and progression.

7.1 Cognitive and memory impairments in HD patients and underlying pathology

Huntington’s disease is classically defined as a motor disease—the clinical onset of HD is still defined by the treating clinician with the aid of the United Huntington’s Disease Rating Scale, a rubric that evaluates the presentation and
severity of motor symptoms. However, cognitive deterioration, including impairments in executive function, are a critical clinical feature of HD (158, 211, 220, 309). Some cognitive changes also precede the motor onset of HD (149, 158, 300, 309), and may be amongst the earliest symptoms of manifest disease (149). Furthermore, disabling psychiatric disturbances and dementias are pervasive amongst the HD population (309, 402). Patients and their carers report that quality of life is adversely affected by cognitive and psychiatric disturbances (including depression, irritability, and apathy) (300), often more so than motor deterioration (our clinical observation, Imogen Rose Neurology Clinic, BRC, Cambridge, UK). Because pathologic malfunction of memory systems may be amongst the earliest changes in the progression of HD, identifying the underlying causes of degeneration in substrates critical to memory function, including the hippocampus, is important not only to our understanding of disease progression, but may also contribute to early diagnosis and an improved treatment of patients suffering from HD, and subsequently an improvement in the quality of life for patients and their carers.

Neuronal degeneration of the caudate and putamen are classic hallmarks of HD (58, 85, 301, 305, 403, 404). However, pathology is wide spread and loss of whole brain volume occurs in early- to mid-stages of HD (327). Cortical and hippocampal degeneration occurs alongside striatal degeneration (85, 301, 327, 360, 403), and may underlie specific cognitive deficits, including memory impairment, in HD. Of particular interest, hippocampal volume is decreased by 9% in mid-stage HD patients (327), a finding that is consistent with my own work using the R6/2 transgenic mouse (see Chapter 4).
While investigating hippocampus-dependent memory in HD patients has not been the focus of any study to date, memory tasks have been a component of several studies designed to investigate early cognitive changes in HD (44, 158, 211, 220, 323, 354, 417). Memory impairments often precede general cognitive deterioration and the appearance of psychiatric signs (149, 158, 220). Deficits in both recall and recognition, characteristic of episodic memory deficits, have been reported in early HD (149, 158, 264, 279). Long-term memory deficits are thought to reflect, in part, a deficit in retrieval (323, 417). Short-term memory deficits, while apparent in presymptomatic patients, decline precipitously around the time of clinical onset of HD and continue to deteriorate throughout disease progression (158, 354). Short-term memory deficits may reflect an impairment in learning (44). HD patients are particularly poor at trial-and-error learning and tasks requiring manipulation of egocentric space, but are unimpaired at unintentional/incidental learning, with the putamen likely acting as substrate for the latter (43, 44). Specific impairments in spatial and pattern recognition memory as well as deficits in spatial working memory in mild to moderate HD have been shown by more than one group using the CANTAB battery of neuropsychological tests (158, 211, 212, 279).

7.2 Cognitive and memory impairments in HD transgenic mice and hippocampal dysfunction

Similar to findings in HD patients (327), a 10% loss in hippocampal volume is observed in R6/2 mice (Chapter 4). In addition to cellular abnormalities in R6 mice, neurons expressing the mutant transgene also exhibit functional abnormalities and
aberrant plasticity (202, 241, 274). Neurons in the striatum of symptomatic mice are more excitable, exhibiting a depolarized resting membrane potential and decreased membrane time constants, but respond with an attenuated intensity to stimulation compared to neurons from WT mice (274), findings that may apply to cortical and hippocampal neurons. LTP induction and consolidation is impaired in hippocampal slices from both the R6/2 and Hdh knock-in models of HD (127, 241, 274). Impairments in LTP at granule cell synapses in both the DG and CA1 are age dependent, with abnormalities appearing first at CA1 and prior to the appearance of overt motor deficits (274).

Hippocampal degeneration and dysfunction likely contribute to memory deficits in HD transgenic mice. Similar to the onset of cognitive dysfunction in HD patients, the onset of cognitive impairments in several HD transgenic rodents occurs prior to the onset of motor symptoms (232, 280, 294, 401). Learning and memory deficits have been observed in R6/2, R6/1, and YAC128 mouse models and the transgenic HD rat line (81, 142, 232, 252, 269, 271, 280, 294, 401). During free exploration of a T-maze preceded by a 1 minute exploration of a ‘familiar’ arm and a 5 minute delay, memory of the familiar location (spontaneous alteration) was impaired by 50% in 14 week old R6/1 mice compared to WT littermates, and further impairments were observed at 20 weeks of age (142, 294). Similarly, R6/1 mice showed no preference for novel versus familiar arms in a Y-maze test for spatial memory after a 1 hour delay (294). Cognitive plasticity was also shown to be impaired in R6/1 mice during sensory associative learning (81, 252).
Abnormalities in adult hippocampal neurogenesis may, at least partially, contribute to specific memory deficits in the context of HD. Treatment with selective serotonin reuptake inhibitors like fluoxetine and sertraline in WT or HD transgenic mice have been shown to improving behavioral outcomes in tests measuring affect and memory and also to rescue deficits in neurogenesis by increasing the pool of NPCs, accelerating maturation of newborn cells, promoting neuronal differentiation, enhancing neurogenesis-dependent LTP (96, 104, 142, 303, 408, 413). For example, in R6/1 mice, spontaneous alteration in the T-maze was improved to levels of untreated WT mice following 10 weeks of treatment with the antidepressant fluoxetine from 10 to 20 weeks of age (142). Targeting deficits in neurogenesis may ameliorate cognitive and memory impairments in HD (70).

8. Role of microenvironment in neurogenic abnormalities in HD

8.1 The neurogenic niche (and the role of the microenvironment) in mediating abnormalities in neurogenesis in HD

The microenvironment of the neurogenic niche plays a key role in maintaining the neurogenic potential of the adult brain (175, 228, 293, 341, 358). The microenvironment also plays a role in fate restriction of endogenous NSCs in neurogenic regions (229, 345, 351, 371). NPCs derived from WT embryonic and adult neurogenic regions can differentiate into fully functioning neurons when transplanted into adult regions of ongoing neurogenesis (SGZ and the SVZ-RMS) (273, 359). In contrast, grafting of these cells into non-neurogenic sites, such as the cerebellum, resulted in no neuronal differentiation, demonstrating that regions of the adult brain
differ in their capacity to support neurogenesis (371). Heterogeneity between astrocytes in the neurogenic niche and those that reside in other brain regions may underlie the capacity for basal neurogenesis in specific brain regions (175). Furthermore, the microenvironment plays a key role in differential fate determination even within neurogenic regions. Jessberger and colleagues demonstrated that expression of the bHLH transcription factor Ascl1/Mash1 redirects adult generated hippocampal progenitors to an oligodendrocyte fate in the hippocampus, neuronal fate \textit{in vitro}, and had no effect in the SVZ (172).

Several factors including signaling molecules such as Wnts, SHH, and VEGF and growth factors, such as BDNF, are important in maintaining the neurogenic potential of the SC niche (21, 228, 381, 412). Ectopic expression of pro-neurogenic growth factors such as BDNF and FGF, enhances survival and differentiation of NPCs (61). The release of mitogenic factors is tightly controlled by glial constituents of the neurogenic niche, particularly astrocytes and endothelial cells (174, 344). In addition, the cerebral microvasculature probably plays a key role in maintaining the neurogenic potential of the SC niche by stimulating self-renewal of NSCs and inhibiting differentiation of NPCs (147, 344).

Dysregulation of the local microenvironment in the degenerating brain may result in a non-permissive environment for neurogenesis (15, 162). Despite the expression of mutant Htt, the proliferation, longevity, differentiation, and survival of NPC derived from the hippocampus and SVZ of transgenic HD mice is similar to that of WT-derived NPCs \textit{in vitro} (142, 308). This finding suggests that abnormalities in neurogenesis \textit{in vivo} are due to the microenvironment in which the NPC resides, and
not the intrinsic properties of the precursor itself. While there is no change in basal SVZ NPC proliferation or maturation in a rodent model of HD (96, 307, 308), the absence of increased proliferation of SVZ NPC in response to quinolinic acid indicates deficits in SVZ microenvironments (308).

8.2 Glial constituents of the neurogenic niche

8.2.1 Astrocytes

The extent to which functional abnormalities of astrocytes contribute to disease progression and dysregulation of neurogenesis in HD is not understood. However, astrocytes are a key component of the neurogenic niche and are known to be potent regulators of both adult neurogenesis and synaptic transmission within normal neuronal networks. In neurogenic regions, astrocytes interact with NSCs and NPCs (341, 358) to promote proliferation and fate determination (175, 358), and may stimulate the maturation and integration of newborn neurons (358, 359). Astrocytes in the SVZ and SGZ promote neurogenesis in part by the release of NPC mitogens like SHH and trophic factors like CNTF, whereas astrocytes from other brain regions are nonpermissive to neurogenesis (and do not express SHH) (174, 175, 430).

Astrocytes also have a well characterized role in modulating synaptic connectivity and transmission (68). Astrocytes may participate in the activity-dependent regulation of newborn neurons directly, or by modulating synaptic transmission within neurogenic regions. Astrocytes in the DG molecular layer and other hippocampal regions express synaptic machinery (14, 37, 299, 436) and release the excitatory neurotransmitter glutamate in a calcium-dependent manner (14, 35-37,
75, 92, 179, 299). Glutamate release by astrocytes can enhance short and long term plasticity in the hippocampus and synaptic strength between perforant path afferents and granule cells, and increase the probability of transmitter release between CA3-CA1 synapses (179, 304). Immature astrocytes promote synaptogenesis and neurite outgrowth through protein signaling and spontaneous astrocytic calcium oscillations. The propensity for astrocytes to promote axonal and dendritic growth decreases with age, and aged and reactive astrocytes show loss of calcium oscillations (68, 183). However, astrocyte contribution to synaptic activity in the context of HD has not been investigated.

Increases in cortical GFAP and the presence of astrocyte inclusions in HD transgenic rodents (240, 347) corresponds with cortical astrocyte proliferation and the presence of nuclear astrocytic inclusions in HD postmortem tissue (347, 350, 404, 423). Diseased astrocytes may adversely affect the health and viability of neurons directly or by impairing protective responses to insult and neurotoxicity (112, 347). Expression of mutant Htt in glia promotes excitotoxicity and cell death in WT neurons (347). Conditioned media from R6/2 derived cortical astrocytes cause deficits in neurite maturation, migration, and synaptic response of primary cortical neurons, an effect mediated by the chemokine CCL5/RANTES in vitro (67). Mutant Htt progressively reduces the expression of astrocytic glutamate transporter GLT-1, reduces expression of astrocytic glutamine synthetase, and impairs glutamate reuptake in vitro and in vivo in HD transgenic mice and Drosophila (29, 231, 347).

8.2.2 Microglia
Microglia appear to be activated in HD postmortem tissue (331, 350) and some types of activated microglia may reduce neurogenesis (101, 262). Cortical microglia density decreases with age in R6/2 mice and microglia are morphologically abnormal from as early as 7 weeks in the striatum and thalamus (Ma et al., 2003).

Investigations of the interaction between microglia and neurogenic regions have predominantly focused on the detrimental effects of activated microglia on neurogenesis. (101, 262). Neuroinflammation induced decreases in neurogenesis can be restored through treatment with nonsteroidal anti-inflammatories and restoration of neurogenesis correlates with a decrease in microglia (263). However, contradictory evidence supports a role for activated microglia in promoting neurogenesis (27). The discrepancy in the regulation of neurogenesis by microglia may depend upon the type and duration of stimuli used to induce microglial activation. Proinflammatory cytokines such as IL-1, IL-6 and TNF-alpha, produced by acute induction of microglia by the endotoxin lipopolysaccharide (LPS) for less that 24 hours, inhibit neurogenesis by impairing differentiation and survival of NPCs (51, 395). Radiation treatment also decreases neurogenesis (263). However, chronic activation of microglia for more than 24 hours induces neuronal differentiation and survival of NPCs (51). Similarly, microglia stimulated by IL-4 or interferon gamma promotes neurogenesis (49, 50). Microglia likely modulate neurogenesis through secreted molecules which promote anti-inflammatory neuroprotective phenotypes and upregulate growth factors such as IGF-1 (51, 66). Coupling between the microglial environment and newborn neurons have been shown to be impaired in models of neurodegenerative disease (66) and may be a relevant consideration in the context of HD.
8.3 Regulation of neurogenesis and maintenance of the neurogenic niche by signaling and trophic proteins

8.3.1 Brain Derived Neurotrophic Factor (BDNF)

The trophic molecule BDNF has been shown to be a potent regulator of adult hippocampal neurogenesis, promoting the survival and maturation of NPC and immature neurons in vitro and in vivo (60, 61, 227). The presence of BDNF is also thought to promote spatial memory formation (136). BDNF and its receptor TrkB are strongly expressed in the GCL and SGZ of the DG (72, 227). TrkB mRNA and receptors are expressed on adult generated transit amplifying progenitors (but absent on immature neurons) (93, 227). Mice lacking TrkB receptors in adult NPCs exhibit impaired proliferation (increased exit of cell cycle in NPCs) and decreased neurogenesis (34, 227), whereas deletion of TrkB only in differentiated neurons does not effect neurogenesis (227). Deletion of full-length TrkB receptors in adult generated progenitors via Cre-induced recombination leads to reductions in dendritic growth, complexity, and spine density, and impaired LTP, and cell death (34). Furthermore, BDNF signaling may provide a physiological link between neural-promoting stimuli and NPC survival as mice lacking TrkB in adult hippocampal NPCs do not respond to stimuli known to promote neurogenesis including chronic administration of antidepressants or activity wheel running (227).

Deficits in BDNF and its receptor in HD have been well characterized. Overexpression of WT Htt potentiates BDNF transcription and translation in vitro and in vivo, while the expression of mutant Htt has the opposite effect (440). Progressive reductions in BDNF transcripts, protein expression, and transport, as well as reduced
expression of the BDNF receptor TrkB, have been well documented in the striatum, cortex, and hippocampus of transgenic HD mice and in vitro in HD models (87, 95, 133, 240, 431, 440-442). Reduction in both BDNF protein and TrkB levels have also been observed in the striatal and cortical regions of postmortem HD brain (95, 113, 133, 440). Cell-cell interactions are important to mutant Htt mediated abnormal BDNF expression as BDNF is transported anterogradely from the cortex to the striatum and overexpression of forebrain BDNF can ameliorate striatal pathology and motor dysfunction in R6/1 mice (126)

The hippocampus is particularly impaired in transcriptional BDNF response to physiologic stimuli such as running in HD (294). Administration of BDNF to hippocampal slices from HD knock-in mice reversed deficits in LTP induction and consolidation (241). The stimulating effects of antidepressants and environmental enrichment on neurogenesis in HD transgenic mice correlated with normalization of BDNF transcription and protein expression (96, 303, 362).

The mechanism by which mutant Htt transcriptionally dysregulates BDNF is well delineated. WT Htt indirectly binds and sequesters REST/NRSF through RILP/dynactin in the cytoplasm, preventing the REST/NRSF complex from binding the NRSE. WT Htt acts as a positive regulator of NRSE-regulated genes, including BDNF (346, 440, 442); sequestration of REST/NRSF by WT Htt leads to BDNF expression. The Htt mutation weakens the ability of Htt to bind REST/NRSF, thereby preventing both effective REST/NRSF sequestration-induction of transcriptional activation and adequate BDNF expression (442).
8.3.2 Wnt

Wnt signaling has not directly been investigated in HD, but Wnt proteins are important regulators of both embryonic and adult neurogenesis. In the embryo, Wnt proteins are secreted factors that activate target genes both acutely and chronically to direct cell proliferation and fate determination, segmentation, limb development, and patterning of the CNS (19, 62, 242, 422, 439). Wnts are crucial for the correct development of cortical structures and particularly for the development of the hippocampus and DG (120, 143, 219, 243, 382). Deletion of Wnt 3a from the dorsal telencephalon that gives rise the hippocampus prevents the formation of a hippocampus and DG (219). Blockage of Lef1, a mediator of canonical Wnt signaling, or beta-catenin, a transcription factor integral to Wnt signaling pathways, results in similar phenotypes (120, 243). The secretion of Wnt/Wg is controlled by the transmembrane protein Wntless/Evi (23, 25).

Similar to its function mediating embryonic development, Wnt signaling appears to be a key regulator of adult hippocampal neurogenesis by promoting both the proliferation of NPCs and their neuronal differentiation (228). Reduced canonical Wnt signaling in the hippocampus from E11 severely impaired the development of the DG, reduced the size of all hippocampal fields, decreased neurogenesis, and increased cell cycle length (357). Wnt downregulation with age may account for decreases in NSC regulation and neurogenesis associated with aging and Wnts themselves may play a role in cellular aging (39, 233). Conditional knock-out of beta-Catenin in adult generated hippocampal neurons severely perturbs their dendritic formation and
branching and cells that do not extend dendrites die within one month of birth (121), further demonstrating the necessity of physiologic input on cell survival.

8.3.3 Sonic hedgehog (SHH)

SHH, a signaling molecule whose activity influences cell differentiation within the neural tube and limb bud, also plays a role in neuronal differentiation and precursor proliferation in the developing midbrain and ventral forebrain (42, 63, 105, 166, 328). SHH regulates adult NPC proliferation in vivo and in vitro, and hippocampal cells exhibit high expression of the SHH receptor Patched (208). However, SHH mRNA is not apparent in the hippocampal neurons; it appears that SHH is produced in the basal forebrain and transported in an anterograde fashion through the fornix to the hippocampus (386, 387). SHH may directly regulate cell cycle, and, in addition, SHH and Patch indirectly regulate cell cycle by acting on cyclins (40, 328). SHH may also induce cell proliferation transcriptionally through regulation of growth factors such as IGF-2 or pro-neural transcription factors like Mash 1 (150, 316, 409). Viral delivery of SHH to the DG increased cell proliferation by greater than 3-fold, an effect that was reversed by administration of a SHH inhibitor dependent upon the integrity of the fimbria-fornix (208). Blocking SHH transduction leads to a decrease in NPC proliferation and neurogenesis, and an increase in cell cycle exit, demonstrating the importance of SHH signaling in maintaining the neurogenic niche (21, 40). Additionally, induction of SHH signaling pathways was sufficient to induce neurogenesis in non-neurogenic pathways (174).
The central hypothesis of this research is that adult hippocampal neurogenesis plays a behaviorally accessible functional role within the broader context of hippocampal processing and that enhancing or impairing this process will produce functionally relevant alterations in behavior. This work seeks to address this hypothesis by developing behavioral techniques to probe the function of neurogenesis in learning and memory and by subsequently applying these techniques to models in which neurogenesis has been altered, including in murine populations in which neurogenesis has been artificially ablated, increased by environmental stimulation, and impaired by long term expression of the mutant Huntingtin gene.

- To demonstrate that adult hippocampal neurogenesis plays a functional role in spatial pattern separation
- To examine whether modulation of the number of mature granule cells through environmental enrichment can modulate the proposed functional role of adult hippocampal neurogenesis in spatial pattern separation
- To examine neurogenesis dependent function in the context of neurodegenerative disease (Huntington’s) in which there is long term dysfunction in adult neurogenesis
Figure 1.1 Regions of constitutive neurogenesis in the adult rodent brain. (A) Neural stem and progenitor cells reside in the SGZ of the DG (orange) of the adult rodent brain. Newborn neurons (blue) migrate into the granule cell layer of the DG where they mature and integrate into the existing circuitry. (B) The subventricular zone of the adult rodent brain consist of the lateral walls lining the lateral ventricles, a region of ongoing neurogenesis throughout adulthood. Newborn cells migrate through the rostral migratory stream to the olfactory bulb where they become fully functioning neurons. DG indicates dentate gyrus; CA3, cornu ammonis 3; CA1, cornu ammonis 1; SVZ, subventricular zone; RMS, rostral migratory stream; GCL, granule cell layer; GL, glomerular layer
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CHAPTER 2

A FUNCTIONAL ROLE FOR ADULT HIPPOCAMPAL NEUROGENESIS IN SPATIAL PATTERN SEPARATION
Abstract

The dentate gyrus (DG) of the mammalian hippocampus is hypothesized to mediate pattern separation—the formation of distinct and orthogonal representations of mnemonic information—and also undergoes neurogenesis throughout life. How neurogenesis contributes to hippocampal function is largely unknown. Using adult mice in which hippocampal neurogenesis was ablated we found specific impairments in spatial discrimination using two behavioral assays: a spatial navigation radial arm maze task and a spatial, but non-navigable, task in the mouse touch screen. Mice with ablated neurogenesis were impaired when stimuli were presented with little spatial separation, but not when stimuli were more widely separated in space. Thus, newborn neurons may be necessary for normal pattern separation function in the DG of adult mice.

Introduction

The dentate gyrus (DG) is thought to contribute to spatial or episodic memory by functioning as a pattern separator (3, 16, 23). Pattern separation is the formation of distinct representations of similar inputs (19). At the cellular level, pattern separation is achieved through the dispersion of cortical inputs from the entorhinal cortex onto a greater number of dentate granule cells (DGCs) with small place fields. By virtue of low firing rates (15) and sparse connectivity between DGCs and CA3 pyramidal cells (2), DGCs are particularly adapted to maintain and transmit orthogonalized information. This ability to pattern separate, or differentially encode small or weak changes derived from increasingly similar or interfering inputs, is particularly
important for the accuracy of memory encoding. Similarly, at the behavioral level, the
ability to form and use memories derived from very similar stimuli that are closely
presented in space and/or time depends upon the ability to pattern separate incoming,
and often complex, information (9, 10, 16). Lesions of the complete DG circuitry
result in impaired pattern separation dependent memory (9, 10, 12).

The DG is also one of two sites where neurogenesis is ongoing throughout life
(35). Adult born neurons integrate into DG circuitry (14, 29, 31) and are thought to
play a role in learning and memory (11, 30, 31), but their contribution to hippocampal
function remains unclear, in part due to the limited availability of behavioral assays
probing this question. We have empirically investigated whether newborn neurons in
the DG contribute to spatial pattern separation function by studying the effects of
manipulating adult neurogenesis on two novel spatial pattern separation tasks (a
delayed non-matching to place task in the RAM and a two-choice spatial
discrimination task in the mouse touch screen). Mice in which hippocampal
neurogenesis had been decreased by focal X-irradiation showed similar impairments
on both tasks. We then confirmed this behavioral pattern separation memory deficit
using a second independent model in which neurogenesis was knocked down by
localized lentiviral expression of a dominant negative Wnt protein in the DG.

Methods

Mice

All mice were housed in standard cages in a temperature controlled (22°C)
room under diurnal condition (12h light/dark cycle). Food and water were provided ad
libitum unless otherwise noted for behavioral experiments. A total of 74 female C57Bl/6 mice was used. For the irradiation experiments, mice were purchased from Harlan (UK, Ltd.). Mice for the dnWnt experiment were purchased from Harlan (San Diego, CA).

Irradiation Procedure

Eight week old female C57Bl/6 mice were anesthetized using a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine i.p. and exposed to focal X-irradiation (n=23) or sham exposed (anesthetization, but no irradiation) (n=20) 2 months prior to behavioral testing. Mice were exposed to a (low) 5-Gr focal X-irradiation dose to a 1 cm gap in the head while sparing most of the brain (including the SVZ) and the snout and eyes using lead shields (24). Mice were irradiated 3 times on day 1, 5 and 9 using a Pantak X-ray machine. All irradiation procedures were carried out at the University of Cambridge Veterinary School in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses. Mice were allowed to recover for 8 weeks before testing began to allow recovery from inflammation (21). All mice were injected with BrdU 50 mg/kg i.p. once daily for 3 days beginning 5 days prior to testing in the RAM or touch screen. Mice were then divided into three groups. Test IR mice (n=3) and test sham controls (n=2) were killed on the day RAM and touch screen testing began so that histological markers of inflammation at the time testing commenced could be analyzed. IR mice (n=10) and sham controls (n=9) were used in the RAM pattern separation testing and IR mice (n=10) and sham controls (n=9) were used in the pattern separation reversal
task in the touch screen.

**Lentiviral Vectors and Injection**

We used the previously described lentiviral vectors expressing CMV-driven dnWnt followed by an internal ribosomal entry site (IRES)-green fluorescent protein (GFP) from the same vector. The control vector was CSC.cPPT.hCMV.eGFP.Wpre (GFP expressing). The dnWnt-IRES-GFP vector was made by inserting dnWnt upstream of the IRES-GFP in the bicistronic cassette in the CSC.cPPT.hCMV.GFP.Wpre vector as described (13, 18). The packaging plasmids used were pMDLg/pPRE, pRSV-Rev, and pMD2.G. Concentrated lentiviral stocks were produced by calcium phosphate transfection into 293T cells, filtration through a 0.22 mm filter, and purification by ultracentrifugation as previously described (5, 6, 28). Expression titers, determined by limiting dilution onto 293T cells were $4 \times 10^9$ (LV-dnWnt) and $6 \times 10^{10}$ (control LV-GFP) transducing units (TU)/ml. Viral stocks were stored at -80°C until use. Eight-week old C57Bl/6 female WT mice were stereotaxically injected with 1 µl per DG of either dnWnt expressing lentivirus (n=16) or GFPcon lentivirus (n=15) at a rate of 0.1-0.3 µl/min, at coordinates from Bregma: -2 anteroposterior, -1.5 mediolateral, -2.3 dorsoventral. All injection procedures were carried out in accordance with protocols approved by the Animal Care and Use Committee, Salk Institute for Biological Studies.

Following the conclusion of the experiment, 4 dnWnt and 1 GFPcon mice were discarded from analysis due to a missed viral injection unilaterally (total viral coverage < 50% DG area). Mice were also injected with BrdU 50 mg/kg i.p. once
daily for 4 days, 1 month prior to testing in the RAM (1 month after viral injection). Six weeks after viral injection, mice were shipped via air courier to the University of Cambridge, Department of Experimental Psychology and allowed to recover for 1 week prior to the commencement of behavioral testing.

Histology

Immunohistochemistry

Animals were anesthetized with sodium pentobarbital (Merial, Oxford, UK) and perfused transcardially with 0.9% NaCl solution, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were post-fixed in 4% PFA for 24 h, followed by equilibration in 30% sucrose for an additional 24 h. Brains were sliced on a sliding tabletop microtome into 40 µm coronal section series. Immunohistological staining was performed on free-floating sections. For BrdU detection, sections were pretreated with 2 N HCl for 30 min at 37°C and washed in 0.1 M borate buffer, pH 8.5, for 10 min. Immunofluorescent double labeling was completed as previously described by Jessberger and Kempermann (13). The primary antibodies used were: rat anti-BrdU (1:500, Harlan Sera-Lab, Loughborough, UK; Accurate, Westbury, NY), sheep anti-BrdU (1:500, Abcam, Cambridge, UK), goat anti-doublecortin (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-doublecortin (1:250, Cell Signaling, UK), rabbit anti-Ki67 (1:500, AbCam, Cambridge, UK; Vector Labs, Burlingame, CA), and mouse anti-NeuN (1:500, Chemicon, Watford, UK; Chemicon, Temecula, CA). Secondary antibodies were produced by Invitrogen/Molecular Probes, Paisley, UK or Jackson ImmunoResearch,
West Grove, PA and used at a 1:250 dilution. Sections were also stained with Hoechst (1:1000, Sigma, Gillingham, UK) or DAPI (1:5000, Sigma, St. Louis, MO).

Quantification of labeled cells

A one-in-twelve series of 40 µm sections (240 µm apart) from each animal was immunohistologically stained (see above) and analyzed by fluorescent and/or confocal microscopy. Immunolabeled cells were counted using a 40x or 63x objective (Leica, Leica FW4000 software; Eclipse E800, Nikon) or by confocal analysis throughout the rostrocaudal extent of the granule cell layer (GCL) and subgranular zone (SGZ) or throughout the rostrocaudal extent of the subventricular zone (SVZ) lining the lateral walls of the lateral ventricles. Resulting numbers were multiplied by 12 to obtain the estimated total number of immunolabeled cells per hippocampus (and divided by 2 to obtain the total number of immunolabeled cells per DG). For colocalizations, 1 to 6 immunopositive cells were randomly chosen from either the SGZ or GCL per section or slide and, via confocal microscopy, determined to overlap with other immunofluorescent makers in the same cell. The presence of overlapping fluorescent signal in any plane of a single cell was considered a double positive cell. Fifty to 100 cells were counted for each experiment. To analyze the area of the DG and coverage of the GCL by viral expression we traced the NeuN+ GCL and the area covered by GFP expression in every 12th section throughout the rostrocaudal extent of the hippocampus using StereoInvestigator software (MicroBrightField; Nikon Eclipse E600 microscope).

Statistical Analysis
Behavioral performances between groups were compared using repeated measures ANOVA (alpha = 0.05). Normality was examined using Q-Q plots and Shapiro-Wilkes tests (alpha=0.05). For the pattern separation tasks in the RAM and mouse touch screen, group (between subject factor) x separation (within subject factor) was examined using repeated measures (alpha = 0.05) and planned comparison, Bonferroni corrected post-hoc two-tailed independent samples t-tests were used to compare group performance at each separation (alpha = 0.025). Two-tailed independent samples t-tests were used to compare means between groups for histological data (alpha = 0.05). All statistical analyses were carried out using SPSS 14.0 for Windows.

Behavioral Protocols

Delayed Non-Matching to Place RAM Learning and Memory task

All behavioral testing was carried out in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses in the University of Cambridge, Department of Experimental Psychology. All mice were food restricted for one week prior to testing and maintained at 85-90% body weight for the duration of testing. Water was provided for the duration of the experiment ad libitum. All testing occurred during the light phase of the diurnal light/dark cycle.

The testing apparatus was a rat-sized wooden 8-arm radial arm maze (RAM). Arms were 14 cm wide by 76.2 cm long and the walls on each arm were 0.5 cm thick and 2.5 cm high. Each side of the center octagon was 15.9 cm. Mice were started in
the start arm ~13 cm from the center octagon and blocked from the rest of the start arm by a block of wood. The apparatus was painted white, and identical and removable cardboard ‘gates’ of 14 cm in length (2.5 cm height) were used to block entries to arms not included in any given trial. The top of the walls were 22.5 cm from the ground (the base of the apparatus was 20.5 cm from the ground). All arms were > 1.09 m from the wall, and all cues were 0.5 to 0.8 m from the arms.

On the morning before testing commenced mice were habituated to the maze. The habituation phase consisted of a 1h group exposure (by cage) to the RAM in which all arms were unblocked and all wells at the end of arms contained several pellets (approximately 20/well). Mice were allowed to explore the maze freely during this habituation session. In the afternoon of the same day, mice were given an individual habituation to ensure that each mouse would retrieve pellets from the baited arm wells. All 8 arms of the maze were unblocked and baited with 1 pellet/well. Mice were allowed to retrieve 2 pellets from any 2 arms (approximately 1-2 minutes) before being removed from the maze.

Following habituation, all testing began between 9 a.m. and 10 a.m. Cage order was randomized throughout testing. Mice were tested for their ability to separate sample (familiar) from correct (new) arms (delayed non-matching to sample, DNMP) in the RAM. Mice received 4 trials/day for 15 consecutive days (60 trials total with 20 trials of each separation). Mice received one trial (consisting of a sample phase + choice phase) and were returned to a holding cage, and all other mice from the home cage (9-10 mice) were tested before the first mouse began the second trial (to maximize intertrial interval). During the sample phase, all arms except a start arm and
the sample arm were blocked off. The mouse was permitted to visit the sample arm and retrieve a food pellet reward. Mice were retrieved from the maze after either (1) spending 10 seconds in the sample arm after retrieving the pellet or (2) exiting the sample arm. During the choice phase, arms in the start and sample (unrewarded) locations and an additional correct (rewarded) location were open. Correct arms varied in distance from the sample arm by a spatial separation of 2, 3, or 4 arms. Mice that entered the correct (rewarded) arm were considered to have made correct choices. Mice that made incorrect choices (i.e., entered the sample/rewarded arm) were allowed to self-correct and enter the correct arm and retrieve the pellet before being removed from the maze [thus, equal (and maximum) numbers of pellets were consumed by all mice on all days of testing]. Mice received 4 trials (sample phase + choice phase) per day of pseudo-randomly presented combinations of start+sample+correct arms for 15 consecutive days (60 trials total, 20 trials of each spatial separation).

We controlled for the number of arm-pair combinations used during choice phases so that novelty for low separation pairs (there were more possibilities for separation 2 than for separation 4) would not be a confounding variable. Four maximum combinations for separation 4 two-arm pairs were possible and we therefore chose 4 combinations for separations 2 and 3. Sample and correct arms were randomized for each combination such that sample arm was either to the left or right of the start arm, and for each combination the start arm could be located in one of two locations perpendicular to either the correct or sample arms. Sample-correct pairings were pseudo-randomly chosen for each day from the pool of possible combinations of
sample+start+correct arms so that there was minimum overlap in the presentation of arms both within one day of testing and across the entire testing duration and so that there was no pattern to whether the correct arm was to the left or right of the start arm. It was determined during pilot experiments that wild type mice performed separation 1 (in which the sample and correct arms were adjacent) at chance levels and so this separation was excluded from the experiments described here. To eliminate the ability of mice to use odor as a facilitatory intramaze cue, the RAM apparatus was rotated on wheels between sample and choice presentations such that the locations of the start and sample arms, but not the arms themselves, were held constant during each trial. The rotation took approximately 20 seconds. Repeated measures ANOVAs between group and separations were carried out using SPSS for each experiment (alpha=0.05). Preplanned Bonferroni corrected post-hoc two-tailed independent samples (Student's) t-tests were used to compare group performance at S2 and S3+S4 (alpha = 0.025).

Mouse touch screen

The testing apparatus consisted of a sound-attenuating box containing a standard modular testing chamber fitted with an infrared touch screen (Craft Data Ltd., Bucks, UK), a pellet receptacle with light illumination and head entry detectors, a 14-mg pellet dispenser, a 3W house light, and a tone generator (Med Associates Inc., Vermont, USA). All trials were mouse initiated and independent of the experimenter. The infrared sensors eliminated the need for force for nose poke detection. A Perspex ‘mask’ containing 2 windows (for pokey training), 3 windows (for PAL) or 5 windows (for two-choice spatial discrimination) approximately 1.6 cm from the floor of the chamber positioned in front of the touch screen allowed the presentation of stimuli on
the touch screen to be spatially localized and prevented the mouse from accidentally triggering the touch screen (e.g., with its tail).

Mice were pre-trained, through several iterative stages, to touch stimuli on the screen to obtain a reward as described in (22). Mice were initially habituated to the apparatus and then learned to associate pellet delivery with the sound of the tone and the onset of the magazine light. Finally, mice learned to nose poke to stimuli as one stimulus at a time was presented in order to obtain a reward and then to initiate trials through the magazine.

**Paired Associates Learning (PAL) testing in the mouse touch screen**

All testing began at 5 p.m. during the light phase of the light-dark cycle. Following habituation training to the touch screen as described above, mice were tested for 55 days on the PAL touch screen test for object-in-place learning (27). For PAL, a 3 window Perspex mask was used to create three distinct spatial locations. Three black and white images (flower, plane, spider) were associated with a particular spatial location (left, center, right, respectively). On each trial, two images were presented, one in its correct location and one in an incorrect location. Mice had to choose the correct object in its correct location to obtain a reward (pellet, receptacle light on, tone, and house light off). If an incorrect object was chosen, mice were punished by the absence of a pellet, no tone, and house light on (time-out) for 5 seconds. Following each incorrect choice, correction trials were administered until the mouse made the correct choice (these trials were not counted in the total trials administered). Testing was stopped following completion of 36 trials or 1 hour (whichever occurred first). There was no difference in the number of trials completed
between groups, and all mice completed 36 trials/day by the 18th day of testing. Mice were tested until the group average reached a criterion of 70% for 2 consecutive days.

**Two-choice spatial discrimination in the mouse touch screen**

Following completion of PAL, mice were tested for 32 days on a two-choice spatial discrimination pattern separation task (20). Mice were required to touch one of two illuminated squares (e.g., the left most square) until a criterion of consecutive touches was reached. Upon reaching criterion, a reversal occurred and the other location (e.g., now the right most square) was designated correct. During an initial 20 day training period, mice were allowed only 1 reversal/day with a maximum of 41 trials/day and a criterion of 9 out of 10 consecutive touches. The separation of the illuminated boxes on the touch screen could either be presented with a high degree of separation (i.e., separation 4 = 3 empty/dark locations between the two illuminated locations) or a low degree of separation (i.e., separation 2 = 1 empty/dark location between the two illuminated locations). Mice were tested for 5-day blocks on each separation and groups were counterbalanced across the separations. Following the completion of 20 days training (10 days total at each separation), the protocol was modified for testing in the following ways: mice were allowed unlimited reversals in a 1h period (up to 81 trials/day) for a further 12 days of testing and the criterion was set at 7 of 8 correct consecutive touches. All mice completed 81 trials/day. Data were analyzed in the following way: only reversals in which the mouse reached criterion were included in the analysis. The last incomplete reversal that the mouse was on when it reached its 81st trial was excluded from the analysis. If a mouse failed to complete its first reversal, a maximum score of 41 trials was used for this trial. This
maximum score was a conservative estimate representing half of the total trials possible per session. Failure to complete one reversal occurred in a total of 12 of 228 sessions (3 times from the sham group and 9 times from the IR group). All mice completed all acquisitions.

**Results**

*Mice with ablated neurogenesis following focal X-irradiation show impaired spatial memory for similar, but not distinct, spatial locations in the radial arm maze.*

Exposure to low dose X-irradiation (IR) leads to a dramatic reduction in the number of newborn neurons in the DG, as previously shown (24, 25). We used this approach to focally ablated neurogenesis in the hippocampus of 8-week old adult female C57Bl/6 mice while sparing the rest of the brain, including the Subventricular Zone (SVZ) (Fig. 2.1 A and B). Focal X-irradiation resulted in a greater than thousand-fold decrease in the numbers of proliferating cells and newborn neurons in the hippocampus compared to sham control mice (Figs. 2.2 A-E, 2.4 A-G, 2.6 A-G). To confirm that newborn neurons had been ablated as well as to examine the extent of inflammation in the hippocampus following a 2 month recovery period post irradiation treatment, we analyzed the brains of IR and sham ‘test’ mice (n=5) that were killed the day behavioral testing commenced. IR ‘test’ mice did not show differences in Iba1+ microglia numbers or morphology compared to sham controls (Fig. 2.1 C and D), but they did show a significant reduction in total numbers of both immature neurons and proliferating cells in the hippocampus (Fig. 2.2 A-E).

Two months after irradiation, IR (n=10) and sham mice (n=9) were tested in a
delayed non-matching to place (DNMP) radial arm maze (RAM) task that we developed to test spatial pattern separation dependent memory (Fig. 2.3). As we had hypothesized that deficits resulting from a knock-down of neurogenesis might be subtle, we purposely designed a challenging spatial task by using a large 8-arm RAM and ensuring the use of external spatial cues in forming spatial memories while eliminating odor as a facilitatory intramaze cue. The difficulty of this task was reflected in lower performance levels by sham mice compared to other RAM tasks (4). Mice were tested for the ability to select from a choice of two arms that had not been presented in a previous sample phase (DNMP) (Fig. 2.3 B). During the sample phase all arms except a start arm and the sample arm were blocked off. The mouse was permitted to visit the sample arm and retrieve a food pellet reward. To eliminate the ability of mice to use odor as a facilitatory intramaze cue, the RAM apparatus was rotated on wheels between sample and choice presentations, such that the location of the start and sample arms, but not the arms themselves, were held constant during each trial (Fig. 2.3 C). The rotation took approximately 20 seconds. During the choice phase, arms in the start and sample (unrewarded) locations and an additional correct (rewarded) location were open. Correct arms varied in distance from the sample arm by a spatial separation of 2, 3, or 4 arms (Fig. 2.3 D). Mice that entered the correct (rewarded) arm were considered to have made correct choices. Mice that made incorrect choices (i.e., entered the sample/unrewarded arm) were allowed to self-correct. Mice received 4 trials (sample + choice phases) per day of pseudorandomly presented combinations of start+sample+correct arms for 15 consecutive days (60 trials total, 20 trials of each spatial separation).
We analyzed pattern separation dependent memory by testing whether mice could differentiate between locations that were presented closely in space (S2) versus those that were more highly separated (S3 and S4). IR mice were selectively impaired at low separations (S2) but not at high separations (S3+S4) (significant interaction, repeated measures ANOVA: F(1,17)=4.57, p=0.047; Bonferroni corrected independent samples t-tests: S2: t(17)=2.55, p=0.021; S3+S4: t(17)=0.03, p=0.974) (Fig. 2.3 E). These results suggest that adult hippocampal neurogenesis was not required to perform the task in which sample and correct arms were presented with a high degree of spatial separation (S3,S4) but was required to correctly discriminate between choice and sample arms when presented in close spatial proximity.

Mice with ablated neurogenesis due to focal X-irradiation show impaired spatial pattern separation but are not impaired on associative object-in-place spatial memory in the mouse touch screen.

To further examine whether loss of adult hippocampal neurogenesis results in global hippocampal deficits or specific pattern separation memory deficits, we tested a naive cohort of IR (n=10) and sham (n=9) mice on a challenging hippocampus-dependent spatial learning task and a two-choice spatial discrimination (pattern separation) task in the mouse touch screen (Fig. 5). The mouse touch screen is useful in that all trials are directed by the mouse through an initiation process and all testing is independent of the experimenter. The testing apparatus (Fig. 2.5 B) consisted of a standard modular chamber fitted with an infrared touch screen, a pellet dispenser, and receptacle with light illumination and head entry detectors, and a tone generator. Mice
were pre-trained through several iterative stages to nose-touch stimuli on the screen to obtain a reward (22). Mice were then trained on a paired associates learning (PAL) object-in-place task (27) that tests the ability to associate correctly three objects (flower, plane, spider) with their correct spatial locations on the screen (left, middle, right, respectively) (Fig. 2.5 C). Mice were only rewarded when they identified the correct object in its correct location during a choice between two objects: one object in its correct spatial location and one object in one of two incorrect locations. Mice were given 36 trials/days plus correction trials over 55 days. Both IR and sham mice learned the task at the same rate (repeated measures ANOVA, F(53,742)=0.18, p=0.671) (Fig. 2.5 D). The demonstration that the performance of IR mice was not different from that of sham mice on this hippocampus-dependent spatial task indicates that mice without neurogenesis are still capable of acquiring, at a normal rate, a complex task involving spatial information in the touch screen.

These IR and sham mice were next tested for spatial discrimination ability in the touch screen using a hippocampus-dependent two-choice spatial discrimination paradigm (20) (Fig. 5 E). Briefly, mice were required to choose the correct spatial location between 2 illuminated boxes in 2 of 5 possible locations until a criterion (7 of 8 consecutive touches) was reached. Once criterion was met, the correct and incorrect locations automatically switched. Similar to the DNMP task in the RAM, pattern separation was tested by varying the distance between choice locations. Lit choice boxes were either far apart, i.e., separated by 3 unlit ‘spaces’ (high separation, S4; Fig. 2.5 E) or close together, i.e., separated by 1 unlit ‘space’ (low separation, S2; Fig. 2.5 E). Spatial separations were held constant during each testing session/day but were
varied across testing days.

In agreement with our findings using the RAM, IR mice were significantly impaired at low (S2) but not high (S4) separations during acquisition (significant interaction, repeated measures ANOVA, average trial to criterion: F(1,17)=6.04, p=0.025; Bonferroni corrected independent samples t-tests: S2: t(17)=2.54, p=0.020; S4: t(17)=0.63, p=0.540) (Fig. 2.5 F). IR mice were not impaired during the reversal trials of the session (F(1,17)=0.65, p=0.431), although interestingly, the pattern was similar, i.e., numerically poorer performance was observed on low separations (Fig. 2.5 G). These data show that the impairment due to our manipulation of adult hippocampal neurogenesis was not in reversal learning per se but in discriminating similar spatial locations, a function that requires spatial pattern separation.

Ablating neurogenesis using focal X-irradiation induces impairments consistent with a deficit in pattern separation in two independent tasks carried out in two very different testing situations. This impairment appears to be specific, as IR mice were capable of learning difficult object-place associations (PAL) at the same rate and to the same performance level as sham mice. Furthermore, the spatial memory deficits observed were similar in both the navigable RAM and non-navigable touch screen.

*Mice with decreased neurogenesis due to targeted lentiviral expression of dominant negative Wnt show impaired spatial memory for similar, but not distinct, spatial locations in the radial arm maze in a similar pattern to that seen in IR mice.*
Despite the 2-month period given to IR mice to recover post-treatment to mitigate possible inflammatory effects or IR, it is nonetheless conceivable that our low-dose focal irradiation protocol may have caused off-target effects. We therefore employed a second, independent method to knock down neurogenesis using lentiviral expression of a dominant negative Wnt (dnWnt) protein and then tested these mice on the same RAM protocol used in the first experiment described above (Fig. 2.7). We used the previously described lentiviral vectors expressing CMV-driven dnWnt followed by an internal ribosomal entry site (IRES)-green fluorescent protein (GFP) from the same vector or a control vector expressing only GFP (GFPcon) (18). Inhibition of Wnt signaling locally in the DG reduces the number of newborn neurons without affecting progenitor proliferation in other brain regions (13, 18). Eight-week old C57Bl/6 female mice received bilateral stereotaxic injections of 1 µl of either dnWnt-expressing lentivirus (n=16) or GFPcon lentivirus (n=15) into the DG (Fig. 2.7 A), resulting in a significant reduction in proliferating cell and neurogenesis (Figs. 2.7 B and C, 2.8 A-F). Behavioral testing in the RAM commenced 2 months after viral injection.

Similar to the pattern separation deficit observed in IR mice, dnWnt mice were impaired at low (S2) but not high (S3+S4) separations compared to GFPcon mice (significant interaction, repeated measures ANOVA: F(1,24)=4.51, p=0.044; Bonferroni corrected independent samples t-tests: S2: t(24)=3.02 , p=0.006; S3+S4: t(24)=0.46, p=0.926) (Fig. 2.7 D). Thus, mice with decreased neurogenesis due to expression of the dnWnt protein are impaired at spatial pattern separation or the ability to correctly distinguish rewarded from nonrewarded spatial locations only when
stimuli to be discriminated are presented closely in space.

**Discussion**

This study provides experimental evidence of a role for newborn neurons in the adult DG in pattern separation function. Ablating newborn neurons using two independent approaches impaired the ability of mice to select a rewarded location when it was presented in close spatial proximity to a non-rewarded location but not when the stimuli to be distinguished were sufficiently spatially distinct. We used two independent behavioral tasks to assess spatial pattern separation—a delayed non-matching to place task in the RAM and a two-choice spatial discrimination task in the mouse touch screen—and results from both behavioral models were in agreement. It is interesting to note that the observed spatial memory deficit was similar in these two very distinct testing contexts. Importantly, mice with ablated neurogenesis were not impaired on all tasks but showed a selective impairment specific to memory performance depending on pattern separation. For example, IR mice with reduced neurogenesis were capable of learning object-in-place associations using the hippocampus-sensitive PAL task (26), indicating that mice were able to learn complex associations in which space was a component.

Previous studies involving rodent lesions of either the dorsal hippocampus (20) or the DG (9, 10, 12) suggest that regions outside of the DG are responsible for disambiguating memories derived from spatially distinct inputs (comparable to the large separations used in this study). In addition, it has been suggested that recruitment of independent cell populations in the CA3 alone, presumably via direct input from the
entorhinal cortex (7), may be sufficient to disambiguate memories for more distinct spatial inputs (16, 17, 32) or make associations between objects and space (8). In this context it is interesting to note that the impairments following ablation of neurogenesis described here are parameter-sensitive (i.e., specific to conditions with a high premium on pattern separation), which may help to explain the variable and sometimes contradictory results from other neurogenesis-ablation studies in which this parameter was not explicitly considered (13, 25, 33, 34).

Given the concerns regarding the specificity of methods used to knock-down neurogenesis, we chose to employ two independent strategies to ablate neurogenesis with a long recovery period post irradiation treatment to offset inflammatory responses (21). Both focal X-irradiation and overexpression of dominant negative Wnt resulted in similar patterns of impairment on the delayed non-matching RAM task. It would seem unlikely, though not impossible, that both models produce off-target effects that would lead to similar behavioral consequences. Furthermore, using a lentiviral strategy, we have reduced neurogenesis by inhibiting Wnt, a protein critical in the production of adult born neurons (18), only in the DG and without affecting other brain regions. It is interesting to note that, although we achieved roughly a 50% decrease in neurogenesis using the dnWnt strategy, we saw a pattern of impairment similar to that seen in the IR group in which neurogenesis was almost completely ablated. Our data suggest there may be a critical threshold for the amount of neurogenesis that is behaviorally significant, a finding that is supported by previous data showing a level-dependent requirement of adult neurogenesis for hippocampus-dependent learning in rats (13). In addition, our RAM task may be sufficiently
challenging that even partial manipulation of newborn neuron numbers is adequate to impair performance.

The DG has been shown to be important for pattern separation and our results show that adult neurogenesis appears to be important for the ability of the DG to perform that function optimally. It remains to be investigated whether immature neurons contribute to pattern separation directly or whether they contribute in more complex ways to a circuit necessary for normal DG function, as suggested by recent modeling studies (1), and whether the function of immature neurons is distinct from that of mature granule cells.
Figure 2.1. The irradiation procedure that was used specifically knocked down hippocampal neurogenesis while sparing SVZ neurogenesis and inflammation was not detected in test mice 2 months after irradiation nor in mice examined at the conclusion of behavioral testing. (A) The number of Dcx+ immature neurons in the SVZ was not different between irradiated (IR) (B, right) and sham (B, left) groups that underwent the touch screen behavior (experiment 2) (independent samples t-test: t(17)=1.58, p=0.58). (C) Test IR mice (n=3) (D, right) (killed the day behavioral testing started) do not show increased numbers of Iba-1+ microglia compared to sham controls (n=2) (D, left) (independent samples t-test: t(3)=1.56, p=0.90). Morphology of Iba+ microglia (D, green) did not appear different between groups. (E) The numbers of Iba1+ microglia did not differ significantly between IR (F, right) and sham (F, left) conditions killed at the conclusion of behavioral testing in the mouse touch screen (experiment 2) (independent samples t-test: t(17)=2.04, p=0.06). Error bars represent SEM. Scale bars represent 50 µm. GCL indicates granule cell layer; HL, hilus; LV, lateral ventricle.
Figure 2.2. Test irradiated mice killed the day behavioral testing started showed a significant decrease in markers of cell proliferation and neurogenesis. We confirmed the effectiveness of our irradiation procedure at the time behavioral testing commenced by examining the brains of 3 test IR and 2 sham mice killed 2 months after irradiation. Test IR mice had a significant decrease in: (A) the total numbers of Dcx+ immature neurons in the DG (independent samples t-test: t(3)=8.21, p=0.004); (B) the total numbers of proliferating cells as shown by total number of BrdU+ cells 3 days after the last BrdU injection (independent samples t-test: t(3)=5.83, p=0.01); and (C) the total numbers of proliferating Ki67+ cells (independent samples t-test: t(3)=6.11, p=0.009). (D, left) Confocal image of the DG from a sham mouse showing Dcx+ cells (red) and BrdU+ (cells) green (quantified in A, B). (D, right) Confocal image of the DG from an IR mouse with few Dcx+ cells (red) and no BrdU+ cells. (E) Confocal images from sham (E, left) and IR (E, right) mice showing proliferating Ki67+ cells (arrows) (quantified in C). Error bars represent SEM. Scale bars represent 25 µm. ** p<0.01. GCL indicates granule cell layer; HL, hilus.
Figure 2.3. Mice with ablated neurogenesis due to focal X-irradiation show impaired spatial memory for similar, but not distinct, spatial locations in the radial arm maze. (A) Mice were irradiated 2 months prior to behavioral testing. (B) Each trial of the DNMP protocol consisted of a sample phase (left) and a choice phase (right). The mouse had to non-match to the novel location. (C) Odor was eliminated as a facilitatory intramaze cue by rotating the RAM between sample and choice phases of the DNMP protocol such that arm locations in the room remained constant but arms themselves differed within trial phases. (D) Pattern separation was tested using a DNMP protocol in the RAM by varying the distance between sample and correct arms: S2/low, S3 and S4/high (S=start arm). (E) IR mice were impaired at low (S2) but not high (S3+S4) separations in the DNMP task. Dashed line represents chance. Error bars represent SEM. Scale bars represent 25 µm. ** p<0.01, *p<0.05. GCL indicates granule cell layer; HL, hilus.
Figure 2.4. Histological analysis confirming the effectiveness of the neurogenesis knock-down from focal X-irradiation in mice tested in the radial arm maze. IR and sham mice were killed at the conclusion of 15 days of RAM testing. (A, B) Irradiation significantly reduced the total numbers of immature Dcx+ cells in irradiated (IR) mice (B, right, white arrows) compared to sham controls (C, left) (independent samples t-test, t(17)=29.82, p<0.001). (C) Significantly fewer BrdU+ cells survived in IR mice compared to sham controls (independent samples t-test: t(17)=6.00, p<0.001). (D) Of those BrdU+ cells that did survive, significantly fewer became neurons in the IR condition (independent samples t-test: t(17)=8.21, p<0.001). (E) There was a significant decrease in the total numbers of proliferating Ki67+ cells (independent samples t-test: t(17)=11.226, p<0.001) in IR mice compared to sham controls. (F) Confocal images of the DG from sham (D, left) and IR (D, right) mice showing surviving BrdU+ cells (arrows) (quantified in C). (G) Confocal images of the DG from sham (G, left) and IR (G, right) mice showing proliferative Ki67+ cells (arrows) (quantified in E). Error bars represent SEM. Scale bars represent 25 µm. ** p<0.01. GCL indicates granule cell layer; HL, hilus.
Figure 2.5. Mice with ablated neurogenesis due to focal X-irradiation show impaired spatial discrimination for similar, but not distinct, spatial locations but not impaired associative object in-place memory in the mouse touch screen. Mice were irradiated 2 months prior to behavioral testing as in (A). Following pre-training for 7-10 days in which mice learned to nose-touch stimuli on the infrared touch screen (B) to obtain a reward, mice were trained on an associative object-in-place task (PAL) (C). For example, as in the left panel of (C), mice had to choose flower-left as a correct association over the incorrect association of plane-right in order to obtain a reward. (D) Irradiated mice (IR) learned the PAL task at the same rate as sham controls (dashed line represents chance). (E) Mice were then tested on a 2-choice spatial discrimination task in which mice had to respond to the correct location (e.g., left illuminated box of left screen, E) until a criterion of 7 of 8 consecutive correct touches was recorded before reversing to the previously incorrect location (e.g., right illuminated box of left screen, E). Mice were tested on either the low separation (S2; left screen) or the high separation (S4; right screen) as depicted in (E) during each testing day. (F) IR mice exhibited significantly impaired performance at low (S2) separations but not high (S4) separations during acquisition of this task, consistent with a pattern separation also deficit observed in the first experiment (Fig. 1). (G) Mice were not impaired on the reversal phase of task, but showed a non-significant trend toward a similar pattern of impairment as observed in the acquisition phase. Error bars represent SEM. * p<0.05.
Figure 2.6. Histological analysis confirming the effectiveness of the neurogenesis knock-down from focal X-irradiation in mice tested in the mouse touch screen. IR and sham mice were killed at the conclusion of touch screen testing. (A,B) Irradiation significantly reduced the total numbers of immature Dcx+ cells in IR mice (B, right) compared to sham controls (B, left) (independent samples t-test, t(17)=18.14, \( p<0.001 \)). (C) IR mice showed a significant decrease in the total numbers of surviving BrdU+ cells compared to sham controls (independent samples t-test: t(17)=4.20, \( p<0.001 \)). (D) Of those BrdU+ cells that did survive, significantly fewer became neurons in the IR condition (independent samples t-test: t(17)=7.70, \( p<0.001 \)). (E) Confocal images of the DG from sham (E, left) and IR (E, right) mice showing surviving BrdU+ cells (arrows) (quantified in C). (F) IR mice (G, right) had a significant reduction in the total numbers of proliferating Ki67+ cells compared to sham controls (G, left) (independent samples t-test: t(17)=22.73, \( p<0.001 \)). Error bars represent SEM. Scale bars represent 25 µm. ** \( p<0.01 \). GCL indicates granule cell layer; HL, hilus.
Figure 2.7. Mice with decreased neurogenesis due to targeted lentiviral expression of dominant negative Wnt show impaired spatial memory for similar, but not distinct, spatial locations in the radial arm maze in a similar pattern to that seen in irradiated mice. (A) 8-week old female C57Bl/6 mice were stereotaxically injected with either a lentivirus expressing dnWnt-IRES-GFP or a control lentiviral vector expressing GFP only 8 weeks prior to the commencement of behavioral testing in the RAM. (B, C) Dominant negative Wnt expression (B, right) significantly reduced the total numbers of immature Dcx+ cells in dnWnt mice (C) compared to GFP controls (B, left) (independent samples t-test: t(24)=3.47, p=0.002). Single channel images depicting Dcx+ cells are shown below triple channel images in (B). (D) Pattern separation was tested using a DNMP as in Fig.3. Dominant negative Wnt mice were impaired at low (S2) but not high (S3+S4) separations in the DNMP task. Error bars represent SEM. Scale bars represent 50 µm. ** p<0.01, * p<0.05. GCL indicates granule cell layer; HL, hilus.
Figure 2.8. Additional histological analysis confirming the effectiveness of the neurogenesis knock-down from local expression of dominant negative Wnt in the DG. (A) The total numbers of surviving BrdU+ cells 6 weeks after BrdU injection were significantly decreased in dnWnt mice compared to GFPcon mice (independent samples t-test: t(24)=3.45, p=0.002). (B) Significantly fewer surviving BrdU+ cells co-localized with the neuronal marker NeuN in dnWnt mice compared to GFPcon mice (independent samples t-test: t(17)=4.76, p<0.001). (C) Confocal images of surviving BrdU+ cells (arrows) in GFPcon (D, left) and dnWnt (D, right) mice quantified in (A). (D) Total numbers of proliferating Ki67+ cells were significantly decreased in dnWnt mice compared to GFPcon mice (independent samples t-test: t(23)=2.36, p=0.027). (E) The percentage of the area of the DG overlapping with LV-driven GFP expression (viral coverage) was not significantly different between groups (independent samples t-test: t(24)=0.095, p=0.925). (F) Confocal images of proliferative Ki67+ cells in GFPcon (F, left) and dnWnt (F, right) mice (viral driven GFP expression is shown in green). Error bars represent SEM. Scale bars represent 50 µm. ** p<0.01, * p<0.05. GCL indicates granule cell layer; HL, hilus.
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Chapter 2 has been submitted for publication: Clelland CD, Choi M, Romberg C, Clemenson GD Jr., Fragnier A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ. “A functional role for adult hippocampal neurogenesis in spatial pattern separation.” The dissertation author was the primary author and investigator of this paper.
CHAPTER 3

ENVIRONMENTAL ENRICHMENT MAY NOT AFFECT SPATIAL PATTERN SEPARATION PERFORMANCE
Abstract

Whether behavioral improvements following periods of exposure to an enriched environment are dependent on intact or increased rates of hippocampal neurogenesis in the adult mammalian brain remains controversial. We sought to address this question by comparing the behavioral performance of mice with and without intact adult neurogenesis on a neurogenesis-sensitive spatial discrimination task following a 4 week period of exposure to an enriching environment. Despite an increase in the total number of surviving neurons following enrichment, the performance of enriched sham mice was not significantly different from that of non-enriched sham mice on a 2-choice spatial discrimination task. Focal ablation of neurogenesis significantly reduced the proliferation and survival of neural precursor cells in both standard and enriched housed mice and impaired spatial discrimination for closely presented stimuli, but not for stimuli presented in more distinct spatial locations, replicating the results presented in Chapter 2. Further investigation is necessary to resolve the outstanding questions of whether modulating the number of mature neurons in the DG alters behavioral capacity in tasks for which intact neurogenesis is necessary for normal performance, and whether immature and mature neurons contribute differentially, synergistically or in an overlapping fashion to hippocampal processing.

Introduction

The tenet that the adult brain is not static, but rather undergoes plasticity in response to external changes, rests in part upon early observations that the adult brain
undergoes anatomical changes in response to environmental complexity or stimulation (5, 6, 11, 18-20). Environmental “complexity” was described by Rosenzweig as “a combination of complex inanimate and social stimulation” (18), and is generally termed “environmental enrichment” in the current literature. More recently, environmental enrichment has been shown to be a potent modulator of the rate of adult hippocampal neurogenesis (2, 4, 10, 28) and has been associated with behavioral learning and memory changes (2, 4, 10). However, whether the links to behavioral improvements following changes in the rate of adult hippocampal neurogenesis (2, 10, 22, 24, 25, 28) are causal is not fully understood.

Exposure to an environment with stimulating and changeable features, typically toys, tubes, nesting material, and houses, often for prolonged periods of four weeks or longer, has striking effects on the survival and differentiation rates of adult generated hippocampal neurons (2, 4, 10, 27). Even a short, one week exposure to an enriched environment may be sufficient to alter spatial learning and memory abilities (8). While the positive effects of environmental enrichment on learning and memory of spatial or object-dependent information have correlated with increases in the rate of neurogenesis (2, 4, 10) and ablation of neurogenesis has been reported to abolish the effects of environmental enrichment (2, 4), one study has reported that improvements in spatial learning and modulation of affective behavior are independent of adult hippocampal neurogenesis (14). One explanation for this discrepancy is that the behavioral tasks employed by different experimenters to test the contribution of neurogenesis to behavior are not, or not consistently, themselves dependent on intact neurogenesis. While ablation of adult hippocampal neurogenesis resulted in
impairments in a reference water maze task in one enrichment study (4), it did not impair performance in a similar task in an analogous study (14).

In order to address these discrepancies we sought to address the question of whether environmental enrichment alters behavioral performance on a neurogenesis-sensitive spatial pattern separation task (see Chapter 2) and whether this effect is mediated by an increase in the rate of adult hippocampal neurogenesis. Eight week old adult, female C57Bl/6 mice were irradiated 2 months prior to re-housing in an enriched environment. Irradiated (IR) and sham mice were housed in either a larger cage containing nesting materials, housing structures, and toys that were changed several times a week (EE) or standard housing (NE). The enrichment period lasted 4 weeks. Following the conclusion of the enrichment period all mice were housed in standard housing for the remainder of the experiment and trained and tested on a touch screen task for spatial discrimination. In agreement with our previous findings (see Chapter 2), the performance of IR (EE+NE) mice was impaired only when stimuli were presented in close proximity, but not when presented in more spatially distinct locations, as compared to sham (EE+NE) mice. However, environmental enrichment (EE) had no effect on the performance of mice in either the IR or sham conditions. While EE increased the number of survival and neuronal differentiation of BrdU-positive cells born prior to the start of enrichment in EE sham mice, it did not have an effect on the numbers of proliferating cells and immature neurons as assessed by post-mortem histological analysis compared to NE sham mice, suggesting that newborn neurons at critical immature stages may play a role in spatial discrimination.
Methods

Mice

Mice were purchased from Harlan (UK, Ltd.). All mice were housed in a temperature controlled (22 °C) room under diurnal condition (12-h light/dark cycle). Food and water were provided ad libitum unless otherwise noted for behavioral experiments. A total of 43 female C57Bl/6 mice was used. All animal handling was in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses.

Irradiation Procedure

Eight week old female C57Bl/6 mice were anesthetized using a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine i.p. and exposed to focal X-irradiation (n=22) or sham exposed (anesthetization, but no irradiation) (n=21) at the University of Cambridge Veterinary School, 2 months prior to re-housing in an enriched environment. Mice were exposed to a (low) 5-Gr focal X-irradiation dose to a 1 cm gap in the head while sparing most of the brain (including the SVZ) and the snout and eyes using lead shields (22). Mice were irradiated 3 times on day 1, 5 and 9 using a Pantak X-ray machine. All irradiation procedures were carried out at the University of Cambridge Veterinary School in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses. Mice were allowed to recover for 8 weeks before re-housing to allow recovery from inflammation (14). All mice were injected with BrdU 50 mg/kg i.p. once daily for 4 days beginning 4 days prior to housing in EE or NE cages. Standard
housed ‘test’ IR mice (n=2) and ‘test’ sham controls (n=2) were killed one week after EE commenced so that histological markers of inflammation could be analyzed. The remaining 39 mice were divided between EE and NE conditions as described under *Housing*.

**Housing**

Prior to the enrichment period, all mice were housed in standard cages measuring 45 x 28 x 12 cm. EE IR (n=10) and EE sham control (n=10) mice were housed in larger (55 x 38 x 25 cm) cages containing nesting material, tubing, platforms, toys, and housing structures for a 4 week period beginning 2 months after the final IR dose. All toys were changed several times per week. Following the 4 week enrichment period all EE mice were returned to standard cages. NE IR (n=10) and NE sham (n=9) mice were housed in standard cages throughout the duration of the experiment. All mice were housed in groups of 9-10 mice. IR and sham groups were counterbalanced between cages.

**Histology**

**Immunohistochemistry**

Perfusion, fixation, and immunofluorescent labeling and detection were completed as described in Chapter 2. Primary antibodies used were: rat anti-BrdU (1:500, Accurate, Westbury, NY), rabbit anti-doublecortin (1:200, Cell Signaling, UK), rabbit anti-Ki67 (1:500, Vector Labs, Burlingame, CA), and mouse anti-NeuN (1:500, Chemicon, Temecula, CA). All secondary antibodies were raised in donkey
and used at a 1:250 dilution (Jackson ImmunoResearch, West Grove, PA). Sections were also stained with DAPI (1:5000, Sigma, St. Louis, MO).

Quantification of labelled cells

A one-in-twelve series of 40 µm sections (240 µm apart) from each animal was immunohistologically stained (see above) and analyzed by florescence and/or confocal microscopy. Immunolabeled cells were counted using a 40x or 63x objective (Eclipse E800, Nikon) or by confocal analysis throughout the rostrocaudal extent of the granule cell layer (GCL) and subgranular zone (SGZ). Resulting numbers were multiplied by 12 to obtain the estimated total number of immunolabeled cells per DG. For colocalizations, 1 to 6 immunopositive cells were randomly chosen from either the SGZ or GCL per section or slide and, via confocal microscopy, determined to overlap with other immunofluorescent markers in the same cell. The presence of overlapping fluorescent signal in any plane of a single cell was considered a double positive cell. Fifty to 100 cells were counted for each experimental condition.

Statistical Analysis

Histological data were analyzed using one-way ANOVA (alpha = 0.05). Behavioral performance between dependent variables (housing and treatment) and separation were compared using repeated measures ANOVA (alpha = 0.05). Significant interactions were further analyzed using Bonferroni corrected independent samples t-tests (alpha = 0.025). For histological analysis, a one way ANOVA was used to compare cell counts between groups (housing and IR treatment) and
significant effects were further analyzed using Bonferroni corrected post-hoc analysis. All statistical analyses were carried out using SPSS 14.0 for Windows.

**Behavioral Protocol**

**Modified Mouse 2-Choice Spatial Discrimination Touch Screen Task**

All behavioral testing was carried out in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses in the University of Cambridge, Department of Experimental Psychology. All testing occurred during the light phase of the diurnal light/dark cycle. All mice were food restricted for one week prior to testing and maintained at 85-90% body weight for the duration of testing. Water was provided for the duration of the experiment *ad libitum*. EE mice were returned to a standard cage 2 days prior to measuring ‘free feeding’ body weight in order to obtain accurate body weight measurements. Testing began at 5 p.m. during the light phase of the dark-light cycle.

The mouse touch screen testing apparatus used was described in Chapter 2. A Perspex ‘mask’ approximately 1.6 cm from the floor of the chamber and positioned in front of the touch screen, allowed the presentation of stimuli on the screen to be spatially localized and prevented mice from accidentally triggering the touch screen. The mask contained either 5 windows (for pokey pre-training/shaping) as described in Chapter 2, or 6 windows (for training and testing). The 6 window mask measured 249 mm total width x 127 mm total height and windows were positioned 15 mm from the bottom of the mask. Each of 6 windows measured 24 x 25 mm (w x h) with two, 38 mm gaps on the outer edges of the 6 windows and 5, 6 mm gaps between the 6
windows. Using shaping, mice were pre-trained to touch the screen to obtain a pellet reward, to associate pellet delivery, the sound of a tone, and onset of a magazine light, and to initiate trials via a nose poke in the magazine apparatus fitted at the back of the testing chamber (see Chapter 2, Methods).

The two-choice spatial discrimination mouse touch screen task used was similar to that described in Chapter 2, Methods. During both training and testing, mice were required to touch one of two illuminated squares (e.g., the left most square) until a criterion was reached (7 of 8 correct consecutive touches). Upon reaching criterion, a reversal occurred and the other location (e.g., now the right most square) was designated correct. Mice received 81 trials/day or a 1 h period (whichever came first). Mice were trained on an intermediate separation (separation 3/S3 = 2 empty/dark locations between the two illuminated locations) until each mouse reached an individual criterion of 2 or more reversals on 3 of 4 consecutive training sessions. Once criterion was met, mice advanced individually to testing stages. The number of days/sessions to criterion was recorded for each mouse during training. During testing, stimuli were presented in one of two possible configurations: the separation of the illuminated boxes on the touch screen could either be presented with a high degree of separation (i.e., separation 5/S5 = 4 empty/dark locations between the two illuminated locations) or a low degree of separation (i.e., separation 1/S1 = 0 empty/dark location between the two illuminated locations). On each day of testing (session) the configuration of stimuli was static (i.e., either separation 5 or separation 1). Mice were tested for 2 days on S5 followed by 2 days on S1 and so on; groups were counterbalanced with respect to the configuration presented on the first day of testing.
Mice were tested for a total of 28 days (14 sessions S1 + 14 sessions S5). The first 8 days of testing (4 sessions S1 + 4 sessions S4) were excluded from analysis.

Data were analyzed in the following way: only reversals in which the mouse reached criterion were included in the analysis. The last incomplete reversal that the mouse was on when it reached its 81st trial was excluded from the analysis. If a mouse failed to complete its first reversal, a maximum score of 41 trials was used for this trial. This maximum score was a conservative estimate representing half of the total trials possible per session. Failure to complete one reversal occurred in a total of 53 of 1092 sessions at (16 times from the EE IR group, 8 times from the EE sham group, 18 times from the NE IR group, and 11 times from the NE sham group).

**Results**

As shown in Chapter 2, exposure to low dose X-irradiation (IR) leads to a dramatic reduction in the number of newborn neurons in the DG. Eight week old adult female C57Bl/6 mice were exposed to focal IR 2 months prior to the beginning of the environmental enrichment, resulting in a significant reduction in adult hippocampal neurogenesis compared to sham controls (Fig. 3.4). To confirm that newborn neurons had been ablated as well as to examine the extent of inflammation in the hippocampus following a 2 month recovery period post irradiation treatment, we analyzed the brains of standard housed IR and sham ‘test’ mice (n=4) that were killed one week after the enrichment of experimental animals commenced. IR ‘test’ mice showed a significant reduction in proliferating Ki67-positive (t(2)=17.47, \( p=0.003 \)) and BrdU-positive
(t(2)=4.35, p=0.049) cells, but did not differ in the number of Iba 1-positive microglia in the DG and SGZ (t(2)=0.75, p=0.53) (Fig. 3.1 A-F).

Two months after IR treatment, IR (n = 20) and sham mice (n =19) were divided into two groups: standard housed animals (NE, n = 19) remained in their ‘home’ cages, and EE mice (n = 20) were re-housed into larger cages which included toys, platforms, and plastic houses that were changed regularly (Fig. 3.2 A and B). Groups were counterbalanced between cages. After a 1 month enrichment period, EE mice were returned to standard cages for the duration of the experiment. Therefore EE was considered a ‘treatment’ rather than a permanent change in housing context.

Behavioral testing commenced the 3 days after the conclusion of the EE period. IR and sham mice were tested for spatial discrimination ability using a 2-choice spatial discrimination task in the mouse touch screen similar to that described in Chapter 2. Briefly, mice were pre-trained through a shaping paradigm to nose-touch stimuli on an infrared touch screen to obtain a reward (16). Mice were then trained to touch one of two illuminated squares (e.g. left most square) until a criterion of 7 out of 8 correct consecutive touches was achieved. Upon reaching criterion, a reversal occurred and the previously unrewarded location (e.g. now the right most square) was designated correct. Mice were trained to distinguish between two stimuli presented at an intermediate separation (S3) (Fig. 3.2 C) until a session/training criterion of 2 or more reversals on 3 of 4 consecutive days was reached. Mice were moved individually from training to testing phases once the training criterion was reached. There was no difference between IR and sham mice on sessions to criterion during the training phase (F(1,39)=0.54, p=0.47), though there was a non-significant decrease in the number of
sessions/days required to reach criterion for EE compared to NE mice (F(1,39)=3.34, $p=0.08$) (Fig. 3.3 A).

During the testing phase, the rules of the task remained constant, but the spatial distance between stimuli presented varied between high separation (S5) and low separation (S1) (Fig. 3.2 C). Stimuli were presented in only one configuration on each day of testing (either high/S5 or low/S1), but varied across testing days. Mice were counterbalanced with respect to stimuli configuration presented on the first day of testing. All mice completed all trials (maximum of 81 trials/session) by the 5th day of testing.

In agreement with our findings reported in Chapter 2, IR mice were significantly impaired at the low separation (S1), but not at the high separation (S5) (significant interaction of IR treatment x separation, F(1,35)=6.14, $p=0.018$; IR treatment main effect F(1,35)=7.25, $p=0.011$; Bonferroni corrected independent sample t-tests: S1, t(37)=3.51, $p=0.001$; S5, t(37)=0.17, $p=0.87$) (Fig. 3.3 B and C). The housing condition did not significantly alter performance at either separation (housing x separation interaction, F(1,35)=0.51, $p=0.48$; housing x IR treatment x separation interaction F(1,35)=0.13, $p=0.73$) (Fig. 3.3 B). Irradiation treatment significantly impaired performance during the acquisition phase of the task (significant interaction IR treatment x separation, F(1,35)=6.14, $p=0.018$) (Fig. 3.3 D) but not the reversal phase (no interaction IR treatment x separation F(1,35)=2.30, $p=0.14$) (Fig. 3.3 E). Across sessions at the low separation (S1), there was a main effect of IR treatment (F(1,37)=5.40, $p=0.026$) (Fig. 3.3 F). IR and sham mice were
not significantly different at the high separation (S5) across sessions (F(1,37)=0.001, 
\( p=0.98 \)) (S5) (Fig. 3.3 G).

Post-mortem histological analysis confirmed that environmental enrichment
increased the number of neurons that survived in the DG and also confirmed the
efficacy of the ablation method used. There was a significant effect of housing
treatment on the number of surviving BrdU-positive cells (F(1, 35)=4.85, \( p=0.034 \))
(Fig. 3.4 A and B). Sham mice housed in an enriched environment had more BrdU-
positive cells at the conclusion of the experiment than standard, NE sham mice
(Bonferroni corrected independent samples t-test, t(17)=2.70, \( p=0.015 \)). Housing also
affected the number of surviving BrdU-positive cells that became mature neurons
(F(1, 35)=4.85, \( p=0.034 \)) (Fig. 3.4 C). A significantly greater number of BrdU-positive
cells co-localized with the neuronal marker NeuN in EE compared to NE sham mice
(Bonferroni corrected independent samples t-test, t(17)=2.70, \( p=0.015 \)). Housing did
not effect the numbers of proliferating Ki67-positive cells (F(1, 35)=0.048, \( p=0.49 \))
(Fig. 3.4 D) or Dcx-positive immature neurons (Dcx, F(1, 35)=2.33, \( p=0.14 \)) (Fig. 3.4
E).

IR treatment significantly reduced the numbers of surviving BrdU-positive
cells (F(1, 35)=414.33, \( p<0.001 \)) (Fig. 3.4 A and B) and the percentage of BrdU-
positive cells that became NeuN-positive neurons compared to sham mice (F(1, 
35)=441.33, \( p<0.001 \)) (Fig. 3.4 C). IR treatment also significantly reduced the
numbers of proliferating Ki67-positive cells (F(1, 35)=50.06, \( p<0.001 \)) (Fig. 3.4 D)
and Dcx-positive immature neurons (F(1, 35)=179.63, \( p<0.001 \)) (Fig. 3.4 E).
Discussion

Environmental enrichment has been shown to have cognitively enhancing effects (2, 4, 12-15, 28, 29) and has also been shown to increase the survival of newborn neurons (1, 2, 4, 10, 12, 14, 21, 23, 28, 30). While several studies have correlated enrichment induced increases in the rate of neurogenesis with enhanced behavioral performance (3, 4, 8), few have addressed whether this relationship is causal (2, 4, 14). One study found that enrichment induced long term memory enhancement was be prevented by blocking neurogenesis with the antimototic agent MAM (2). MAM treatment, however, has been criticized for its confounding off-target effects (14). In contrast, a recent study showed that the behavioral improvements following a long term exposure to an enriching environment did not require intact hippocampal neurogenesis using a reference version of the Morris water maze (14). It is not possible to resolve these contradictory results on the basis of experimental design as the performance of non-enriched mice with reduced neurogenesis in both studies was comparable to that of non-enriched controls, suggesting that intact neurogenesis was not required for behavioral performance. While it is recognized that the effects of environmental enrichment are widespread (12) and the region(s) underlying enhanced behavioral performance following enrichment are likely equally disperse, these studies leave unresolved the question of whether enrichment induced increases in neurogenesis mediate behavioral improvements. We sought to address this question by comparing the behavioral performance of mice with and without intact neurogenesis on a neurogenesis sensitive spatial discrimination task following 4 weeks of exposure to an enriching environment.
The data presented here do not support our initial hypothesis that environmental enrichment would improve behavior on a neurogenesis sensitive task for spatial discrimination. Exposure of adult mice to an enriched environment did not affect the behavioral performance of mice in either the IR or sham groups. This was a surprising finding given the well known association between enrichment and enhanced performance on learning and memory tasks. While the task used in this study is sensitive to deficits in neurogenesis, and the results presented here replicate those presented in Chapter 2 (Fig. 3.3 A and B), the outcomes raises additional questions regarding the role of enrichment in modulating both neurogenesis and behavior.

Interpretation of these results must account for the difficulty of the 2-choice spatial discrimination task used in this study. The task differed in two important ways from the task described in Chapter 2: mice were trained on an intermediate separation (S3) prior to testing, and the stimuli used during testing were smaller in size and presented more closely in space in the low spatial separation condition (S1 was used here, S2 in Chapter 2), rendering an appreciable increase in difficulty of the task. The difficulty of the task is reflected in the performance of mice at small separations. While mice reached criterion in an average of 15 trials in the acquisition phase of the touch screen experiment presented in Chapter 2, in this study the performance of sham mice during acquisition phases was, on average, between 25 and 30 trials. The poorer performance of mice on the more difficult task utilized here raises the possibility that a ceiling effect (Fig. 3.3 F) may obscure potential real effects.

These results do, however, underscore the question of whether immature and mature neurons in the adult brain play dissociable roles in mediating learning and
memory. While enrichment produces long lasting changes in the structure of the DG by increasing the number of integrating neurons from a pool born prior to or during the period of enrichment, the duration of behaviorally relevant enrichment dependent effects is unknown. It is possible that the cognitively enhancing effects of enrichment observed by others occur within a defined temporal window and that testing that occurs outside of that window would therefore not detect behavioral changes. The possible existence of a critical period generated by temporally controlled exposure to an enriching environment implies a transient functional contribution of neurogenesis, a hypothesis that is supported by recent evidence suggesting that newborn neurons preferentially incorporate into memory circuits at critical periods during their development (9, 26).

Other investigators have reported an increase in the number of proliferating cells during or immediately following a period of enrichment (2, 4, 14). However, we did not find a significant difference in the numbers of proliferating cells or immature neurons in sham EE versus NE mice 2 months after the mice were removed from enrichment cages (3 month after the commencement of enrichment and following the conclusion of behavioral testing). It is not possible from the data presented here to parse between three possible explanations for this observation. It is possible that enrichment had no effect on proliferation, that it increased proliferation transiently but this effect returned to baseline following a period of removal from the stimulating environment, or that proliferation rates were increased by enrichment to a stable asymptote and touch screen testing (a potential form of ‘enrichment’ itself) also increased proliferation in NE sham mice to a similar asymptote.
One explanation for the finding that the ablation of neurogenesis impairs spatial discrimination performance, as presented here and in Chapter 2, is that immature neurons, whether by their own action or via circuit modification, underlie pattern separation performance. In this case, it would be predicted that an increase in the pool of immature neurons would be both sufficient and necessary to modulate behavioral performance. A recent study has reported the number of Dcx-positive immature neurons following a partial knock down of neurogenesis correlated with learning and memory performance in rats (7). In this context, increasing the number of mature dentate GCs would not affect spatial discrimination performance. This hypothesis is in line with the finding that despite a doubling in the number of surviving BrdU-positive cells and a 20% increase in their neuronal differentiation in sham EE compared to sham NE mice, there was no associated effect on behavioral performance. If the rate of survival of BrdU-positive cells remained constant throughout the duration of the enrichment period, enrichment would result in the integration of several thousand more new neurons in the DG of sham EE compared to sham NE mice.

It is important to note that enrichment procedures are not standardized. While some studies have include running wheels within enrichment cages (10, 14), others have excluded running wheels (2). Given that enrichment may primarily increase the rates of neuronal differentiation and survival, while other physiologic stimuli, such as running, may primarily alter the rate of cellular proliferation (17) comparisons across enrichment studies in which parameters of the enriched environment vary can be difficult.
The data presented here suggest that despite an increase in the total number of integrating dentate GCs, the performance of EE sham mice on a spatial discrimination task was not significantly different from that of NE sham mice. Focal ablation of adult hippocampal neurogenesis did, however, impair the performance of mice in a pattern similar to that reported in Chapter 2 and consistent with an impairment in spatial pattern separation function. Further work is required to determine the duration of the effect of enrichment that is behaviorally relevant as well as to determine whether immature and mature neurons contribute in differential, overlapping, or synergistic ways to hippocampal processing.
Figure 3.1. Test irradiated mice killed the at the beginning of the enrichment period show a significant reduction in markers of cell proliferation but do not show differences in a marker of inflammation. We confirmed the effectiveness of our irradiation procedure at the time behavioral testing commenced by examining the brains of 2 test IR and 2 sham mice killed 2 months after irradiation. Test IR mice had a significant decrease in proliferating cells in the DG as assessed by (A) the total numbers of Ki67+ proliferating cells and (B) the total numbers of proliferating BrdU+ cells 1 week after the last BrdU injection. (C) Confocal image of the DG from a sham mouse showing BrdU+ cells (green) and NeuN+ (blue) from sham (left) and test IR (right) mice (quantified in B and D). (D) Of those BrdU+ cells, significantly fewer overlapped with the neuronal marker NeuN in IR compared to sham mice (t(2)=10.26, p=0.009). (E) IR (F, right) and sham (F, left) did not differ in the number of Iba1+ cells in the GCL and SGZ of the DG. Error bars represent SEM. Scale bars represent 50 µm. ** p<0.01, * p<0.05. GCL indicates granule cell layer; HL, hilus.
Figure 3.2. Experimental design of environmental enrichment and behavioral testing protocols. (A) IR mice (n=20) were irradiated 2 months prior to re-housing in an enriched environment (B, left), a larger cage consisting of housing structures, a platform, and toys that were changed regularly. IR and sham mice were injected with BrdU (50mg/kg 1x daily) for 4 days prior to beginning enrichment. Following a 1 month enrichment period, EE mice were re-housed in standard cages. NE mice were housed for the duration of the experiment in standard cages (B, right). (C) Schematic of the 2 choice spatial discrimination behavioral task. Mice were trained on an intermediate separation (separation 3). The spatial discrimination performance of mice was compared between groups and across spatial separations. Mice were tested for 28 days on low (separation 1) or high (separation 5) separations. Scale bar represents 20 cm.
Figure 3.3. Mice with ablated neurogenesis due to focal X-irradiation show impaired spatial discrimination for similar, but not distinct, spatial locations, an effect that is resistant to long term exposure to an enriched environment. (A) The number of sessions/days to training criterion was not statistically different between groups. (B) Environmental enrichment had no effect on spatial pattern separation performance across all separations. (B and C) IR mice (EE+NE) were significantly impaired at spatial discrimination when stimuli were presented in close spatial proximity (S1), but not when stimuli were presented at more spatially distinct locations (S5) across all phases of testing (combined acquisition and performance) (B) or during acquisition phases of the task (F(1,37)=5.41, p=0.026) (C). Performance across acquisition (D) and reversal (E) phases of the task. IR mice showed impaired performance across sessions at the low (F) but not high (G) spatial separations. Error bars represent SEM. * p<0.05.
Figure 3.4. Environmental enrichment stably increased the number of adult born neurons, but did not affect the number of proliferating cells and immature neurons as assessed by post-mortem histological analysis. Mice were killed at the conclusion of behavioral testing, 5 months after IR treatment and 2 months after the conclusion of a 1 month period of environmental enrichment. (A) Environmental enrichment increased the number of surviving BrdU+ cells in EE sham (B, top left) compared to NE sham (B, bottom left) mice. IR significantly reduced the number of surviving BrdU+ cells in both EE and NE mice (B, right) 5 months after IR treatment. (C) Enrichment also significantly increased the percentage of BrdU+ cells that became NeuN+ neurons in sham EE compared to sham NE mice. IR significantly reduced the percentage of surviving BrdU+ cells that co-localized with NeuN. IR significantly impaired the numbers of proliferating Ki67+ cells (D) and Dcx+ immature neurons. Enrichment did not significantly alter the number of Ki67+ or Dcx+ cells in either treatment condition. Error bars represent SEM. Scale bars represent 50 µm. ** p<0.01, *p<0.05. GCL indicates granule cell layer; HL, hilus.
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CHAPTER 4

DEFICITS IN ADULT HIPPOCAMPAL NEUROGENESIS AND BEHAVIORAL IMPAIRMENTS IN THE R6 MOUSE MODEL OF HUNTINGTON'S DISEASE
Abstract

Huntington’s disease (HD), caused by polyglutamine expansions in the huntingtin protein, is a progressive neurodegenerative disease resulting in cognitive and motor impairments, dementia, affective disturbances, and death. Whether abnormalities in adult hippocampal neurogenesis or hippocampal dysfunction contribute to disease progression in HD is unclear. While it has previously been shown that cell proliferation and maturation of immature neurons are abnormal in the dentate gyrus (DG) of adult mice transgenic for exon 1 of the mutant human HD gene, the extent of abnormalities across the cell lineage from neural stem cell (NSC) to mature neuron have not been investigated. We quantified cells at various stages in maturation in the diseased HD DG in 12 week old R6/2 mice and found, in addition to deficits in proliferating cells, an increase in Nestin-positive radial-glial like processes and mature astrocytes in the GCL, and decreases in Dcx-positive immature neurons, microglia, and total hippocampal volume. In addition, both R6/1 and R6/2 mice were impaired at spatial discrimination across all spatial separations tested in delayed non-matching to place and delayed alternation RAM tasks and a 2-choice spatial discrimination task in the mouse touch screen. These deficits are greater than can be accounted for by impairments in adult neurogenesis alone and are consistent with widespread pathology observed throughout the hippocampus and the brain of HD patients and mice.
Introduction

Substantial interest has focused upon adult neurogenesis in the context of normal aging and neurodegenerative disease, primarily for two reasons: (1) aging and neurodegenerative diseases have been associated with both deficits in cognitive performance and decreases in the rates of adult neurogenesis (9, 50, 54, 63, 71) (though whether the two are causally related remains the subject of debate) (3), and (2) understanding or harnessing the normal process of adult neurogenesis may lead to brain ‘self-repair’ or cell therapies (10). Understanding differences in the adult neurogenesis in pathologic and non-pathologic states may contribute to understanding both the contribution of adult neurogenesis to cognitive function and neuropathological changes associated with disease states. Huntington’s disease is a relevant model in which to investigate deficits in neurogenesis dependent cognition as abnormalities in adult hippocampal neurogenesis have been shown in both post mortem tissue from HD patients (11, 12) as well as animal models of the disease (20, 35, 52). In addition, unlike other age related neurodegenerative diseases, HD is caused by a single mutated gene (1), making it a desirable model in which to simultaneously investigate disease effects and mechanisms. However, neither the mechanism underlying abnormalities in adult hippocampal neurogenesis nor the functional relevance of these abnormalities is understood.

Huntington’s disease (HD), caused by polyglutamine expansions in the huntingtin protein, is a progressive neurodegenerative disease resulting in cognitive and motor impairments, dementia, affective disturbances (25). There are currently no disease modifying treatments available to treat HD (53). The disease presents in mid-
life, with progression lasting for approximately 20 years and eventually resulting in
death (16). Pathological CAG expansions within exon 1 (the coding region) of the
huntingtin (htt) gene on the short arm of human chromosome 4 results in the
progressive degeneration of the basal ganglia (caudate nucleus and putamen), cerebral
cortex, brainstem, spinal cord, thalamus, and hypothalamus (1). The hallmark of HD
is the progressive loss of medium-spiny GABAergic neurons in the striatum (7, 51, 68,
69), but degeneration of cortical and hippocampal neurons (55, 59, 68) also
contributes to disease progression and cognitive deficits observed in HD, and probably
occurs in parallel with striatal loss from disease onset.

Because pathological malfunction of the hippocampus may precede the onset
of motor and cognitive symptoms in HD, identifying the causes of neuronal
dysfunction may contribute to early diagnosis and/or improved early care of patients
suffering from HD. Adult neurogenesis is abnormal in both HD patients (11, 12) and
mice transgenic for exon 1 of the mutant human huntingtin protein (R6 line) (20, 35,
52). The generation of a transgenic mouse line, R6, expressing exon 1 of the human
mutant htt protein driven by an endogenous promoter, has facilitated examination of
neuropathological changes in the adult murine CNS, presumably reflecting changes
that also occur in human HD patients (6, 43). However, the mechanism by which the
mutant huntingtin protein affects neurogenesis is not known.

R6/2 mice exhibit progressive motor (6, 41, 43) and cognitive deterioration
(18, 41, 47, 60), including impaired hippocampal LTP (18, 47). Ubiquitinated nuclear
and cytoplasmic inclusions of mutant htt (14) in the R6/2 line are similar to neuronal
intranuclear inclusions observed in human HD (15) and are found in high
concentrations within the hippocampus of transgenic mice (46). Interestingly, it has been shown that the onset of impaired spatial cognition (18, 41, 47, 60) and hippocampal dysfunction (47) in R6 mice may be delayed by environmental manipulations, such as long-term exposure to an enriched environment (26, 35, 36, 61, 65), a manipulation that has been shown to have an effect on adult neurogenesis (32, 66).

Abnormalities in adult neurogenesis as exhibited by both transgenic HD mice (19, 20, 35, 36, 52, 64) and humans (11-13) may have clinical relevance for patients afflicted with HD. While striatal pathology contributes to some motor and cognitive defects of HD patients (2), non-striatal pathology, especially that of the hippocampus, may mediate aspects of affective and cognitive deficits in HD (11, 49, 55).

The proliferation of DG born NPCs is reduced in R6/1 and R6/2 mice (19, 20, 35, 36, 52), especially later in disease progression (19, 35). In the DG of R6/1 and R6/2 mice, immature neurons have smaller and irregularly shaped soma, shorter neurites, and migrate shorter distances into the granule cell layer (36, 52). Fate determination of precursor cells, at least on a cursory level, has been reported to be unchanged (36). Environmental enrichment or physical activity, which modulate the proliferation and survival of newborn neurons, improve motor and some cognitive deficits in transgenic HD mouse models (48, 61, 65). However, it remains to be clearly delineated whether a partial restoration of neurogenesis resultant from environmental enrichment directly contributes to behavioral changes in HD mice.

In contrast to the proliferative abnormalities observed *in vivo*, the proliferation, longevity, and survival of NPC derived from the hippocampus and SVZ of transgenic
HD mice appear similar to that of WT-derived NPCs in vitro (21, 52). This finding suggests that abnormalities in neurogenesis in vivo may be due to the microenvironment in which the NPC resides, and not the intrinsic properties of the precursor itself. While there is no change in basal SVZ NPC proliferation or maturation in a rodent model of HD, the absence of increased proliferation of SVZ NPC in response to quinolinic acid further indicates deficits in SVZ microenvironments in dysregulation of adult neurogenesis (52). Local conditions of the microenvironment play a crucial role in regulating neurogenesis (40, 58) and dysregulation of this environment in the degenerating brain may result in a non-permissive environment for neurogenesis (3, 27).

The behavioral impact of rescuing or restoring adult neurogenesis in HD rodent models (8, 21, 30) has shown promise for the therapeutic potential of endogenous NPCs and further indicates that abnormalities in adult neurogenesis may underlie some cognitive deficits in HD. However, it remains to be demonstrated that abnormalities in adult hippocampal neurogenesis contribute to behavioral dysfunction in HD.

A decrease in proliferating cells in the DG of both the R6/2 and R6/1 mouse models has been reported (20, 35). In the R6/2 this decrease in proliferation has been associated with a loss of Dcx-positive NPCs (52). However, the earliest changes in cell lineage from stem cell to fully mature neuron have not been investigated. In addition, the glial constituents in the neurogenic niche have not been examined, and given that hippocampal NPCs appear to behave normally in vitro (52), non-neuronal components of the neurogenic niche may play an important role in neurogenesis.
dysregulation in transgenic HD mice. To pinpoint abnormalities in neurogenesis in cell lineage and/or the neurogenic niche in adult R6/2 hippocampus, we further characterized the cell lineage and glial constituents of the neurogenic niche in the R6/2 DG. We show that abnormalities in adult hippocampal neurogenesis occur at selective stages of cellular development and also that the numbers of glial cells in the DG and SGZ differ from those of WT animals. In addition, we have tested R6/2 and R6/1 mice on neurogenesis sensitive, hippocampal-dependent tasks described in Chapter 2. HD mice show deficits in these tasks to a greater extent than which deficits in adult neurogenesis alone can account.

Methods

Mice

R6 mice transgenic for exon 1 of the human huntingtin protein (43) were purchased directly from Jackson Laboratories (Bar Harbor, Maine) or bred in the Cambridge Centre for Brain Repair animal colony from breeder pairs purchased from Jackson Laboratories (from the strains: R6/2 B6CBA-Tg(HDexon1)62Gpd/3J, 150 CAG repeats; R6/1 B6CBA-Tg(HDexon1)62Gpd/1J,115 CAG repeats; C57Bl/6/CBA background). All genotyping of in house bred transgenic and WT littermate mice was confirmed by PCR Laragen (Los Angeles, CA). All mice were housed in a temperature controlled (22 °C) room under diurnal condition (12-h light/dark cycle). Food and water were provided ad libitum unless otherwise noted for behavioral experiments. All animal handling, testing, and procedures were carried out in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under
appropriate Home Office personal and project licenses or in accordance with protocols approved by the Animal Care and Use Committee, Salk Institute for Biological Studies.

The following animals were used for histological analysis and behavioral experiments: **Baseline Proliferation R6/2**: 12 week old female R6/2 (n = 12) and WT littermate mice (n = 11) (17.5-24.1g) received daily injections of bromodeoxyuridine (BrdU) (50mg/kg i.p.) for 3 consecutive days. Mice were killed 4 days after the last BrdU injection.

**Baseline Nestin/Ki67 confirmation**: 13 adult male R6/2 (and 12 adult male WT littermates were killed between 10 and 16 weeks of age.

**Baseline Maturation R6/2**: 8 week old female R6/2 (n = 10) and WT littermate mice (n=10) received daily injections of BrdU (50mg/kg i.p.) for 3 consecutive days. Mice were killed 4 weeks after the last BrdU injection.

**Dentate Volume by MRI in R6/2**: 13 week old R6/2 (n = 15) and WT (n = 12) mice were assessed for hippocampal and DG volume using MRI adapted for rodents. Mice were assessed at a second time point at 19 weeks of age (in collaboration with Josef Priller, Charite-Universitaetsmedizin, Berlin, Germany).

**R6/2 RAM Spatial Discrimination Task**: 8-9 week old female R6/2 (n = 7) and WT littermate (n = 8) were injected with BrdU 50 mg/kg i.p. once daily for 2 days beginning 7 days prior to testing in the RAM. Mice were killed 4 weeks after the commencement of behavioral testing.
R6/1 RAM Spatial Discrimination Separation Task: 8 week old female R6/1 (n = 9) and WT littermate (n = 10) were tested for 15 d and killed following the conclusion of behavioral testing.

R6/1 Touch Screen Spatial Discrimination Task: 9 week old adult female R6/1 (n = 10) and WT littermates (n = 10) were tested for 24 d and killed at the conclusion of behavioral testing.

Histology

Immunohistochemistry

Perfusion, fixation, and immunofluorescent labeling and detection were completed as described in Chapter 2. The primary antibodies used were: rat anti-BrdU (1:500, Harlan Sera-Lab, Loughborough, UK), sheep anti-BrdU (1:500, Abcam, Cambridge, UK), goat anti-doublecortin (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-doublecortin (1:250, Cell Signaling, UK), rabbit anti-Ki67 (1:500, AbCam, Cambridge, UK), mouse anti-NeuN (1:500, Chemicon, Watford, UK), mouse anti-GFAP (1:400, Sigma, Gillingham, UK), rabbit anti-GFAP (1:400, Dakocytomation, Ely, UK), rabbit anti-S100β (1:1000, Swant, Bellinzona, Switzerland), mouse anti-nestin (1:500, Pharmogen), rabbit anti-Sox2 (1:100, Chemicon, Watford, UK), Goat anti-Endoglin (1:500, R&DSystems), and Sheep anti-huntingtin/Hdh (1:500, a generous gift from Dr. Gillian Bates, King’s College London, UK). The secondary antibodies used were: Donkey anti-rat 488, donkey anti-sheep 488, 568, donkey anti-goat 488, 568, 647, donkey anti-rabbit 488, 647, donkey anti-rabbit 567, donkey anti- mouse 555, donkey anti-mouse 647. All secondaries
were produced by Invitrogen/Molecular Probes, Paisley, UK and used at a 1:250 dilution. Sections were also stained with Hoescht (1:1000, Sigma, Gillingham, UK).

Quantification of labelled cells

A one-in-twelve series of 40 μm sections (240 μm apart) from each animal was immunohistologically stained (see above) and analyzed by florescent and/or confocal microscopy. Immunolabeled cells were counted using a 40x or 63x objective (Leica, Leica FW4000 software; Eclipse E800, Nikon) or by confocal analysis throughout the rostrocaudal extent of the GCL and SGZ. The SGZ was considered to be the single inner cell layer of the DG bordering the hilus plus the width of two cells into the hilus. Resulting numbers were multiplied by 12 to obtain the estimated total number of immunolabeled cells per hippocampus (and divided by 2 to obtain the total number of immunolabeled cells per DG). For colocalizations, 1 to 6 immunopositive cells were randomly chosen from either the SGZ or GCL per section or slide and, via confocal microscopy, determined to overlap with other immunofluorescence markers in the same cell. The presence of overlapping fluorescent signal in any plane of a single cell was considered a double positive cell. Fifty to 100 cells were counted for colocalizations for each experiment.

Statistical Analysis

For histological analysis, independent samples t-tests were used to compare means between groups (e.g. genotype) (alpha = 0.05). Behavioral performances in the RAM and mouse touch screen were analyzed by comparing group x separation using
repeated measures ANOV (alpha = 0.05). Post-hoc comparisons between groups were assessed using Bonferroni corrected independent samples t-tests (alpha = 0.025). All statistical analyses were carried out using SPSS 14.0 for Windows.

Behavioral Protocols

All behavioral testing was carried out in accordance with United Kingdom Animals (Scientific Procedures) Act 1986, under appropriate Home Office personal and project licenses in the University of Cambridge, Department of Experimental Psychology. All testing occurred during the light phase of the diurnal light/dark cycle. All mice were food restricted for one week prior to testing and maintained at 85-90% body weight for the duration of testing. Water was provided for the duration of the experiment ad libitum.

Delayed Alternation RAM Task

The testing apparatus and room were described in Chapter 2. The habituation phase consisted of a 1-h group exposure (by cage) to the RAM in which all arms were unblocked and all wells at the end of arms contained several pellets (approximately 10/well) 2 days before testing commenced. Mice were allowed to explore the maze freely during this habituation session. On the following day mice were given an individual habituation to ensure that each mouse would retrieve pellets from the baited arm wells. All 8 arms of the maze were unblocked and baited with 1 pellet/well. Mice were allowed to retrieve 2-4 pellets from any 2-4 arms before being removed from the maze.
Following habituation, mice began testing daily between 9 a.m. and 10 a.m. Cage order was randomized throughout testing. Mice were tested for their ability to alternate between an arm presented on a previous trial and an arm not presented on a previous trial. Two fixed arms locations were chosen each for training (arm locations 2 and 7) and each separation of the testing phases (S2: arm locations 2 and 8, S4: arm locations 2 and 6). Mice were started at the end of a start arm (location 4) ~13 cm from the center octagon. To start each day of training/testing all of the arms were blocked with cardboard gates except a ‘sample’ arm. On the first day of training/testing this arm was randomly chosen from one of two possible locations. On each subsequent day of training/testing the ‘sample’ arm location was the arm location not visited on the last trial of the previous day. On each trial two arms were unblocked (the previously visited incorrect/unrewarded arm and a correct/rewarded arm) and the mouse had to choose the arm not visited on a previous trial in order to obtain a pellet reward. For trial 1, the previously visited arm was the ‘sample’ arm (sample exposure was not considered as a trial). Mice were retrieved from the maze after entering and traversing the length of a chosen arm. Mice that entered the correct (rewarded) arm were considered to have made correct choices. Mice that made incorrect choices (i.e., entered the sample/rewarded arm) were not allowed to self-correct and no correction trials were given. Thus all mice entered a maximum of 11 arms (1 sample arm + 10 choice arms) during each training/testing session. To eliminate the ability of mice to use smell as a facilitatory intermaze cue, the RAM apparatus was rotated on wheels between each trial and the sample presentation and trial 1 such that the locations of the
start and sample arms, but not the arms themselves, were held constant during each trial. The rotation took approximately 20 seconds.

During training mice received up to 10 trials/day, 6 days/week for 24 days on an intermediate separation 3 (arm locations 2 and 7). Mice were then tested on separation 4 (high separation; arm locations 2 and 6) for 2 days, followed by 2 days testing at separation 2 (low separation; arm locations 2 and 8) (Fig. 6A), and so on until 12 further days of testing were completed. Pattern separation was tested by varying the distance between choice arms: location 2 was held constant during training and testing while the second arm location changed between locations 7 (S3, training), 6 (S4, testing) and 8 (S2, testing).

Delayed Non-Matching to Place RAM Task

The DNMP protocol replicated that described in Chapter 2.

Two-Choice Spatial Discrimination Mouse Touch Screen Task

The mouse touch screen testing apparatus used was described in Chapter 2. A Perspex ‘mask’ containing 5 windows approximately 1.6 cm from the floor of the chamber and positioned in front of the touch screen allowed the presentation of stimuli on the screen to be spatially localized and prevented mice from accidentally triggering the touch screen. Using shaping, mice were pre-trained to touch the screen to obtain a pellet reward, to associate pellet delivery, the sound of a tone, and onset of a magazine light, and to initiate trials via a nose poke in the magazine apparatus fitted at the back of the testing chamber (see Chapter 2, Methods). Testing began at 5 p.m. during the light phase of the light-dark cycle.
The two-choice spatial discrimination mouse touch screen task used was similar to that described in Chapter 2, Methods. Mice were required to touch one of two illuminated squares (e.g., the left most square) until a criterion was reached. Upon reaching criterion, a reversal occurred and the other location (e.g., now the right most square) was designated correct. The separation of the illuminated boxes on the touch screen could either be presented with a high degree of separation (i.e., separation 4/S4 = 3 empty/dark locations between the two illuminated locations) or a low degree of separation (i.e., separation 2/S2 = 1 empty/dark location between the two illuminated locations). Mice were allowed a maximum of 61 trials/day or 2 hours per session and criterion was 7 of 8 correct consecutive touches. Mice were trained for 10 days on 5-day blocks of separation 4 or separation 2. Following training, mice were tested for a further 14 days on 7-day blocks of separation 4 or separation 2 (groups were counterbalanced with respect to starting separation).

Data were analyzed in the following way (as in Chapter 2, Methods): only reversals in which the mouse reached criterion were included in the analysis. The last incomplete reversal that the mouse was on when it reached its 61st trial was excluded from the analysis. If a mouse failed to complete its first reversal, a maximum score of 31 trials was used for this trial. This maximum score was a conservative estimate representing half of the total trials possible per session. Failure to complete one reversal during testing occurred in a total of 21 of 280 sessions (16 times from the R6/1 group and 5 times from WT group).
Results

Abnormalities in neural cell lineage and glial constituents of the neurogenic niche in the R6/2 adult DG

Proliferating cells were significantly reduced in the DG and SGZ of 12 week old R6/2 HD transgenic mice compared to WT littermate controls (Ki67: t(10.15)=0.07, p=0.001; baseline BrdU: t(18)=3.04, p=0.007) (Fig. 4.1 A-C and F), a finding reported by others for both the R6/2 (20) and R6/1 mouse lines (35). This deficit in proliferating cells appeared stable across time in that decreases in BrdU-positive cells 4 weeks after BrdU administration were also observed in an independent cohort of mice killed at 12 weeks of age (t(15)=3.27, p=0.005) (Fig. 4.1 D). In addition, there was a trend toward a decrease in the expression of neuronal fate in 4 week old BrdU-labeled cells in the DG (79% BrdU+/NeuN+ cells compared to 52% BrdU+/NeuN+ cells) though this difference was not statistically significant (t(15)=1.69, p=0.53)) (Fig. 4.1 E).

Quantification of hippocampal volume using structural MRI revealed a decrease in both total brain (Wilcoxon rank sum, p=0.005) and hippocampal (Wilcoxon rank sum, p=0.003) volume in 13 week old R6/2 mice compared to age-matched WT littermates (Fig. 4.2 A and B), a time point at which deficits in proliferation were observed (Fig. 4.1). These findings are in agreement with analysis of structural MRI data from HD patients in early- to mid-disease stages in which a 9% decrease in hippocampal volume was reported (55). Decreases in total brain (Wilcoxon rank sum, p=0.0002) and hippocampal (Wilcoxon rank sum, p=0.0001) volume were also observed in the same mice at 19 weeks of age (Fig. 4.2 C). The
decrease in hippocampal volume at both time points was not significantly greater than
the loss of total brain volume and the rate of decrease in total brain and hippocampal
volume did not appear to accelerate substantially over time despite disease progression
(Fig. 4.2 B and C). It has been reported that adult neurogenesis contributes to a greater
than 10% increase in DG volume over a 5 to 6 month period (28). It is possible that
observed decreases in proliferative capacity may, at least in part, contribute to the
deficit in hippocampal volume observed here.

To further examine abnormalities in the lineage from NPC to immature neuron
we quantified cells in the SGZ and GCL of 12 week old R6/2 and WT mice expressing
SC or NPC markers. As an indicator of radial glial-like stem cells, nestin-positive
primary processes (i.e., only larger processes, no smaller diameter collateral processes,
were counted) traversing the DG were quantified. Surprisingly, R6/2 mice had a
significant increase in the number of nestin-positive radial glial-like processes in the
DG as compared to WT littermates (t(17)=3.45, p=0.003) (Fig. 4.3 A and B) a finding
that was confirmed in an independent cohort of mice (mean ± SEM processes/DG:
R6/2, 4043 ± 647; WT, 1961 ± 454; t(13)=2.36, p=0.03). Nearly all (98.65%) of the
nestin-positive processes examined co-localized with GFAP (Fig. 4.3 B). While the
quantification of primary nestin-positive processes may represent the total number of
nestin-positive cells, it is important to note that primary processes do not necessarily
exist in a 1 to 1 relationship with cell bodies.

Radial glial-like cells are thought to represent a quiescent stem cell population
in the adult brain (17, 31, 34, 44); however, recent evidence suggests that a distinct
population of Sox 2-positive cells with self-renewing capacity may constitute the stem
cell population of the adult DG (62). We therefore quantified the number of Sox 2-positive cells in the GCL and the SGZ of the DG. The SGZ was defined as the area corresponding to a single cell layer of the DG adjacent to the hilus and the width of two cells layers of the hilus bordering the DG. There were no differences in the numbers of Sox-2 positive cells in the DG (DG, t(16)=0.23, p=0.81; SGZ, t(16)=0.49, p=0.63; GCL, t(16)=0.64, p=0.53) of R6/2 mice compared to WT littermates (Fig. 4.3 C and D). However, there was a significant decrease in Dcx-positive NPCs in the DG of R6/2 mice (t(17)=3.05, p=0.007) (Fig. 4.3 E and F). These data raise the interesting possibility that although the numbers of Sox 2-positive putative stem cells does not differ between groups, cells may adopt more quiescent properties consistent with a nestin-positive radial glial fate at the expense of proliferation and/or formation of Dcx-positive immature neurons.

It is unclear whether fate determination of NSC/NPCs is affected by expression of the mutant htt transgene (cell intrinsic effect) or whether cell extrinsic factors in the diseased HD microenvironment contribute to or underlie abnormalities in adult neurogenesis, as suggested by data showing a lack of difference in proliferation rates between R6/2 and WT derived NPCs in vitro (52). We therefore examined glial constituents and indicators of disease state in the DG neurogenic niche. While there was no significant difference in the number of mature S100β-positive astrocytes in the SGZ (t(10)=0.22, p=0.829) or overall difference in the number of astrocytes in the DG (SGZ+GCL) (t(10)=1.54, p=0.141) of R6/2 compared to WT mice, there was a significant increase in the number of mature astrocytes in the R6/2 GCL (t(10)=2.73, p=0.016) (Fig. 4.4 A and B). These data further support the hypothesis that mutant htt
may directly or indirectly affect differentiation by potentially promoting gliogenesis at
expense of neurogenesis. Furthermore, there were significantly fewer microglia in the
DG (SGZ + GCL) of R6/2 compared to WT mice at 12 weeks of age (t(8)=2.81 ,
p=0.023) (Fig. 4.4 C and D), a finding that is consistent with a reported decrease in
microglia in other cortical regions of the R6/2 brain (42). Though it has been
suggested that microglia may play a role in adult neurogenesis (45) a direct
relationship between microglia and neurogenesis has not been shown. Finally, disease
progression does not appear homogenous across all cell types in the hippocampus. The
formation of intranuclear and intracytoplasmic inclusions of mutant Htt protein have
been considered hallmarks of HD disease progression at the cellular level (14), though
debate remains concerning whether inclusions containing mutant Htt are cytotoxic or
cytoprotective (4, 24, 56). We report that inclusions occur in fewer than 25% of
NeuN-positive cells in the GCL of the DG; in contrast, 93% of NeuN-positive neurons
in CA1 contained inclusions (Fig. 4.5), indicating that many mature neurons in the DG
do not exhibit overt signs of disease pathology (compared to other nearby regions)
despite the substantial disturbance in adult neurogenesis occurring in close proximity.

*R6/2 mice transgenic for exon 1 of the mutant human HD gene show impaired spatial
discrimination during delayed alternation across all separations in the RAM*

Eight to 9 week old adult, female R6/2 and WT mice were trained for 24 days
(up to 10 trials per day) on a delayed alternation version of the RAM on an
intermediate separation 3 (Fig. 4.6 A). The performance of groups did not differ across
training sessions (F(1,13)=0.66, p=0.43) (Fig. 4.6 B). Mice were then tested for 12
days (10 trials per day) on both high (S4) and low (S2) separations. One arm location (arm location 2) was held constant during training and both separations of testing (Fig. 4.6 A); only the spatial distance between choice arms varied between each trial type. As in Chapter 2, odor was eliminated as a possible facilitatory intramaze cue by rotating the maze between trials during both training and testing such that arm locations remained constant but the arms themselves were irrelevant in solving the task. The performance of the R6/2 mice during testing phases was significantly different from that of WT mice (main effect of group F(1,13)=9.26, \( p=0.009 \)) with a greater difference in performance occurring at the low/S2 separation (Bonferroni corrected independent samples t-tests: S2, \( t(13)=3.63, \ p=0.003 \); S4, \( t(13)=1.57, \ p=0.14 \)) (Fig. 4.6 C).

Post-mortem histological analysis revealed a significant decrease in both the number of Dcx-positive immature neurons (\( t(13)=5.54, \ p<0.001 \)) and the number of BrdU-positive cells (\( t(13)=7.11, \ p<0.001 \)) in the DG of R6/2 compared to WT control mice (Fig. 4.6 D-F). There was a non-significant trend toward the presence of fewer BrdU-positive cells colocalizing with the neuronal marker NeuN (\( t(13)=2.00, \ p=0.069 \)) (Fig. 4.6 G).

*R6/1 mice transgenic for exon 1 of the mutant human HD gene show impaired spatial discrimination across all separations in a DNMP RAM task*

Eight week old adult, female R6/1 and WT mice were tested for spatial discrimination ability using a DNMP RAM protocol described in detail in Chapter 2, Methods. R6/1 mice were impaired at performing the task across all separations (main
effect of group F(1,18)=47.19, \( p<0.001 \). R6/1 mice were impaired at both the low/S2 separation (Bonferroni corrected independent samples t-tests, \( t(18)=4.34, \ p<0.001 \)) and the high/S4 separation (Bonferroni corrected independent samples t-tests, \( t(18)=4.81, \ p<.001 \)) (Fig. 4.7 A). It is interesting to note that the duration of testing lasted 15 days, by which time the mice were no older than 10.5 weeks of age. The deficits reported here occur before the onset of overt motor deficits previously reported in these mice (43).

Following behavioral testing, mice were killed and the numbers of proliferating cells and immature neurons in the DG were quantified. The number of proliferating Ki67-positive cells was not statistically significantly different between groups (\( t(18)=1.80, \ p=0.09 \)), though there was a non-significant trend toward a decrease in Ki67-positive proliferating cells in the DG of R6/1 mice compared to WT littermate controls (Fig. 4.7 B and D). There were significantly fewer Dcx-positive immature neurons in the DG of R6/1 mice compared to WT littermates (\( t(18)=6.27, \ p<0.001 \)) (Fig. 4.7 C and D).

*R6/1 mice transgenic for exon 1 of the mutant human HD gene show impaired spatial discrimination across all separations in the 2-choice spatial discrimination task in the mouse touch screen*

Nine week old adult, female R6/1 mice were trained for 10 days on 5-day blocks of separation 4 and separation 2. Performance of mice during training did not differ (trials to criterion, acquisition + reversal, \( F(1,18)=0.03, \ p=0.87 \)). Mice were then tested for an additional 14 days on 7-day block trails of separation 4 or separation 2.
R6/1 mice showed impaired performance across both separations during the acquisition phase (trials to criterion, main effect of group, F(1,18)=11.18, p=0.004) compared to WT littermates, with a significant difference between group performance at the low/S2 separation (Bonferroni corrected independent samples t-tests: S2, t(1,18)=3.45, p=0.004; S4, t(1,18)=2.18, p=0.043) (Fig. 4.8 A). R6/1 mice were also impaired across both separations of reversal phases (F(1,18)=6.14, p=0.023) (Fig. 4.8 B).

Post-mortem histological analysis revealed a significant decrease in both the numbers of proliferating Ki67-positive cells (t(18)=2.96, p=0.008) (Fig. 4.8 C and E) and the numbers of Dcx-positive immature neurons (t(18)=6.54, p<0.001) (Fig. 4.8 D and E).

**Discussion**

The data presented here indicate that abnormalities in adult hippocampal neurogenesis in the R6/2 line expressing exon 1 of the mutant human HD transgene are more widespread than the deficits in cellular proliferation previously reported (20, 35). We demonstrate that deficits in adult hippocampal neurogenesis occur early in the cellular lineage from NSC to neuron. While there is an upregulation in nestin-positive primary processes traversing the DG GCL, suggesting a concomitant increase in nestin-positive cells, and a decrease in the number of Dcx-positive immature neurons, the number of Sox 2-positive NSC/NPCs is similar between groups. This finding suggests that mutant Htt may contribute, directly or indirectly, to abnormalities in neuronal differentiation. At least one recent finding indicates that Sox 2-positive cells
may have the capacity to become nestin-positive radial glial-like cells (62). It is interesting to note that the numbers of Dcx-positive immature neurons in the R6/2 DG are equivalent to roughly half of the number observed in the DG of WT mice, a decrease that occurs concomitantly with an increase in radial glial-like nestin-positive processes, suggesting that Sox 2 positive cells, of which total numbers are similar between R6/2 and WT DGs, may adopt a more quiescent state at the expense of the formation of new neurons. Increased numbers of mature astrocytes in the GCL of the DG, but not in the SGZ, further support the hypothesis that differentiation deficits occur in the adult neurogenic niche. It remains to be tested whether Sox 2-positive NSCs can ‘de-differentiate’ into radial glial like quiescent NSCs in the HD brain.

A deficit in neuronal progenitor proliferation or neuronal differentiation may, in part, contribute to loss of DG volume overall. We found a significant reduction in hippocampal volume that occurred relatively early in disease state in the R6/2 mice and remained stable over disease progression. A similar finding has been shown in HD patients (55). A loss of hippocampal volume early in disease progression may have implications for hippocampal processing and capacity, a hypothesis that has not been directly testing with HD patients to date.

It has been reported that cell-cell interactions are necessary for the emergence of significant neurodegeneration in cortical regions outside of the hippocampus and may contribute to functional deficits (22, 23, 70). The expression of the mutant Htt protein has been shown to have both cell autonomous and cell non-autonomous effects in the adult brain, and it is currently not clear whether the abnormalities in adult neurogenesis observed here are due to cell intrinsic effects (e.g. dysregulation of the
cell cycle) or due to pathologic cell-cell interactions. It is likely that a combination of both cell autonomous and non-autonomous factors play a role in cellular abnormalities in HD. Inclusion formation, a processes that is thought to be cell autonomous (23), occurs in less than 30% of NeuN-positive neurons and Sox 2-positive precursor cells in the R6/2 DG, compared to over 95% of NeuN-positive neurons in the CA1 hippocampal subregion. These data suggest that a majority of neurons in the DG are overtly normal, and in addition to the findings discussed above, further indicate that cell non-autonomous effects may contribute to HD hippocampal pathology.

Understanding the mechanism underlying abnormalities in adult hippocampal neurogenesis in the diseased and/or degenerating brain may contribute to the development of therapeutic targets and therapies. However, whether the abnormalities described here are relevant in terms of hippocampal function has not been explored. Such an investigation may be not only applicable to HD, but also to conditions or pathologies in which neurogenesis is downregulated, such as aging, depression, AD or PD (3, 29, 33, 54, 57, 63, 71). We therefore employed the behavioral tasks that we developed that are sensitive to ablation of adult hippocampal neurogenesis (Chapter 2) in testing the behavioral performance of R6 mice transgenic for exon 1 of the mutant human HD gene.

The ability of R6 mice to discriminate two spatially varied stimuli in order to obtain a food reward was impaired across all spatial separations tested. This impairment was consistent across both the R6/2 and the R6/1 model. These models differ by the number of CAG repeats (an approximate difference of 40 repeats) in the mutant gene and by the both the timing of symptomatic onset and the rate of disease
progression (43). While behavioral testing occurred between 8 and 11 weeks of age for the R6/2 mice and 8 and 12 weeks of age for the R6/1 mice, a period prior to, or coinciding with the earliest stages of motor impairment (5, 6, 43), the observed pattern of impairments was comparable and preceded overt motor deficits. This pattern of impairment was also consistent across tasks. R6/1 mice showed impaired spatial discrimination in both the non-navigable mouse touch screen and the navigable RAM.

The comparable performance of R6/2 and WT mice on discriminating rewarded from nonrewarded locations that were separated by 2 blocked arms (S3) during the training phase of the RAM delayed alternation task, with subsequent deficits in both the large (S4) and small (S2) separations during the testing phase, raises the possibility that deficits in the testing phase of the task may be a function of disease state. However, R6/1 mice, in which motor symptoms appear later and progress more slowly, were tested at similar ages to the R6/2 mice and well before the appearance of overt motor symptoms, showed a similar impairment across both high and low separations, suggesting that these impairments are not a consequence of motor impairment. Impairment on hippocampus sensitive tasks used here may indicate abnormalities in hippocampal function that precede motor abnormalities. In addition, to limit the contribution of motor deficits as an outcome measure of cognitive performance, the behavioral performance of mice was measured as the number of correct/incorrect choices, rather than a motor dependent measure such as latency. While it is not possible to completely separate motor function from behavioral performance, the data presented here, deriving from groups of animals at different
disease states and across two different behavioral paradigms, indicate a consistent and early cognitive impairment in R6 HD transgenic mice.

R6 mice showed impaired discrimination across all spatial separations presented. The deficit reported here is greater than that which would be expected due to a deficit in neurogenesis alone (see Chapter 2). While deficits in performance at the low separations of the task may result from abnormalities in DG function (consistent with the impairments following ablation of adult neurogenesis reported in Chapter 2), impairments in performance at the large separations (S3/4) may result from more global impairments, or dysfunction of other hippocampal subregions, such as CA3. CA3 appears to play a role in spatial discrimination or pattern separation function when objects or contexts are more spatially distinct (37-39, 67). The interpretation that mice show greater deficits in spatial discrimination than would be predicted by deficits in adult hippocampal neurogenesis is consistent with the observation that pathology is widespread within the HD brain (1) and is supported by histological analysis that revealed a high rate of inclusion formation in the CA1 hippocampal subregion and structural MRI analysis revealed a loss of 10% total hippocampal volume.
Figure 4.1. A reduction in the numbers of proliferating cells in the DG of R6/2 mice at 12 weeks of age compared to WT littermates. (A) The number of proliferating Ki67+ cells was significantly decreased in 12 week old R6/2 mice compared to WT littermates. (B) Representative confocal image of Ki67+ cells (arrows) in the R6/2 DG. The numbers of BrdU+ cells were also decreased in 12 week old R6/2 compared to WT mice 1 week (C) or 4 weeks (D) after BrdU injection. (E) There was a non-significant trend toward a decrease in the percentage of BrdU+ cells that co-localized with the neuronal marker NeuN 4 weeks after BrdU injection in R6/2 mice compared to WT littermates. (F) Confocal images of BrdU+ cells (green) in the WT (left) and R6/2 (right) DG. Arrows point to example BrdU+ cells. Error bars represent SEM. Scale bars represent 25 µm. ** p<0.01. GCL indicates granule cell layer; HL, hilus.
Figure 4.2. Hippocampal and total brain volumes are decreased in R6/2 compared to WT mice at 13 and 19 weeks of age. (A) T2-weighted MRI of one animal with the hippocampal region highlighted as green overlay (left), 3D reconstruction (right). (B) 13 week old R6/2 mice have significantly smaller hippocampal and whole brain volumes (Wilcoxon rank sum test, p=0.003, p=0.005, respectively). (C) Repeated measurements at 19 weeks of age again reveal significantly smaller volumes of the hippocampus and whole brain in R6/2 compared WT littermates (Wilcoxon rank sum test, p=0.0001, p=0.0001, respectively)
Figure 4.3. Abnormalities in stem and progenitor cells in the DG of 12 week old R6/2 mice compared to WT littermates. (A) The number of nestin+ primary processes (B, red) crossing the GCL of the DG was significantly increased in R6/2 mice (B, bottom) compared to WT littermates (B, top). 98.7% of nestin+ processes co-localized with GFAP (B, right, green). (C) The number of Sox2+ putative NSCs was not different between R6/2 (D, right) and WT mice (D, left). (E) There was a significant decrease in the number of Dcx+ immature neurons in the DG of R6/2 mice (F, right) compared to WT littermates (F, left). Error bars represent SEM. Scale bars represent 25 µm. **p<0.01. GCL indicates granule cell layer; HL, hilus.
Figure 4.4. Abnormalities in the numbers of astrocytes and microglia in the DG of 12 week old R6/2 mice compared to WT littermates. (A) There was a significant increase in the number of S100β+ astrocytes in the GCL, but not in the SGZ, of 12 week old R6/2 mice (B, right) compared to WT littermates (B, left). There was a significant decrease in the number of Iba1+ microglia in the DG of R6/2 (D, right) compared to WT (D, left) mice. Error bars represent SEM. Scale bars represent 50 µm. * p<0.05. GCL indicates granule cell layer; HL, hilus.
Figure 4.5. Expression of inclusions positive for htt occurs at a lower rate in the DG than CA1 in the hippocampus of 12 week old R6/2 mice. (A) The number of NeuN+ neurons colocalizing with htt-containing inclusions was significantly greater in CA1 than the DG of R6/2 mice (paired t-test $t(9)=7.77, p<0.001$). A greater number of mature neurons in the DG colocalized with S830/htt+ inclusions than immature Dcx+ and Sox2+ precursor cells, suggesting that cell extrinsic factors may contribute to abnormalities in neurogenesis in adult R6/2 mice. (B) A confocal image of htt+ inclusions (top, blue) in the neurogenic region containing Nestin+ (green) and Sox2+ (red) precursors. A single channel image showing a high rate of inclusion formation in the hilus and outer GCL, but with fewer inclusions in the inner GCL and SGZ (B, bottom) in the R6/2 mouse. (C) Confocal images of S830/htt+ (green) inclusions in the NeuN+ neurons (red) in the DG (left) and CA1 (right) of a 12 week old R6/2 mouse. Error bars represent SEM. Scale bars represent 25 µm. ** $p<0.001$. GCL indicates granule cell layer; HL, hilus.
Figure 4.6. R6/2 mice show impairments in spatial discrimination across all spatial separations tested compared to WT littermates on a delayed alternation RAM task. (A) Mice were to discriminate between rewarded and non-rewarded locations in a delayed alternation RAM task on an intermediate separation (separation 3, locations 2 and 7, training). During testing phases the choice arms were presented either more closely together (separation 2, locations 2 and 8) or farther apart (separation 4, locations 2 and 6). (B) The performance of R6/2 and WT mice was comparable across training sessions. (C) The performance of R6/2 mice was significantly lower than that of WT mice across all separations during testing phases. The numbers of Dcx+ immature neurons (D) and surviving BrdU+ cells (E) were significantly reduced in the DG of R6/2 mice compared to WT controls. (F) Confocal images of WT (left) and R6/2 (right) DG showing immunofluorescent signal for Dcx+ (red) and BrdU+ (green) cells. (G) There was no significant difference in the percentage of surviving BrdU+ cells that co-localized with the neuronal marker NeuN. Error bars represent SEM. Scale bars represent 50 µm. **p<0.01. GCL indicates granule cell layer; HL, hilus.
Figure 4.7. R6/1 mice show impairments in spatial discrimination across all separation tested compared to WT mice on a DNMP RAM task. (A) The performance of R6/1 mice was significantly lower than that of WT mice across separations tested. (B) The numbers of Ki67+ proliferating cells in the DG were not statistically significantly different between R6/1 and WT mice tested in the RAM. (C) The number of Dcx+ immature neurons was significantly reduced in R6/1 mice compared to WT littermates. (D) Confocal images showing Dcx (red) and Ki67 (green, arrow) in the DG of WT (left) and R6/1 (right) mice. Error bars represent SEM. Scale bars represent 25 µm. ** p<0.01. GCL indicates granule cell layer; HL, hilus.
Figure 4.8. R6/1 mice show impairments across all separations in spatial discrimination compared to WT mice in a touch screen 2-choice spatial discrimination. R6/1 mice showed significantly impaired performance (higher numbers of trials to criterion) across all separations of both acquisition (A) and reversal (B) phases of a 2-choice spatial discrimination touch screen task. There was a significant decrease in the numbers of Ki67+ proliferating cells (E, green) and Dcx+ immature neurons (E, red) in the DG of R6/1 (E, right) mice compared to WT littermates (E, left). Error bars represent SEM. Scale bars represent 25 µm. ** p<0.01. GCL indicates granule cell layer; HL, hilus.
References


Chapter 4 will be submitted for publication: Clelland CD, Choi M, Fraginier A, Clemenson GD Jr., Armstrong M, Tyers P, Priller J, Bussey TJ, Saksida LM, Gage FH, Barker RA. “Behavioral deficits and characterization of the neurogenic niche in Huntington’s disease R6 transgenic mice.” The dissertation author was the primary author and investigator of this paper.
CHAPTER 5

CONCLUSION
Despite substantial interest and inquiry, the functional role of adult hippocampal neurogenesis remains unclear. Over the past several years, several lines of evidence have shown correlations between both the rate and total amount of neurogenesis in the adult hippocampus with behavioral performance on learning and memory tasks (14, 16, 17, 20, 48, 50, 51). Physiologic stimuli that promote learning and memory generally correlate with an increase in the rate of neurogenesis (21, 48-51, 53), while pathologic stimuli or stimuli that correlate with decreases in learning and memory performances have also been associated with decreases in adult hippocampal neurogenesis (3, 5, 12, 22, 25, 29, 30, 34, 44, 46, 53). The general consensus is that enhanced adult neurogenesis improves cognitive functioning (and conversely that impaired adult neurogenesis may impair cognitive functioning) but recent studies examining the behavioral consequences of ablating neurogenesis have produced variable and sometimes contradictory results (16, 17, 40, 41, 43, 51, 52). An understanding of the specific functional role of adult hippocampus, with respect to the function of the dentate gyrus (DG) and hippocampus, as well as in the context of the normal and diseased brain, is warranted.

Adult hippocampal neurogenesis has also garnered recent attention in the context of the degenerating and diseased brain. Insofar as adult neurogenesis contributes to hippocampal function, abnormalities in neurogenesis observed in a variety of disease models, affective disturbances, and normal aging, may underlie some cognitive deficits associated with these states. Eliminating or reducing deficits in adult hippocampal neurogenesis may therefore alleviate or reverse associated
cognitive impairments (3, 6), and adult hippocampal neurogenesis may serve as a relatively malleable and accessible therapeutic target.

This thesis first addresses the question of whether adult hippocampal neurogenesis plays a functional role in spatial pattern separation. The data presented in Chapter 1 support a role for adult hippocampal neurogenesis in spatial pattern separation, a process important for learning and memory. We showed that mice in which adult hippocampal neurogenesis had been ablated were selectively impaired at spatial discrimination for stimuli presented in close spatial proximity but not for stimuli presented at more distant spatial locations. Modulating the number of mature neurons through environmental enrichment does not appear to alter this function (Chapter 2). While we show a function for adult neurogenesis in spatial discrimination consistent with a role in spatial pattern separation, it is unclear whether newborn neurons themselves perform this role or whether they contribute, in more complex ways, to a network optimized for this function.

The second question addressed by this thesis is whether rodent models of neurodegenerative diseases, in which abnormalities in adult hippocampal neurogenesis appear to be a feature of disease progression, show impairments on cognitive tests sensitive to the changes in adult neurogenesis. Huntington’s disease is a suitable model in which to probe questions regarding degeneration and changes in plasticity in the degenerating brain given that: (1) the HD causing gene is known, (2) animal models of the disease exhibit reproducible symptoms that replicate to some degree clinical features of HD patients, and (3) neurogenesis and other forms of plasticity have been shown to be abnormal in both HD patients and HD rodent models. We
show that transgenic R6 mice expressing exon 1 of the human mutant HD gene express consistent patterns of impairments on hippocampus and neurogenesis sensitive spatial discrimination tasks (Chapter 4). The performance of R6 mice was impaired to a greater extent than WT mice in which neurogenesis had been ablated, a finding that is consistent with the widespread pathology characteristic of HD. These results are a first step toward probing cognitive performance in models of neurodegeneration without relying solely on motor measures as indicators of performance. Although this work addresses some questions regarding the function of adult hippocampal neurogenesis and abnormalities in adult neurogenesis and behavior in the R6 transgenic HD mouse model, it highlights an array of questions which remain to be addressed.

*What is pattern separation?*

The DG acts as an interface between the entorhinal cortex (and other regions of the brain) and CA3 to differentially encode small or weak changes in inputs (4, 26, 28, 31). This ability to maintain or expand the difference between similarly transmitted inputs has been termed pattern separation by computationalists (28). The term ‘pattern separation’ has also been applied to electrophysiological (e.g. (26)) and behavioral (e.g. (13)) phenomena. A common mechanism underlying cellular and circuit level encoding and behavioral performance has not been shown, but it is theoretically possible that a common mechanism underlies the ability of the DG to maintain and transmit orthogonalized information and that this ability is expressed in behavioral learning and memory correlates.
While the pattern separation function of the DG has been defined as an encoding process (28) (thus most analogous to the behavioral phenomena of ‘learning’), it is not clear whether the DG is also involved in retrieval processes (memory). Examination of immediate early gene expression in DG cells following re-exposure to previously learned testing environments, such as during probe trials of Morris water maze, suggests that some DG cells are also active during memory re-activation (4, 18, 19, 47). While it is at least theoretically possible to dissociate between encoding and memory on a mechanistic level, it is difficult to do so behaviorally. Any task involving learning necessarily involves memory. The converse is also true. It is difficult if not impossible to eliminate the possibility that outcome measures assessed during probe, retention, or memory phases of behavioral tasks reflect new learning, the integration of new information, or associations between new experiences and memories for old experiences. This is particularly confounding when investigating the function of the DG and the experimental evidence provided in this thesis further highlights this issue. It remains to be shown whether deficits in pattern separation dependent function depend on learning, memory, or both, and more specifically whether these deficits result from impairments in encoding or retrieval.

*Do newborn neurons have distinct function(s) to those of mature DG granule cells, or do they contribute to circuitry necessary for pattern separation function?*

Because new neurons are born into a highly specialized region of the adult brain, it is important to consider their contribution at cellular, circuit, and behavioral levels. Newborn neurons represent a highly plastic subpopulation of cells in the DG
that are thought to play a role in learning and memory. It is unclear whether these cells contribute to circuitry necessary for normal pattern separation function or whether these cells contribute in functionally unique ways to hippocampal processing.

Newborn cells that have fully matured appear to contribute to a homogenous functional group of GCs (24), but they may, in contrast, encode and maintain specific contextual information, such as temporal coding or pattern ‘integration’, as suggested by a recent modeling study (2). However, at immature stages, newborn neurons represent a functional group distinct from that of mature GCs. The distinct electrophysiological properties of immature neurons (enhanced LTP amplitude and a lower threshold for LTP induction compared to mature GCs) (10, 42, 54) indicate that these cells represent a robust form of plasticity within the DG, but it remains to be shown how this plasticity contributes to or modifies DG function. The hyperexcitability of newborn immature neurons may serve to overcome interneuron inhibitory input, thus allowing DG to transmit pattern separated representations that are then stored by CA3, as one modeling study suggests (32). Thus while newborn neurons may not pattern separate themselves, they could contribute to circuitry necessary to transmit or utilize orthogonalized information.

Alternatively, newborn neurons may contribute to hippocampal processing in ways distinct from those of mature GCs. Newborn neurons may mediate or participate in pattern separation function directly by virtue of their immature state and unique electrical properties. It is not possible from the experimental data presented in this thesis to resolve this question. The experiments presented in Chapters 2 and 3 involved long term reductions in the pool of immature neurons as well as any mature
neurons that these cells may have produced. In Chapter 3 enriched sham animals showed both an increase in mature neurons and an increase in neuronal differentiation, but did not differ in the numbers of immature neurons and proliferating cells compared to non-enriched sham mice 3 months following the initiation of a 1 month enrichment period. That this finding was not accompanied by a differential behavioral outcome may reflect the fact that the immature population of newborn neurons contribute uniquely to DG processing; that is, modulating or increasing the number of mature dentate GCs may not be beneficial in the context of specific functional capacities such as those demanded by pattern separation, but rather the pool (or size of the pool) of immature neurons may contribute to functional capacity directly or through modulating existing circuitry. This hypothesis is supported by recent experimental evidence involving lentiviral dnWnt expression in rat hippocampus in which a correlation between the number of Dcx-positive immature neurons and behavioral performance on spatial and object recognition tasks has been shown (17). In order to more directly address whether immature neurons serve a distinct functional role from that of mature granule cells, more transient models of reducing or silencing new born neurons is necessary.

The hypothesis that the pool of immature neurons may be important for DG function raises an additional interesting possibility that there may also be a threshold for the number of newborn cells that is functionally important. As reported in Chapter 2, the lentiviral mediated dnWnt expression in the DG of adult mice partially knocked down the number of newborn neurons but resulted in a behavioral impairment similar to that found in IR mice in which adult neurogenesis was almost completely ablated. It
is possible that a partial, subtotal lesion of DG is sufficient to disrupt normal DG function resulting in significant behavioral impairments, but dnWnt mice did not show an intermediate difference in performance between WT and IR mice due to the difficulty of the DNMP RAM task. Conversely, graded impairments following partial reduction in the numbers of newborn neurons have been observed in other spatial or context dependent tasks (17).

*Are newborn neurons pattern separators, pattern integrators, or a combination of both?*

It remains to be resolved how newborn neurons contribute to spatial discrimination and pattern separation dependent function. One interesting possibility is that newborn neurons, by virtue of their electrical properties, ‘integrate’ information, especially across time (1, 2). This hypothesis suggests that while the mature cells in the DG are suited to encode and amplify slight differences in contexts, episodes, etc., newborn neurons may provide information about the relation between inputs, especially for information which occurs coincidentally within a period of weeks (2). An alternative interpretation is that newborn neurons allow the flexible transfer of previously learned and/or stored information into representations in which novelty is a factor or in contexts in which the function required involves a high degree of interference, overlap, or ambiguity, i.e., cases in which CA3 does not perform a pattern separation function optimally. The difference between these two interpretations is both mechanistically and behaviorally relevant. In the former case, newborn neurons would serve a function inverse to that of the proposed function of
the mature dentate GCs and the DG as pattern separators. By encoding information about similarities, it is theoretically conceivable that newborn neurons within the DG would contribute to more highly ‘tuned’ representations and thus the DG would be better able to transmit more highly separated output codes. This arrangement would require a second level of analysis in that a comparison must then be made between the information provided by new versus older neurons (a function that could potentially occur in the DG or in CA3). The alternative hypothesis, that newborn neurons allow association or transference of information, may imply that information that is learned well by other regions, such as CA3, or by newborn neurons themselves, could then be utilized in forming distinct representations in perceptually difficult contexts or between context with a high degree of overlap, a function that may occur in parallel with processing by mature DG cells. In the context of the behavioral tasks described in Chapter 2 these two hypotheses can be summarized in the following ways: the former hypothesis suggests that the encoding of information pertaining to the similarities (temporal or otherwise) between two stimuli presented closely in space allows for inference regarding their differences while the latter hypothesis suggests that discriminations or tasks involving two difficult to distinguish/remember stimuli are not ‘learned’ (or weakly learned) in and of themselves by newborn neurons, but rather learned information is flexibly applied in novel or contextually ambiguous contexts in a neurogenesis dependent manner. This latter hypothesis can by summarized as learning by transference or application. For example, in the context of the behavioral tasks described in this thesis, highly separated stimuli may be learned with or without the participation of the DG, but presumably involving extra-DG regions such as CA3,
and that this ‘knowledge’ (or code) is then applied, via participation of newborn immature neurons, to more difficult cases (small separations) in which the same behavioral rules hold. These potential roles for newborn neurons in plasticity within the context of hippocampal learning and memory need not be mutually exclusive. The role of newborn neurons may be to provide a form of synaptic modification or plasticity that outlasts the episode to be learned/encoded (the initial period when mature dentate GCs are active) thus allowing for the “formation of codes for regularly occurring combinations of active inputs that might need to participate in different episodic memories” (37) and by virtue of this function serve as temporal integrators. The mechanism by which newborn neurons contribute to pattern separation remains to be resolved.

What are the implications of this role for neurogenesis in pattern separation for behavioral testing?

The studies reported here offer some technical insight into the inconsistent and contradictory functional impairments observed after reducing or ablating adult neurogenesis in other studies (16, 17, 40, 41, 43, 51, 52). The deficits reported in Chapter 2 and replicated in Chapter 3, utilizing two methods to ablate neurogenesis and two different, yet comparable, testing contexts, suggest that impairments following ablation of neurogenesis are parameter-sensitive (i.e., specific to conditions with a high reliance on pattern separation). These data suggest that testing contexts in which task difficulty and/or fine discrimination (encoding and/or memory dependent processes) is maximized may require neurogenesis-dependent plasticity, whereas
testing contexts in which, for example, it is difficult to control for the external cues or strategies utilized by animals (such as in the Morris water maze), may or may not require such plasticity.

*Why is adult neurogenesis potentially important in the context of neurodegeneration?*

Given our finding that the adult neurogenesis plays a specific functional role in spatial pattern separation (and may play a similar role in pattern separation for temporal or contextual information), and that adult neurogenesis seems particularly vulnerable to changes associated with neurodegenerative diseases, investigations into the relation between disease-associated environmental changes, abnormalities in adult hippocampal neurogenesis, and cognitive dysfunction is warranted. The link between plasticity in the adult and/or diseased brain and cognitive function is particularly important for understanding both the normal process of learning and memory and abnormalities that may play a role in neurodegenerative diseases.

This thesis makes an initial foray into investigating the link between neurogenesis dysregulation at the cellular level and behavioral performance in the context of a murine HD model. Fundamentally, the question underlying all disease and pathologic contexts in which neurogenesis is affected is whether changes in adult neurogenesis contribute to or are a consequence of pathology and/or dysfunction of the diseased brain. In therapeutic terms, the mechanistic answer to this question may not be as critical as the question of whether restoring neurogenesis may be therapeutically relevant. For example, deficits in adult hippocampal neurogenesis have been correlated with depression but do not appear to be causative of depression (they
may, in fact, be a consequence of the disease state) (9, 36, 38). However, antidepressant treatments appear to, at least in part, require, or coincide with, the recovery of neurogenesis and other forms of plasticity to be effective (36, 39, 45). Thus, understanding changes in plasticity in the context of the diseased or degenerating brain, and the functional consequences of such changes, would not only further our insight into both normal and pathologic neural function, but may also inform our clinical treatment of patients with neurodegenerative diseases. This is particularly relevant in HD given that no disease modifying treatment is currently available (35).

*Is a decrease in plasticity in the degenerating brain relevant to Huntington’s disease on a neuronal and/or functional level and is it possible to behaviorally probe for specific cognitive deficits in animal models of neurodegenerative disease?*

A common feature of many neurodegenerative disease states is a reduction in neural plasticity. In HD, adult neurogenesis has been shown to be affected in both HD patients (7, 8) and rodent HD models (11, 25, 33, 34). We show that, at the cellular level, abnormalities in adult neurogenesis are more complex than previously reported. Our findings suggest abnormalities develop during the differentiation of precursor cells and may further support the hypothesis that neuron non-autonomous defects in the neurogenic niche may contribute to abnormalities in adult neurogenesis (34). It is interesting to note that the increase in nestin-primary processes in R6/2 DG (Chapter 4) may coincide with an increase in quiescent NSCs or an imbalance between proliferative and inactive states, a finding that may help to explain recent reports
showing an absence of the neurogenic response to neurogenesis promoting stimuli in HD transgenic mice (23, 34). Further work regarding the mechanism underlying these abnormalities is required.

Whether deficits in adult neurogenesis contribute to behavioral and functional deficits in the context of neurodegenerative disease has not been conclusively shown. This is a difficult question to approach given the many changes in the degenerating brain have common underlying feature or occur in parallel. In HD, it is difficult to dissociate changes in motor, cognitive, affective, and motivational systems, and this problem is particularly confounding in behavioral paradigms. This thesis does not show a direct causal relationship between abnormalities in adult neurogenesis and behavioral impairments; however, it does take a first step toward developing and applying behavioral tasks with a high premium on cognitive performance (and a low reliance on outcome measures that are motor-dependent) in the context of neurodegenerative disease. Using two behavioral tasks that are both hippocampus and neurogenesis sensitive (Chapter 1), we found that mice show impairments in performance across tasks and in both the R6/2 and R6/1 models which differ by both disease onset and rate of progression (27). This finding alone is not trivial in that it is not yet understood whether hippocampal dysfunction is a feature of HD.

The hypothesis that adult neurogenesis plays a potentially specialized functional role suggests that the contribution to functional impairments are likely equally specialized. Nonetheless, that neurogenesis plays a functional role in spatial pattern separation suggests that abnormalities in neurogenesis could contribute to pathogenesis or disease progression. The investigations reported in Chapter 4 raise
further questions regarding the modeling of neurodegenerative diseases and the behavioral relevance of such models. While the behavioral deficits observed across the three behavioral experiments reported in Chapter 4, despite showing a consistent pattern of impairment, show a more pronounced deficit than which deficits in neurogenesis alone could account, there remains the possibility that restoring neurogenic plasticity within the hippocampus could ameliorate some of the behavioral symptoms. Interestingly, a ten week course of the antidepressant fluoxetine restored levels of neurogenesis in the R6/2 HD transgenic mouse and was associated with improvement in spontaneous alternation in the T-maze (15), a task which may be functionally similar to that used in our studies. While it is difficult, if not impossible, to conclusively isolate selective cognitive features of disease progression, and their underlying mechanisms, in animal models of neurodegeneration, a preponderance of evidence examining impairments utilizing selective behavioral tasks and reversing or ameliorating those impairments by targeting mechanistic pathways and processes will undoubtedly provide insight into complex neurodegenerative disease.

In summary, this thesis provides evidence for a functional role of adult hippocampal neurogenesis in spatial discrimination consistent with a pattern separation function. It is not clear whether newborn neurons contribute directly to this function or to a circuit responsible for normal DG and hippocampal function. Despite the well known correlations between increased rate of neurogenesis and performance on learning and memory tasks, modulating the number of mature dentate GCs, without increasing the pool of immature neurons stably over time, does not appear to affect
this behavior. However, long term deficits in adult neurogenesis, as examined in the context of the neurodegenerative disease HD, may contribute to deficits in spatial discrimination observed in R6 mice expressing the mutant human HD transgene. Further investigation into the mechanism(s) underlying the role of adult neurogenesis in spatial pattern separation and whether abnormalities in this form of plasticity contribute to cognitive dysfunction in HD would provide insight into the biological process of neurogenesis in the adult brain and contribute to our understanding of disease processes and the link between disease and cognition.
References


