Title
MHC I Molecules in the Regulation of Neural Stem Cell Function and Cognition

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Publication Date
2018

Peer reviewed|Thesis/dissertation

MHC I Molecules in the Regulation of Neural Stem Cell Function and Cognition

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

To my parents, Tim and Winny;
And my sister, Kathy

Acknowledgements

I owe my gratitude, first and foremost, to my thesis advisor Saul Villeda, who has helped me to find a voice in a place that often feels noisy and encouraged me to take steps into territory uncharted. Thank you for being an unbelievably supportive mentor, and for having the trust to invite us to start a lab with you. This lab has been an incredible place to grow as a scientist, and I am forever thankful for your patience, motivation, guidance, and friendship over these last years. Thank you for providing us with countless opportunities to learn: the knowledge and experiences I have gained, I will cherish for a lifetime.

I have been fortunate to have many wonderful role models during my time in graduate school at UCSF. As the saying goes, “If I have seen further, it is by standing on the shoulders of giants.” Thank you to my committee members, Arturo Alvarez-Buylla, Daniel Lam, and Susanna Rosi— it has been a true pleasure to learn from you. Your expertise and advice have instrumentally guided my work, and I have seen all the further because of it. Thank you for the excellent examples you have set, you are the scientists we all aspire to become. I must also thank Rushika Perera and Georgia Panagiotakos for not only the help with experimental design and talk preparations, but also for the invaluable career and life advice you have shared. I am also grateful to Mario Merlini, one of the first mentors I had as a rotation student at UCSF—what I have learned from you, both in lab and in life, still have a bearing on me today.

I also want to extend a heartfelt thank you to all the members of the Villeda Lab, past and present. This work would not have been possible without the knowledge and

endless support you have provided, but more importantly, the “saul-idarity” and friendships that have developed both in the lab and outside. Thanks to Kris Plambeck for your help in the mysterious world of molecular cloning, to Jill Bouchard for your mentorship in the lab and for your advice and encouragement in pursuing teaching, to Cedric Snethlage, Cesar Sanchez, and Brit Ventura who have kept the lab running as smoothly as it does, to Jeremy Shea for teaching me how to prepare RNA-seq libraries, to Geraldine Gontier for your friendship and advice of all sorts, and to Joe Udeochu for sharing your endless knowledge. A special thank you to Luke Smith and Liz Wheatley, for the stimulating scientific discussions, the laughter, the WCWs, and your friendship. To Manasi Iyer, Shelly Fan, Hank Garcia, Buddy White, Alana Horowitz, and Kirsten Chui, your enthusiasm and senses of humor have made with lab a wonderful place to work. It has been a true pleasure to be a part of this lab.

There have been many others who have contributed greatly to my thesis work. Thank you to Greg Bieri at Stanford for your assistance with the design and generation of the lentiviral constructs that have become a critical part of the project. Thank you to Soren Muller, whose expertise in bioinformatics has hugely shaped this work, and kept us ahead of the game. Thank you to Sandy Canchola, who keeps the Parnassus behavior core in such great shape. I am also very grateful for several members that run the Neuroscience Graduate Program: Anatol Kreitzer, Roger Nicoll, and Lou Reichardt who have directed it, and Pat Veitch and Lucita Nacionales for your superb administrative support.

Finally, I am eternally grateful for my parents, Tim and Winny. I cannot thank you enough for your endless love for me and for the support and encouragement you have ceaselessly provided my whole life. And to my dear sister, Kathy: thank you for being a constant source of encouragement, inspiration, and laughter in my life.

It really does take a village to raise a child, and I am forever indebted to the community that has supported me in every possible way to see the completion of this work.

Abstract

MHC I Molecules in the Regulation of Neural Stem Cell Function and Cognition

by

Kai Lin Lin

Proteins of the major histocompatibility complex class I (MHC I), previously known mainly for antigen presentation to cells of the immune system, have recently been shown to play a crucial role in both developmental neural refinement and adult synaptic plasticity. However, their function in non-neuronal cell populations in the brain or the impact of cellular changes regulated by MHC I molecules on behavioral outputs have yet to be investigated. In this dissertation, I identify novel, non-immune roles for two specific classical MHC I molecules (out of as many as 50 encoded in the murine genome) in the brain. I demonstrate that H2-K^b is a negative regulator of proliferation in neural stem cells (NSCs), a unique cell type amenable to regenerative biology. Using genetic mouse models as well as primary NSC cultures, I show that deletion or knockdown of H2-K^b molecules causes increased proliferation and neurogenesis in the adult hippocampus *in vivo* as well as in a cell-type specific environment *in vitro*. Transcriptome analysis of NSCs lacking H2-K^b reveals that this enhanced proliferative potential is due to an induction of several growth factor receptor signaling pathways. I found functionally that one mechanism by which H2-K^b may exert its inhibition is through a physical interaction with fibroblast growth receptor 1, putatively dampening its signaling. Though also highly expressed in the hippocampus, I show that the other classical MHC I molecule, H2-D^b, does not play a major role in NSC function. I thus

probed other hippocampal processes and revealed a developmental necessity H2-D^b in preserving normal locomotion, anxiety-related behavior, and spatial and associative memory. Furthermore, I show that its acute ablation in the adult hippocampus can remarkably improve hippocampus-dependent memory. The hippocampus, an extraordinary brain structure in its capacity for immense plasticity throughout life, is particularly vulnerable to deleterious conditions such as disease and aging. As MHC I molecules are known to increase in the aged and injured brain, understanding their precise functions in the hippocampus may provide opportunities to seize its neuroplastic and regenerative potential for therapeutic intervention.

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List of Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
β2m	β2-microglobulin
BDNF	brain derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
CD68	cluster of differentiation 68
CNS	central nervous system
$D^{-/-}$	genetic knockout of H2-D^b
Dcx	doublecortin
DG	dentate gyrus
EdU	5-ethynyl-2´-deoxyuridine
EGFR	epidermal growth factor receptor
FGFR	fibroblast growth factor receptor
GFAP	glial fibrillary acidic protein
GO	gene ontology
HLA	human leukocyte antigen
Iba1	ionized calcium-binding adapter molecule 1
IGF1	insulin-like growth factor 1
$K^{-/-}$	genetic knockout of H2-K^b
$KD^{-/-}$	genetic double knockout of H2-K^b and H2-D^b
LGN	lateral geniculate nucleus
LPS	lipopolysaccharide
LTD	long-term depression

LTP	long-term potentiation
MHC I	major histocompatibility complex, class I
NeuN	neuronal nuclei
NK	natural killer
NMDA	*N*-methyl-D-aspartate
NSC	neural stem cell
Pir	paired immunoglobulin-like receptor
RAWM	radial arm water maze
RNAi	RNA interference
shRNA	short hairpin RNA
sh-D	shRNA sequence targeting H2-D^b mRNA
sh-K	shRNA sequence targeting H2-K^b mRNA
SNP	single nucleotide polymorphism
Tap1	transporter associated with antigen processing 1
Tbr2	T-box brain protein 2
TCR	T cell receptor
V1	primary visual cortex
VEGF	vascular endothelial growth factor
WT	wild type

Chapter 1

Introduction

Major histocompatibility complex class I (MHC I) proteins are most well known for their role in antigen presentation and immunological surveillance in the adaptive immune system (Neefjes et al., 2011). Although MHC I molecules are expressed by all nucleated cells, a long-held central dogma in the field of neuroimmunology describes certain organs— such as the central nervous system (CNS)— as being immune-privileged, with this ability to evade normal inflammatory responses being imparted by a near absence of classical immune molecules under healthy conditions (Joly et al., 1991; Murphy, 1923). In the last two decades however, this belief has been challenged following the discovery that immune components are not only present in the CNS, but also play critical roles in brain development and synaptic function (Boulanger, 2009; Shatz, 2009). However, their roles in non-neuronal cell types in the brain or how the cellular changes they regulate influence behavioral outputs have never been elucidated. This dissertation explores non-canonical roles for specific MHC I molecules in the context of previously unstudied cell types and functions of the brain: H2-K^b in the regulation of neural stem and progenitor cell function and hippocampal neurogenesis, and H2-D^b and its modulation of hippocampus-dependent cognitive function.

Structure and Classification of MHC I Molecules

MHC molecules (referred to as human leukocyte antigen [HLA] complex in humans, and H-2 complex in mice) are a set of cell surface proteins critical in facilitating

the immune system's ability to recognize and respond to foreign antigens in the body. The MHC gene family can be classified into two major subgroups based on structure, expression, and function: class I (MHC I) and class II (MHC II). MHC II molecules are normally expressed by specialized immune cells, such as macrophages, B cells, and dendritic cells; they function to present extracellularly derived antigens to CD4-positive T cells, mounting a humoral response. MHC I molecules, which this dissertation will focus on, are expressed on the surface of all nucleated cells and present intracellular peptides to CD8-positive, cytotoxic T cells.

MHC I molecules are trimeric in structure (**Figure 1A**), consisting of a transmembrane heavy chain, a smaller β2-microglobulin (β2m) light chain, and the antigenic peptide being presented (Bjorkman and Parham, 1990). The heavy chain is a 44kD glycoprotein with three extracellular domains (α1-3) and short cytoplasmic tail of ~30 amino acids; the α1 and α2 domains are highly polymorphic and form the peptide-binding groove, while the membrane-spanning α3 domain is relatively conserved and carries sequence similarity to the immunoglobulin superfamily. The α3 domain also interacts with the CD8 co-receptor on T-cells in its classical immune role (Figure 1B). β2m is the invariant, 12kD subunit that is non-covalently bound to the MHC I heavy chain, with the ability to be shed off the cell surface and detected in the circulation. MHC I molecules bind peptides that are 8-10 amino acids long and present them on the cell surface to surveilling immune cells. The proteasome degrades intracellular proteins into these short peptides, which are then translocated from the cytosol into the lumen of the endoplasmic reticulum by the transporter associated with antigen processing 1 (Tap1). Here, the α chain of MHC I molecules can bind the peptide and, after association of

β2m, the complete MHC I complex can be stably expressed on the cell surface (Williams et al., 2002).

MHC I molecules can be further divided into two groups: classical MHC I molecules (HLA-A, HLA-B, and HLA-C in humans) which are the polymorphic, trimeric structures described above, as well as non-classical MHC I molecules. These non-classical counterparts may be less polymorphic in the α1 and α2 domains, or may lack components such as the peptide, β2m, or the α3 domain. These molecules are less well studied but have been ascribed roles as antigen-presenting and antigen-independent ligands in the innate immune system (Allen and Hogan, 2013). This dissertation will focus on the two classical MHC I subtypes found in C57BL/6 mice, H2-K^b and H2-D^b.

Canonical Function of MHC I in the Immune System

MHC I molecules facilitate the ability of the immune system (in particular, T cells) to recognize self versus non-self antigens and to appropriately initiate either autotolerance or destruction of infected host cells. They display proteolytically digested peptides from intracellular proteins on the surface of all nucleated cells for sampling by cytotoxic T cells. Cytotoxic T cells express T cell receptors (TCR) that recognize epitopes presented by MHC I, as well as CD8 co-receptors, which confer binding specificity to the α3 domain of the MHC I complex (**Figure 1B**). When intracellular pathogens or cancerous transformation are detected, the TCR signaling pathway is initiated. Subsequent steps of cell-mediated immunity involve the release of cytolytic proteins by cytotoxic T cells, which trigger the caspase cascade in the infected cell and lead to its apoptosis (Broere et al., 2011).

TCR binding is the canonical interaction exhibited by MHC I molecules, but MHC I molecules have a multitude of receptors in the immune system with which they are capable of interacting. These include receptors found on natural killer (NK) cells, such as paired immunoglobulin-like (Pir) and Ly49 receptors, which can have either activating or inhibitory functions in NK immune synapse formation (Long, 2008).

The roles of MHC I molecules in the immune system have been extensively characterized. In contrast, the expression and function of MHC I molecules outside the immune context, and perhaps independent of antigen presentation, are less well understood. Significant progress, however, has been made in defining their non-canonical roles in other capacities— in particular the CNS— since their discovery in an unbiased screen for genes involved in synapse remodeling two decades ago.

Immune Privilege Reconsidered: Roles of MHC I in the CNS

During critical periods in brain development, early neuronal activity can sculpt the connections maintained in long-lasting circuits through the preservation and strengthening of certain synapses and the elimination of weaker ones. By using the developing visual system as a model, Corriveau et al. sought to screen for the gene expression changes that might translate this initial strengthening of synaptic strength into long-term structural changes in neurons (Corriveau et al., 1998). Surprisingly, it was found that genes affected by neural activity blockade in this system included MHC I, suggesting its involvement in synaptic development and maintenance. This finding was unexpected because until relatively recently, it was thought that neurons did not express MHC I or other classical immune components except during damage or viral infection

(Joly et al., 1991). Beginning with this study, there is now a growing body of literature demonstrating that not only are MHC I molecules present in the healthy CNS, but that their expression can be influenced by neural activity and that they play critical roles in nervous system development and synaptic plasticity.

Expression of MHC I and its receptors in the CNS

MHC I mRNA and protein can be found in the CNS across many species including rodents, cats, marmosets, and humans (Corriveau et al., 1998; Ribic et al., 2011; Zhang et al., 2015), and is controlled in its temporal and spatial distribution. Expression in neurons and glial cells have been observed in the visual and olfactory systems, hippocampus, cerebral cortex, cerebellum, spinal cord, and motor neurons (Ishii and Mombaerts, 2008; Lindå et al., 1998; Massa et al., 1993; McConnell et al., 2009a; Ribic et al., 2011; VanGuilder Starkey et al., 2012). In neurons, MHC I is expressed both pre- and post-synaptically, with distribution being developmentally dynamic. Although present throughout life in the mammalian CNS, MHC I levels are highest during early post-natal development and during aging (in the hippocampus) (Needleman et al., 2010; VanGuilder Starkey et al., 2012).

MHC I expression is also influenced by the activity state of the neuron. When activity is decreased via intracranial infusion of tetrodotoxin, MHC I mRNA correspondingly decreases; consistently, increasing activity by infusing kainic acid increases MHC I mRNA in the hippocampus and cortex (Corriveau et al., 1998).

The discovery of MHC I molecules in the CNS invites the question of whether its known immune receptors are also present, and more interestingly, are their functions in

the CNS comparable or unrelated to their immune roles? Indeed, adapter proteins in the TCR complex are expressed in the brain: CD3ζ is found throughout the brain and CD3ε is expressed in the cerebellum (Baudouin et al., 2008; Nakamura et al., 2007). However, functional TCR receptors themselves do not appear to be present in the CNS. Along with the lack of T cells in the uncompromised CNS, this observation casts doubt that MHC I is signaling through the canonical antigen presentation pathway. NK cells receptors—PirB, Ly49—are also found in hippocampal neurons in vivo, and in cultured cortical neurons, respectively (Syken et al., 2006; Zohar et al., 2008). These receptors may have neuronal functions dependent as well as independent on MHC I binding. In general, the pattern of MHC I / MHC I-related receptor expression in the brain is correlated with regions that exhibit high plasticity.

Activity- dependent visual system development

CNS plasticity refers to the ability of neurons to change the strength of their connections or remodel their organization in response to various stimuli. The first direct evidence of MHC I molecules regulating neuronal plasticity was demonstrated in the mouse developing visual system, which elegantly models both visual input-independent and input-dependent remodeling in the lateral geniculate nucleus (LGN) and the primary visual cortex (V1). The early stage of development involves retinal ganglion cells projecting from both eyes to the thalamic dorsal LGN and forming segregated eye-specific layers; these form due to early spontaneous activity from the retinal cells. LGN neurons then send projections to the V1 where their activity organizes layer IV of the visual cortex into eye-specific patches called ocular dominance columns; visual activity

coming from both eyes is required for proper maturation of synapses in the V1. Mice deficient for cell-surface expression of MHC I ($\beta2m^{-/-}Tap1^{-/-}$) cannot form the tightly localized, eye-specific regions in the LGN (Huh et al., 2000). The ipsilateral projections are expanded and there is improper segregation between left and right eye territories, caused by extraneous, inappropriate synapses that are normally removed during development in WT mice. The inputs are able to make it into the LGN, however, suggesting that MHC I is involved only in regulating the later processes of finer synaptic refinement and pruning rather than early, gross patterning of the developing visual system.

A more specific mouse model, lacking just two of the over 50 MHC I genes (specifically H2-K^b and H2-D^b double knockout, $KD^{-/-}$), show similar aberrant eye-specific segregation in the LGN. In addition, $KD^{-/-}$ mice also show enhanced ocular dominance plasticity compared to WT mice (Datwani et al., 2009). This experimental paradigm is done at the onset of the visual cortex critical period at P22 and tests the ability of the inputs of one eye to expand into binocular zones in the V1 when the other eye is surgically closed or removed. Datwani et al. showed that $KD^{-/-}$ mice had increased expansion of cortical inputs from the open eye as measured by in situ hybridization of the immediate early gene Arc, when compared to WT animals.

Together, these data directly implicate MHC I, specifically the two classical molecules H2-K^b and H2-D^b, as mediators of synapse weakening and removal during retinogeniculate refinement and reveal their role in negatively regulating ocular dominance plasticity.

Neurite outgrowth and establishment of synapses

MHC I molecules have been shown to modulate the structure of individual neurons as well as the connections that form between neurons. They limit the growth of dendrites and axons *in vitro*: axonal extension of cultured retinal ganglion cells towards thalamic explants overexpressing H2-D^b is stunted and addition of exogenous, recombinant MHC I molecules limits neurite outgrowth in retinal explants (Escande-Beillard et al., 2010; Wu et al., 2011b). Synaptogenesis is also affected by MHC I molecules in a similar bidirectional manner. Reduction of surface MHC I (via knockdown of β2m) increased the glutamatergic and GABAergic synapse density in cortical neurons in culture (Glynn et al., 2011). Conversely, overexpression of the H2-K^b isoform in cultured neurons elicited the opposite effect: a decrease in synaptic density. This was further corroborated *in vivo* in the visual cortex where it was observed that $\beta2m^{-/-}$ mice had higher synapse density compared to WT mice at P8, P11, P23 and P60. The inhibitory role that MHC I molecules play in the growth of neurites and establishment of synaptic connections are in line with the overgrowth phenotypes seen in MHC I knockouts during visual system development.

Synaptic plasticity

In additional to structural modulation, MHC I molecules can also functionally regulate transmission of electrical signals between synapses as well as influence synaptic plasticity. In cultured hippocampal neurons and cortical slices from $\beta2m^{-/-}$ $Tap1^{-/-}$ mice, miniature excitatory postsynaptic current frequency (but not amplitude) is increased (Goddard et al., 2007), suggesting an enhancement of presynaptic release

properties. When presynaptic release is correlated with postsynaptic receptor activation for a prolonged time, a type of plasticity called long-term potentiation (LTP) – or an increase in synapse strength— occurs between participating neurons. LTP is widely considered one of the major cellular mechanisms facilitating learning and memory. When two neurons fire in an uncorrelated manner for a prolonged time, long-term depression (LTD) will occur and the weakened synapse is eventually eliminated. In the same $\beta 2m^{-/-}Tap1^{-/-}$ mice, hippocampal LTD is absent while LTP is enhanced (Huh et al., 2000). MHC I molecules function to dampen synaptic strength and plasticity, in line with their inhibitory roles in neuronal structure.

MHC I signaling in the brain

It is clear that MHC I molecules are critical mediators of CNS development, structure, and plasticity, but the underlying mechanisms are less well studied. As these molecules have a short cytoplasmic tail, it is generally assumed that MHC I acts by interaction with other receptors rather than its own intracellular signaling. In the brain, MHC I may engage in *trans*- synaptic interactions with immune-related molecules on neighboring neurons. Animals lacking CD3ζ phenocopy $\beta 2m^{-/-}Tap1^{-/-}$ and $KD^{-/-}$ mice, showing enhancements in retinogeniculate projection, increased retinal ganglion cell dendritic branching, and enhanced LTP with absent LTD (Huh et al., 2000; Xu et al., 2010). Mice deficient of PirB have enhanced ocular dominance plasticity and cultured neurons treated with Ly49 anitbodies have inhibited neurite outgrowth but increased cell survival (Syken et al., 2006; Zohar et al., 2008); as these phenotypes are similar to

MHC I deficient mice, it suggests that MHC I and these immune receptors may be involved in the same pathway.

In addition to *trans* interactions, MHC I can also form complexes or bind to adjacent proteins in *cis* interactions (**Figure 1B**). *Cis*-interacting proteins comprise of immune receptors (such as NK receptors and various cluster of differentiation molecules) but also non-immune components like insulin receptors, insulin-like growth factor receptors, epidermal growth factor receptors (EGFR), luteinizing hormone receptors, amongst others (Arosa et al., 2007). When interacting on the same surface, MHC I has been implicated in regulating trafficking and internalization of growth factor and hormone receptors (Olsson et al., 1994; Schreiber et al., 1984; Solano et al., 1988). Examples of non-immune MHC I interactions in the CNS include the regulation of vomeronasal pheromone receptor trafficking by non-classical MHC I molecules, and the negative modulation of hippocampal synapse density by inhibition neuronal insulin receptors (Dixon-Salazar et al., 2014; Loconto et al., 2003).

A thorough examination of MHC I and MHC I-related receptor expression patterns, both across time and the various cell types in the brain, would provide important clues to how these immune molecules are influencing CNS functions and plasticity. Currently, the field has focused on MHC I in neurons but their expression and roles in non-neuronal CNS cell types have remained completely understudied. Neural stem or radial glial cells (NSCs), capable of generating functional neurons, divide embryonically to populate the CNS during development as well as postnatally to confer additional plasticity to the brain partially throughout life. Chapter 3 of this dissertation will

explore the role of classical MHC I molecules in regulating NSC function and hippocampal neurogenesis.

Hippocampal Neurogenesis

A brief history

Neurogenesis, the process by which NSCs generate new neurons, is most active during embryonic development but was discovered to continue postnatally in a variety of species including rodents, birds, lizards, and humans (Altman and Das, 1965; Eriksson et al., 1998; Garcia-Verdugo et al., 1989; Goldman and Nottebohm, 1983; Gould et al., 1999; Lois and Alvarez-Buylla, 1994). In rodents, the primary regions of neurogenesis are the ventricular-subventricular zone of the lateral ventricles (Altman, 1969; Lois and Alvarez-Buylla, 1993) and the subgranular zone in the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965; Cameron et al., 1993), a structure critical to the formation of new memories. In the subgranular layer of the adult DG, Seri et al. were the first to describe cells expressing glial fibrillary acidic protein (GFAP) as the stem cells that give rise to intermediate progenitors, which then become neuroblasts (Seri et al., 2001). Immature neurons migrate into the inner granule cell layer where differentiation into DG granule cells occurs. In a process that spans about four weeks, new neurons are born that receive inputs from the molecular layer and send projections through the hilus and into the CA3 of the hippocampus. The integration of adult-born neurons into existing hippocampal circuitry suggests their importance in learning and memory. A growing body of evidence, with analyses at the cellular, circuitry, and behavioral levels, have suggested significant contribution of adult hippocampal

neurogenesis to spatial learning and memory, pattern discrimination, trace conditioning and contextual fear conditioning, and even clearance of previous hippocampal traces (reviewed in Deng et al., 2010). However, evidence for specific functions of adult neurogenesis in these processes is still inconsistent between studies and requires further investigation (reviewed in Oomen et al., 2014). Dysregulation of neurogenesis is apparent in variety of neuropsychiatric disorders and aging, and often associated with a decline in cognitive capacity (Haughey et al., 2002; Kuhn et al., 1996; Parent et al., 1997; Toro and Deakin, 2007; van Wijngaarden and Franklin, 2013). Functional ongoing neurogenesis offers the maturing brain a striking plasticity, and understanding its regulators can help harness this innate regenerative capacity of the CNS in the treatment of brain disorders.

Mechanisms regulating adult neurogenesis in the murine brain

Adult neurogenesis is a tightly regulated process, with influences stemming both intrinsically and from the external niche or systemic environment.

- Intracellular

Cell-cycle regulators, transcription factors, and epigenetic factors are three major classes of cell-intrinsic regulators of adult NSC functions. Cell cycle inhibitors such as p16 and p53 can maintain quiescence of adult NSCs, transcription factors like the FoxOs, Sox2 and TLX are important in the maintenance of precursor pools, and epigenetic factors like Tet2 and mixed-lineage leukemia 1 (Mll1) can influence NSC proliferation and differentiation (Ehm et al., 2010; Gontier et al., 2018; Lim et al., 2009; Paik et al., 2009; Qu et al., 2010).

- Extracellular/ Systemic

Morphogens, such as Notch, Shh, Wnts, and BMPs, exert various influences on adult NSCs to fine-tune their behavior, with some regulating cell proliferation while others maintain the stem cell pool (Choe et al., 2016). Growth factors, neurotransmitters, and hormones in the local niche are also major players in the extracellular regulation of NSC function (Zhao et al., 2008). Epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) are required for maintaining NSCs *in vitro*, and also promote proliferation *in vivo* (Kang and Hebert, 2015; Kuhn et al., 1997; Lee et al., 2009). In the dentate gyrus, NMDA-type glutamate receptor activity is inversely correlated with NSC proliferation and GABAergic influence can promote their differentiation (Cameron et al., 1995; Tozuka et al., 2005).

Outside of the niche, systemic or environmental factors can also influence adult neurogenesis. Exercise, enriched living environment, learning, and exposure to young blood are able to increase generation of adult-born neurons, whereas chronic stress, inflammation, diabetes, and aging can impair adult neurogenesis (Kempermann et al., 1997; Kuhn et al., 1996; Mirescu and Gould, 2006; van Praag et al., 1999; Stranahan et al., 2008; Villeda et al., 2011).

Immune regulators of neurogenesis in the murine brain

There is also precedent that the process of adult neurogenesis is sensitive to immune influence, both from resident CNS macrophages called microglia as well as peripheral lymphocytes. Mice injected intrathecally or systemically with lipopolysaccharide (LPS), a potent activator of innate immunity, showed decreases in

hippocampal neurogenesis (Monje et al., 2003). This was attributed to a robust activation of microglia in the brain, leading them to adopt a cytotoxic phenotype and to secrete pro-inflammatory factors like TNF-α and IL-6. A multitude of cytokines such as interleukins, interferons, and tumor necrosis factor, secreted by both CNS and non-CNS cell-types in response to inflammation, are known to bidirectionally regulate various aspects of NSC function (reviewed in Borsini et al., 2015). Peripheral immune cells have also been shown to support adult neurogenesis under physiological conditions. Immune-deficient (both nude and severe combined immunodeficiency [SCID]) mice show impairments in hippocampal neurogenesis, which could be partially restored upon reconstitution of T cells; the secretion of brain-derived neurotrophic factor (BDNF), either directly by T cells or in directly by NeuN-positive neurons via stimulation by T cell-derived cytokines, is thought to underlie this observation (Ziv et al., 2006). The modulation of immune components has, excitingly, been shown to mitigate the neurogenic decline that occurs in aging. β2m, the light chain of MHC I molecules, decreases hippocampal neurogenesis when exogenously added, but aged animals genetically lacking β2m ($\beta2m^{-/-}$) display an increase in newborn neurons compared to their aged WT counterparts (Smith et al., 2015). Moreover, aged $\beta2m^{-/-}$ mice also show enhanced learning and memory in the radial arm water maze (RAWM) and contextual fear conditioning, tasks that are dependent on the hippocampus. Whether or not these cognitive improvements are a direct cause of improved neurogenesis is unclear, but there is strong support that immune modulation of neural plasticity and cognitive function can occur in various ways.

Recent findings in human adult neurogenesis

The pioneering study describing dividing cells in the adult human brain generated excitement, as the prospect of harnessing newborn neurons to promote human health became a potential possibility. In this study, postmortem brain tissue of adult patients who received infusions of the thymidine analog bromodeoxyuridine (BrdU) showed its incorporation into dividing cells in the hippocampus as previously seen in rodents (Eriksson et al., 1998). Subsequent studies used complementary approaches—histological analysis of endogenous markers of immature neurons (Knoth et al., 2010) and radiocarbon dating (Spalding et al., 2013)— to demonstrate similar findings. Though exciting, the existence of adult neurogenesis has been contentiously debated in the past, with flawed methodology being cited (Rakic, 2002). The debate continues today as two prominently published studies demonstrate opposite findings: one report concluded that neurogenesis in the human hippocampal DG drops to undetectable levels during childhood (Sorrells et al., 2018), whereas the other reported lifelong hippocampal neurogenesis in humans (Boldrini et al., 2018). While many questions remain unanswered, these new papers challenge us to look forward, optimizing our research questions regarding plasticity and refining our techniques as they pertain to the human brain.

Cognition

Cognition is an immensely wide-ranging process that involves the acquisition of knowledge, and the use of this knowledge to generate new knowledge. Learning and

memory mechanisms are integral to cognitive function, and thus a brief discussion of a structure intricately involved in the process, the hippocampus, is warranted.

Anatomy and connectivity of the hippocampal formation

Physically, the hippocampus resides both downstream and upstream of cortical association regions of the CNS, making it a prime structure for the integration of multi-modal sensory information (reviewed in Sweatt, 2010). Sensory information from the cortex funnels through the perirhinal and entorhinal cortices into the DG of the hippocampus, and glutamatergic dentate neurons then project through the Cornu Ammonis (CA) regions of the hippocampus, CA3 and CA1. Information leaves the hippocampus through CA1 neurons projecting to the ipsilateral and contralateral entorhinal cortices. The hippocampal formation is also reciprocally connected with the prefrontal cortex, a brain region implicated in planning complex cognitive behavior and decision-making.

Within the hippocampus, changes in the strength of connections between neurons is widely assumed to be the cellular mechanism by which memories are encoded (reviewed in Martin et al., 2000). Long-term potentiation (LTP) or long-term depression (LTD) are two specific processes thought to underlie selective strengthening or weakening of pertinent synapses (Bliss and Collingridge, 1993; Kessels and Malinow, 2009). They occur when repetitive, coincident depolarization of a set of synapses causes activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, resulting in subsequent changes to the trafficking and function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. A large magnitude, transient

activation of NMDA receptors leads to LTP via AMPA receptor insertion, whereas weak, persistent activation of NMDA receptors induces removal of AMPA receptors resulting in LTD. The result is a long-lasting modification of synaptic strength between two or more synapses involved in the circuit, a property thought to be necessary for learning and memory (Kessels and Malinow, 2009).

Hippocampal-dependent cognitive functions

Although there is no unifying model of precisely how the hippocampus encodes a memory, it has been firmly established that the hippocampal formation is critical in facilitating long-term memory consolidation, spatial cognition, temporal information processing, and constructing multi-modal relationships between objects in the environment (reviewed in Sweatt, 2010). The types of studies that lend support to the hypothesis that the hippocampus regulates memory consolidation include lesioning studies, pharmacological infusions, *in vivo* recordings during behavioral testing, observation of molecular changes after learning, and a multitude of genetic engineering and optogenetic studies (Liu et al., 2012; reviewed in Martin and Morris, 2002). The connectivity of the hippocampus puts it in prime position to carry out functions which can be summarized as two main tasks: the processing of a wide variety of information from the environment, and the download of this information for storage as long-term memory.

Immune modulation of learning, memory, and plasticity

Memories, like the neural processes that encode them, are highly malleable. The number of players involved in the regulation of learning, memory, and hippocampal plasticity is immense; but as with hippocampal neurogenesis, these processes are susceptible to influence by immune molecules and cells despite their confinement to the immune-privileged CNS. Under quiescent, physiological conditions, immune components including T cells, inflammatory cytokines (primarily IL-1, IL6, and TNF-α), and prostaglandins can help facilitate learning and memory (reviewed in Yirmiya and Goshen, 2011). These immune-related molecules may exert influence on cognitive function by directly affecting the underlying processes of hippocampal LTP and LTD: blocking IL-1β receptors after the induction of LTP impairs its maintenance (Schneider et al., 1998), blocking TNF-α signaling reduces synaptic strength by decreasing AMPA receptor expression (Beattie et al., 2002), and inhibition of prostaglandin production by ibuprofen resulted in impaired hippocampal LTP (Shaw et al., 2003). However, in conditions of strong immune stimulation (e.g. injury, stroke, autoimmune disorders, pathogens, or aging), the normally pro-cognitive roles these immune molecules and cells play can quickly become detrimental. Indeed, there is ample evidence of the involvement of inflammatory mediators in acute CNS injury (such as brain trauma and epilepsy) and well as chronic neurological conditions (such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, and aging itself) (Lucas et al., 2006; McGeer and McGeer, 2004), many of which present with cognitive decline.

The hippocampus is a remarkable brain structure in its capacity for immense plasticity throughout life, both at the synaptic level as well as in its ability to generate

new neurons. This degree of neuroplasticity might also confer it a marked vulnerability to deleterious conditions such as disease and aging (Bartsch and Wulff, 2015). Given the intricate involvement of inflammatory processes and immune components under these circumstances, understanding how specific molecules shape and misshape our brains in development and disease is of utmost importance. With further knowledge of its modulators, the unique level of neuroplasticity and regenerative potential of the hippocampus provides hope for future therapeutic interventions.

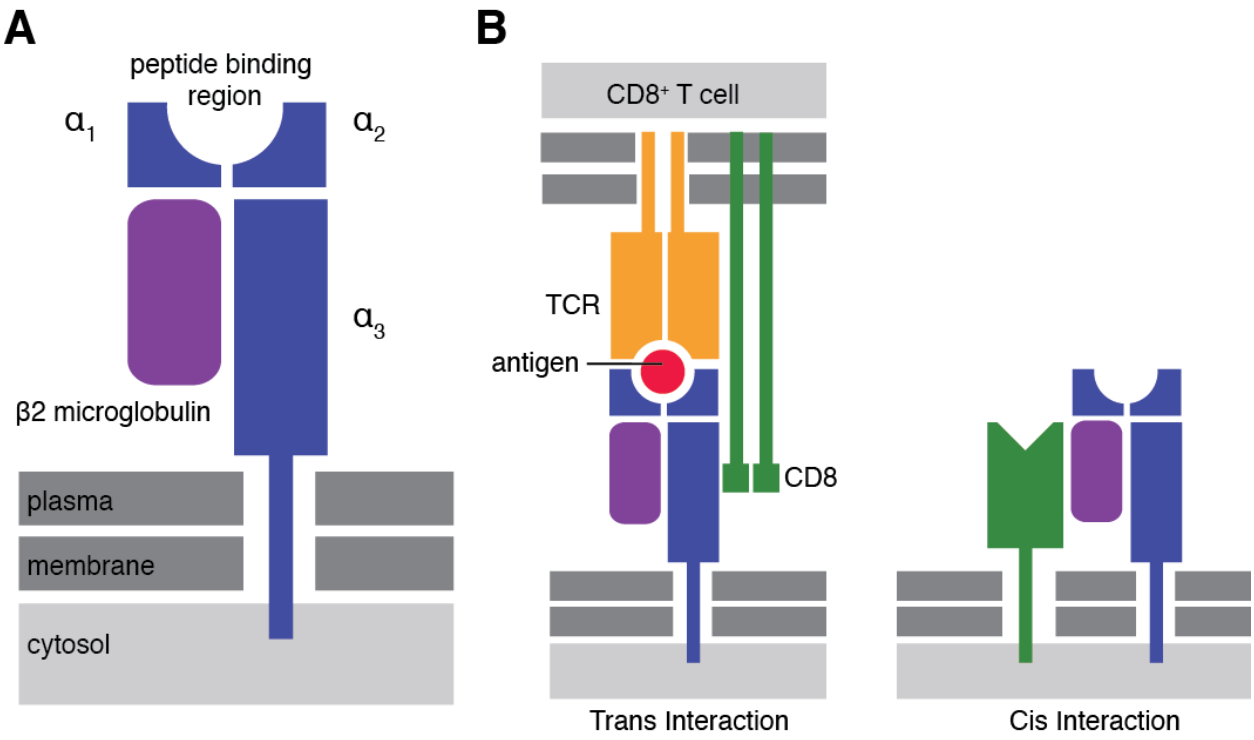

Figure 1. Structure and interactions of MHC I molecules. MHC I molecules are trimeric in structure, consisting of a heavy chain, light chain, and the peptide fragment being displayed. The heavy chain consists of three extracellular domains (α1-3) and short cytoplasmic tail of ~30 amino acids; the α1 and α2 domains are highly polymorphic and form the peptide-binding groove, while the membrane-spanning α3 domain is relatively conserved and carries sequence similarity to the immunoglobulin superfamily. β2m is a subunit, which is non-covalently bound to the MHC I heavy chain (A). MHC I molecules can interact in *trans* with T cell receptors (TCR) on CD8+ T cells which sample the peptide, as well as CD8 co-receptors, which confer binding specificity to the α3 domain of the MHC I complex. MHC I molecules can also interact in *cis* with other putative binding partners and receptors (in green) on the same cell surface (B).

Chapter 2

Materials and Methods

Mouse Models

All animal handling and use was in accordance with institutional guidelines approved by the University of California San Francisco IACUC. All mice were group-housed under specific pathogen-free conditions under a 12 h light-dark cycle with *ad libitum* access to food and water. Mouse strains used are described in Table 1. All studies in adult mice were conducted using male animals, and neural stem cells isolation was from male and female postnatal day 1 (P1) pups. The numbers of mice used to result in statistically significant differences was calculated using standard power calculations with $\alpha = 0.05$ and a power of 0.8. We used an online tool (http://www.stat.uiowa.edu/~rlenth/Power/index.html) to calculate power and samples size based on experience with the respective tests, variability of the assays and inter-individual differences within groups.

Table 1. Mouse strain and relevant information

Strain	Vendor	Description	References
C57BL/6	Taconic	Inbred wildtype from Taconic	
KD$^{-/-}$ (H2-K^{b} H2-D^{b} double knockout)	Taconic	Double knockout mouse: K$^{-/-}$ and D$^{-/-}$ mice were intercrossed	Perarnau et al., 1999
K$^{-/-}$ (H2-K^{b} knockout)	Taconic	Knockout mouse with 2nd and 3rd exons of the H2-K^{b} gene replaced with HPRT minigene	Perarnau et al., 1999
D$^{-/-}$ (H2-D^{b} knockout)	Taconic	Knockout mouse with loss of first three exons of the H2-D^{b} gene	Pascolo et al., 1997

Generation of H2-D^b conditional knockout mice for cell-type specific studies

The strategy to generate a conditional knockout model in the mouse H2-D1 locus involved using a CRISPR/Cas-9 system to insert LoxM2 sequences in the 5'UTR or 3'UTR regions, referred to as 5' insertion site or 3' insertion site, respectively. To achieve this model, three major steps were carried out. First, a mixture containing *in vitro* transcribed active guide RNA molecules (sgRNA) of 5'gRNA and its single-stranded oligo deoxylnucleotide (ssODN) donor and Cas-9 protein was injected into C57BL/6 embryos. The 5' LoxM2$^+$ mice born from this injection were used for a second injection to target the 3' site for LoxM2 insertion. Mice generated from both injections were subjected to PCR and sequencing for potential founders. Third, the potential founder was bred with WT mice to test germline transmission and to produce F1 mice.

C57BL/6 embryos were then injected through cytoplasmic route with a CRISPR targeting cocktail containing 5'g26 with corresponding ssODN donor. Embryos well developed *in vitro* and were transferred into CD1 female mice. Twenty-six mice were born and 19 showed 5' LoxM2 insertion. The female mice were mated with WT C57BL/6 males for targeting the 3' site. A total of 77 oocytes were injected with 3'g3 and corresponding ssODN. Sixty-eight were transferred into three surrogates. Four mice were born, two of which have both LoxM2 insertions, and are therefore potential founders. Both founders were mated with WT mice. The male founder produced two litters of seven F1 mice, with four being positive for floxed H2-D1 sites. In future experiments beyond this thesis, these mice will be crossed with various Cre mouse lines to test the effects of H2-D^b knockout in specific cell types.

Immunohistochemistry

Tissue processing and immunohistochemistry was performed on free-floating sections following standard published techniques (Bachman, 2013). Briefly, mice were anesthetized with a ketamine (100 mg/kg)-xylazine (10mg/kg) cocktail (Patterson Veterinary, Henry Schein) and transcardially perfused with cold PBS. Brains were removed, fixed in phosphate-buffered 4% paraformaldehyde, pH 7.4, at 4°C for 48 h followed by cryoprotection in 30% sucrose, and coronally sectioned at 40 μm with a cryomicrotome (Leica SM2010 R). Sections were washed three times in tris-buffered saline with 0.1% Tween 20 (TBST) and incubated in 3% normal donkey serum (Thermo Fisher Scientific) for 1 h. After overnight incubation in primary antibody (see Table 2 for details) at 4°C, staining was revealed using fluorescence conjugated secondary Alexa antibodies (1:500). Antigen retrieval for BrdU labeling required incubation in 3M HCl at 37°C for 30 min before incubation with primary antibody; Nestin labeling required incubation in Citrate Buffer (Sigma-Aldrich) at 95°C (3 times 5 minutes) prior to incubation with primary antibody. To estimate the total number of immunopositive cells per DG, confocal stacks of coronal sections of the DG (4-6 sections per mouse, 40 μm thick, 240 μm apart) were acquired on a Zeiss LSM 800. Immunopositive cells in the granule and subgranular cell layers were then counted and multiplied by 12 to estimate the total number in the entire DG.

Table 2. Primary antibodies

Antigen	Host	Dilution	Source
BrdU	Rat [BU1/75(ICR1)]	1:1000	Thermo Fisher
CD68	Rat (FA-11)	1:250	Bio-Rad
Cleaved Caspase 3	Rabbit, polyclonal	1:300	Cell Signaling
Doublecortin	Goat, polyclonal	1:500	Santa Cruz
EGFR	Mouse (A-10)	1:500	Santa Cruz
Erk1/2	Rabbit (137F5)	1:1000	Cell Signaling
FGFR1	Rabbit, polyclonal	1:400	Abcam
FGFR3	Rabbit, polyclonal	1:500	Abcam
GAPDH	Mouse (6C5)	1:10000	Abcam
GFAP	Rabbit, polyclonal	1:1000	Dako
H2-K^{b}	Guinea pig, polyclonal	1:500	Covance (custom)
Iba1	Rabbit, polyclonal	1:1000	Wako
Map2	Mouse (HM-2)	1:1000	Sigma-Aldrich
NeuN	Mouse (A60)	1:1000	Millipore
Nestin	Mouse (rat-401)	1:200	Millipore
Phospho Akt (Tyr450)	Rabbit, polyclonal	1:1000	Millipore
Phospho Erk1/2 (Thr202/Tyr204)	Rabbit (D13.14.4E)	1:2000	Cell Signaling
Phospho S6 (Ser235/236)	Rabbit, polyclonal	1:1000	Cell Signaling
Turbo-GFP	Rabbit, polyclonal	1:10000	Thermo Fisher

Western Blot Analysis

Mouse hippocampi (isolated after transcardial perfusion with cold PBS) or primary neural stem cells (NSCs) were lysed in RIPA lysis buffer (500 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Na deoxycholate, 1% NP40, 0.1% SDS, and complete protease inhibitors; Roche). Protein lysates were mixed with 4x NuPage LDS loading buffer (Invitrogen) and loaded on a 4-12% SDS polyacrylamide gradient gel (Invitrogen) and subsequently transferred onto a nitrocellulose membrane. The blots were blocked in 5% milk in Tris-Buffered Saline with Tween (TBST) and incubated with primary antibody at 4°C for 16h. Horseradish peroxidase-conjugated secondary antibodies and an ECL kit

(GE Healthcare/Amersham Pharmacia Biotech) were used to detect protein signals. Exposures were taken using a ChemiDoc imaging system (Bio-Rad) and quantified using ImageJ software (Version 1.8.0_91). GAPDH bands were used for normalization.

RNA Isolation and qRT-PCR

mRNA of NSCs was isolated by lysis with TRIzol Reagent (Thermo Fisher Scientific), separation with chloroform (0.2 mL per mL TRIzol), and precipitated with isopropyl alcohol. mRNA from DG tissue was isolated with the RNeasy Mini Kit (Quiagen). High Capacity cDNA Reverse Transcription Kit (Life Technologies) was used for reverse transcription of mRNA into cDNA and qRT-PCR was carried out using Power SYBR Green PCR Master Mix (Life Technologies) in a CFX384 Real Time System (Bio-Rad). Primers were: H2-K^b forward 5'-CGGCGCTGATCACCAAACA-3', H2-K^b reverse 5'-AGCGTCGCGTTCCCGTT-3'; H2-D^b forward 5' -GTAAAGCGTGAAGACAGCTGC-3', H2-D^b reverse 5'-CTGAACCCAAGCTCACAGG-3'; GAPDH forward 5'-GCATCCTGCACCACCAACTG-3', GAPDH reverse 5'-ACGCCACAGCTTTCCAGAGG-3'.

BrdU Administration and Quantification

For short-term proliferation studies, mice were intraperitoneally injected with BrdU (50mg/kg body weight, Sigma-Aldrich) for 6 days (once per day) before sacrifice. For study of newborn neuron survival, mice were injected with BrdU (50mg/kg) for six days (once per day) and animals were sacrificed 28 days after administration. To estimate the total number of BrdU-positive cells in the DG, we performed fluorescence staining for BrdU on 4-6 hemibrain sections per mouse (40 μm thick, 240 μm apart). The number of BrdU-positive cells in the granule cell and subgranular cell layer of the

DG were counted and multiplied by 12 to estimate the total number of BrdU-positive cells in the entire DG. To quantify neuronal fate and maturation of dividing cells after 28 day survival, BrdU-positive cells across 4-6 sections per mouse were analyzed by confocal microscopy for co-expression with NeuN.

Stereotaxic injections

Animals were placed in a stereotaxic frame and anesthetized with 2% isoflurane (Patterson Veterinary) (2 L per min oxygen flow rate) delivered through an anesthesia nose cone. Fur around the incision area was trimmed and ophthalmic eye ointment was applied to the cornea to prevent desiccation during surgery. Viral suspensions were injected bilaterally into the dorsal hippocampi using the following coordinates: (from bregma) anterior = −2 mm, lateral = 1.5 mm and (from skull surface) height = −2.1 mm. A 2 μL volume was injected stereotaxically over 10 min (injection speed: 0.20 μL per min) using a 5-μL 26s-gauge Hamilton syringe. To limit reflux along the injection track, the needle was maintained *in situ* for 8 min, slowly pulled out half-way and kept in position for an additional 2 min. The skin was closed using silk suture. Each mouse was injected subcutaneously with enrofloxacin (Bayer) antibiotic and buprenorphine (Butler Schein) as directed for pain, single-housed, and monitored during recovery.

Isolation of Primary Neural Stem and Progenitor Cells from the Postnatal Mouse Hippocampus

Primary neural stem and progenitor cell (NSPC) isolation and culture were preformed following previously published techniques (Babu et al., 2011). Hippocampi

were dissected from postnatal day 1 (P1) C57/BL6, $KD^{-/-}$, $K^{-/-}$, or $D^{-/-}$ mice and pooled by genotype for NSPC isolation without distinguishing genders. After removing superficial blood vessels, hippocampi were mechanically dissociated by fine mincing and enzymatically digested for 30 minutes at 37ºC in DMEM media containing 2.5U/ml Papain (Worthington Biochemicals), 1U/ml Dispase II (Boeringher Mannheim), and 250U/ml DNase I (Worthington Biochemicals). NSPCs were purified using a 65% Percoll gradient and cultured (Neural Basal A medium supplemented with 2% B27 without Vitamin A, 1% Glutamax, 1% Penicillin Streptomycin, 10ng/ml EGF, 10ng/ml bFGF) as a monolayer on poly-D-lysine and laminin-coated plates at a density of 10^5 cells/cm^2.

NSPC Proliferation Assay

WT or knockout NSPCs were seeded in 500 µL growth medium (Neural Basal A medium supplemented with 2% B27 without Vitamin A, 1% Glutamax, 1% Penicillin Streptomycin, 10ng/ml EGF, 10ng/ml bFGF) at a density of 10,000 cells/well on PDL/laminin-coated glass coverslips in a 24-well tissue culture plate. 24 h later, cells were treated with 20 µM BrdU or EdU for 6 h prior to fixing with 4% PFA. Acute knockdown of H2-K^b, H2-D^b, or both, was induced by treating plated wildtype NSPCs with shRNA -encoding lentiviruses (6.5×10^6 to 7.5×10^6 viral particles per mL media) for three days prior to BrdU or EdU treatment as described. Cells were washed 3 times with PBS after fixation, blocked in 3% donkey serum, and incubated in primary antibody at 4ºC for 16 h. After 3 washes with PBS, BrdU staining was revealed with fluorescence-conjugated secondary Alexa antibodies. EdU incorporation was revealed using a Click-

iT EdU Alexa Fluor Imaging Kit (Thermo Fisher Scientific) and nuclei were counterstained with Hoechst 33342 (1:10000; Thermo Fisher Scientific). BrdU- or EdU-positive cells were counted per field of view at three randomly determined locations per coverslip. *In vitro* experiments were conducted in triplicates for each condition, and repeated to ensure reproducibility. For inhibitor experiments, NSPCs were pre-treated for one hour with inhibitors (PD 161570, 0.1uM; AG 99, 10uM) prior to the 6 h EdU pulse and immunocytochemistry as described.

NSPC Differentiation Assay

WT or knockout NSPCs were seeded in 500 µL growth medium at a density of 10,000 cells/well on PDL/laminin-coated glass coverslips in a 24-well tissue culture plate. 24 h later, media was removed and replaced with growth factor-free basal media (Neural Basal A medium supplemented with 2% B27 without Vitamin A, 1% Glutamax, 1% Penicillin Streptomycin) to induce differentiation. Half the media was replaced every two days with fresh basal media, and cells were fixed after six days of differentiation in 4% PFA. Immunocytochemistry was conducted as described above and Map2- or GFAP-positive cells were counted per field of view at three randomly determined locations per coverslip. *In vitro* experiments were conducted in triplicates for each condition, and repeated to ensure reproducibility.

Cell Cycle Analysis by Cumulative EdU Labeling

WT and $K^{-/-}$ NSPCs cultured *in vitro* in growth media were treated with 20 µM EdU at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 h prior to fixation. Cells that

incorporated EdU were labeled using the Click-iT Plus EdU Alexa Fluor Flow Cytometry Kit, and percentage of EdU^+ cells was determined by flow cytometry. The labeling index (LI; percent of total cells positive for EdU) was plotted over time, increasing linearly until a plateau (which corresponds with the growth fraction [GF], or maximum percentage of cells actively proliferating) was reached. The data can be fitted with two regression lines (as described in Nowakowski et al. 1989): one line which describes the initial linear increase, and another which describes the horizontal line corresponding to the GF. The time (in the *x* axis) where the two regression lines intersect is equal to Tc - Ts, where Tc is the cell cycle length and Ts is the S phase length. The intersect of the first regression line and the *y* axis is defined as the initial labeling index (Li0) and is equal to GF x Ts/Tc. Using these equations and the regression lines, Tc and Ts were calculated for WT and $K^{-/-}$ NSPCs. Each time point for both genotypes was conducted in duplicates.

Growth Factor Receptor Signaling Pathway Analysis

WT and $K^{-/-}$ NSPCs were plated at 500 000 cells/ well in a 6-well dish in proliferation media for 24 hours. Cells were then subjected to absent (0 ng/mL), low (5 ng/mL), or regular (20 ng/mL) EGF and FGF concentration conditions for one hour before protein was harvested as described above. Western blot analysis for growth factor receptor signaling pathway components (Erk1/2, Akt, ribosomal protein S6; see Table 1) was conducted for each condition.

Viral Plasmids and Viruses

We generated lentiviruses (LVs) encoding shRNAs targeting endogenous H2-K^b (sh-K), H2-D^b (sh-D), or both (sh-KD) using a lentiviral shRNA expression system (pGreenPuro shRNA, System Biosciences) according to the manufacturer's instructions. The targeted sequences were cloned into the pGreenPuro vector (H2-K^b, 5′-GAATGTAACCTTGATTGTTAT-3′; H2-D^b, 5'-ACCACACTCGATGCGGTATTTC-3'; H2-K^b H2-D^b, 5'-ACCCTCAGTTCTCTTTAGTCAA-3'). Plasmids were sequenced and shRNA-mediated knockdown was tested via transfection of a neuro2a cell line and subsequent Western blot analysis for H2-K^b and qPCR analysis for H2-K^b and H2-D^b.

LV vectors were generated at the UCSF Viracore, with viral titers between 1.2 × 10^9 to 1.5 × 10^9 viral particles per mL. LVs were stereotaxically injected into one hippocampus, and a control virus with a scrambled shRNA sequence (sh-control, 5'-GGACGAACCTGCTGAGATAT-3'), based on the same viral plasmid, was injected as an internal control at the same location into the contralateral hemisphere.

Co-Immunoprecipitation

An N2a neuronal cell line (American Type Culture Collection, CCL-131), cultured in DMEM + 10% FBS, was used for co-immunprecipitation experiments. Cells were plated at a confluency of 85–90% and Lipofectamine 2000 (Invitrogen) was used as the transfection reagent. N2a cells were transfected with 3x FLAG-tagged H2-K^b and EGFR, FGFR1, or FGFR3. Protein overexpression was assessed with by Western blot analysis. Cells were lysed (20mM Tris, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X, 2.5mM sodium pyrophosphate decahydrate, 0.5% NP40, 1mM β-glycerophosphate, 10mM NaF, 1ug/mL leupeptin) 48 h after transfection, and protein lysates (1mg) were incubated with Anti-FLAG M2 magnetic beads (Sigma-Aldrich) for 4

hours at 4°C. Beads were collected on a magnetic column, washed 3 times in cold TBS, and eluted directly in Laemmli buffer (Bio-Rad) at 95°C for 3 minutes.

Bioinformatic analysis of single cell RNA-seq data from Shin et al., 2015

Pseudo time assignment and read counts (CPM) for all cells were obtained from Shin et al. 2015. Read counts were log2 transformed (log2(CPM+1)) and scaled between 0 and 1 by dividing the normalized read count for each gene in each cell by the maximum expression value across all cells for the respective gene. Cells were then ordered according to their pseudo time estimate from early to late. Subsequently, a local polynomial regression line was fit alone the trajectory and the 95% confidence interval was calculated using the ggplot2 R package. Time points T1 and T2 were determined by the time of increasing Tbr2 expression (last stationary point of the T*br2* regression line calculated in R). G1 and G2 cell cycle score were calculated as the summed expression of G1 and G2 markers, as published in Tirosh et al. 2016, divided by the median score across cells.

RNA-Seq Library Construction

After RNA isolation, RNA-Seq libraries were constructed using the Smart-Seq2 protocol from Trombetta et al. 2014, with modifications. Briefly, 1 ng high quality RNA was reverse transcribed using SuperScript II (Life Technologies, 18064-014) with a poly-dT anchored oligonucleotide primer, and a template switching oligonucleotide primer that generated homotypic PCR primer binding sites. The cDNA underwent 10

rounds of PCR amplification using KAPA HiFi Hotstart (Kapa Biosystems, KK2601), followed by Ampure bead (Agencourt) cleanup. The quality of the amplified cDNA was tested using qPCR for GAPDH and nucleic acid quantitation. 1ng of high quality amplified cDNA was fragmented with the Tn5 transposase from the Illumina Nextera kit (FC-131-1096) to a median size of ~500bp. The fragmented library was amplified with indexed Nextera adapters (FC-131-1002) using 12 rounds of PCR. Final libraries were purified with Ampure beads, and quantified using a qPCR Library Quantification Kit (Kapa Biosystems, KK4824). Libraries were pooled for sequencing on an Illumina HiSeq 2500.

Bioinformatic Analysis of RNA-seq Data

Quality trimming and adapter removal of raw FASTQ files was carried out with cutadapt (Martin, 2011). Only reads longer than 20 base pairs were kept for downstream analysis. Subsequently, trimmed reads were mapped to the mouse genome (Grchm38) using HISAT2 (Kim et al., 2015). Transcripts given by the GENCODEv25 reference (http://www.gencodegenes.org/releases/current.html) were quantified with featureCounts (Liao et al., 2014), considering only uniquely aligned reads. Differential expression tests were calculated using DEseq2 (Love et al., 2014) and transcripts with an adjusted p-val < 0.05 were considered differentially expressed. ConsensusPathDB (Herwig et al., 2016) was used for calculation of pathway enrichments.

Home Cage Nesting Behavior

This test is a modified version of a previously published protocol (Deacon, 2012). Mice were individually housed and each cage was provided with a fresh nestlet, a 5cm square piece of pressed cotton. The nests were scored on a four-point scale (0-4) based on the following criteria: 0- the nestlet is largely untouched (>90% intact); 1- the nestlet is partially torn up (50-90% intact); 2- the nestlet is mostly shredded but there is no identifiable nest site; 3- a clearly identifiable, cratered nest, with walls higher than mouse body height on 50% of its circumference. Refer to Chapter 4, Figure 1 for representative photos. Mice were scored for nesting after 2, 6, and 12 hours after single-housing.

Open Field Test

Mice were placed in the center of an open 40 cm x 40 cm square chamber (Kinder Scientific) with no cues or stimuli and allowed to move freely for 10 min. Infrared photobeam breaks were recorded and movement metrics analyzed by the MotorMonitor software (Kinder Scientific).

Elevated Zero Maze

Mice were placed on elevated zero apparatus (53.98 cm diameter; 88.27 cm height; 5.08 cm wide racetrack; Kinder Scientific), which has two open sections and two walled sections forming a circle. Animals were placed at the junction of an open and closed arm and allowed to move freely for 10 min. Infrared photobeam breaks were recorded and movement metrics analyzed by the MotorMonitor software (Kinder Scientific).

Radial Arm Water Maze

Our paradigm followed a previously described protocol (Alamed et al., 2006). The goal arm location containing a platform remained constant throughout the training and testing phases, and the start arm was changed during each trial. On day 1 during the training phase, mice were trained for 15 trials, with trials alternating between a visible and a hidden platform. On day 2 during the testing phase, mice were tested for 15 trials with a hidden platform. Entry into an incorrect arm was scored as an error, and errors were averaged over the training blocks (three consecutive trials).

Contextual and Cued Fear Conditioning

Our paradigm followed previously published techniques (Raber et al., 2011). Mice learned to associate the environmental context (fear-conditioning chamber) with an aversive stimulus (mild foot shock; unconditioned stimulus, US), which enabled testing for hippocampal-dependent contextual fear conditioning. The mild foot shock was paired with a light and tone cue (conditioned stimulus, CS) in order to also assess amygdala-dependent cued fear conditioning. Conditioned fear was displayed as freezing behavior. Specific training parameters were as follows: tone duration, 30 s; level, 70 dB, 2 kHz; shock duration, 2 s; intensity, 0.6 mA. On day 1, each mouse was placed in a fear-conditioning chamber and allowed to explore for 2 min before delivery of a 30-s tone (70 dB) ending with a 2-s foot shock (0.6 mA). Two minutes later, a second CS-US pair was delivered. On day 2, each mouse was first placed in the fear-conditioning chamber containing the same exact context but with no administration of a

CS or foot shock. Freezing was analyzed for 2 min. One hour later, the mice were placed in a new context containing a different odor, cleaning solution, floor texture, chamber walls and shape. Animals were allowed to explore for 2 min before being re-exposed to the CS. Freezing was analyzed for 30 sec after termination of the CS. Freezing was measured using a FreezeScan video tracking system and software (Cleversys, Inc).

Data and statistical analysis

Graphed data are expressed as mean ± SEM. Statistical analysis was performed with Prism 5.0 software (GraphPad Software). Means between two groups were compared with two-tailed, paired or unpaired Student's t-test. Comparisons of means from multiple groups with each other or against one control group were analyzed with 2-way ANOVA and Bonferroni or Fisher's LSD post hoc tests. All histology and behavior experiments conducted were done in a randomized and blinded fashion. For each experiment, the overall size of the experimental groups corresponded to distinct animals. Unique samples were not measured repeatedly within the same characterization of a given cohort.

Chapter 3

Role of Classical MHC I Molecules in Regulating Neural Stem and Progenitor Cell Function

Proteins of the major histocompatibility complex class I (MHC I), previously known mainly for antigen presentation in the immune system, have recently been shown to be necessary for both developmental neural refinement and adult synaptic plasticity. However, their roles in non-neuronal cell populations in the brain have yet to be investigated. This study identifies classical MHC I molecule H2-K^b as a negative regulator of proliferation in neural stem and progenitor cells (NSPCs), cell types conferring inherent plasticity to the rodent brain. Adult animals with a genetic deletion ($K^{-/-}$) or acute knockdown of H2-K^b in the dentate gyrus exhibit enhanced cell division and an increased number of adult-born neurons in the hippocampus. This proliferative phenotype is also observed in cultured $K^{-/-}$ progenitor cells devoid of influences from the neurogenic niche or systemic environment. Subsequent transcriptomic analysis of $K^{-/-}$ and wild type progenitors further revealed that endogenous H2-K^b molecules inhibit cell proliferation by dampening growth factor receptor signaling pathways, through a physical interaction with fibroblast growth factor receptor 1. These findings identify H2-K^b as a novel regulator of cell proliferation through the modulation of growth factor signaling.

Introduction

Major histocompatibility class I (MHC I) proteins are most well known for their role in antigen presentation and immunological surveillance in the adaptive immune system (Neefjes et al., 2011). Although MHC I molecules are expressed by all nucleated cells, long-held dogma in the field of neuroimmunology described the central nervous system (CNS) as being immune-privileged, with the ability to evade normal inflammatory responses imparted by a near absence of classical immune molecules under physiological conditions (Joly et al., 1991; Murphy, 1923). In the last two decades however, this belief has been challenged following the discovery that immune components, such as complement, cytokines, and MHC I molecules, are not only present in the CNS but play many critical roles in brain development and function (Deverman and Patterson, 2009; Garay and McAllister, 2010; Shatz, 2009; Stevens et al., 2007). More specifically, the classical MHC I molecules, H2-K^b and H2-D^b, have been demonstrated in the CNS to negatively regulate visual system plasticity, neurite outgrowth, synapse density, and synaptic strength (Huh, 2000; Datwani et al., 2009; Wu et al., 2011; Dixon-Salazar et al., 2014) Notwithstanding, their roles in regulating cellular functions beyond mature neuronal cells types have yet to be explored.

Neural stem cells (NSCs), capable of producing functional neurons during embryonic and perinatal development as well as partially throughout life, represent a unique cell type critical in the formation of the CNS and potentially amenable to regenerative biology. NSCs of the dentate gyrus (DG) reside in a specialized niche that supports their maintenance, proliferation, and differentiation into excitatory granule cells that integrate into hippocampal circuitry and confer plasticity (Christian et al., 2014).

This fine-tuned process is responsive to modulation by cell-intrinsic mechanisms, including transcription factors such as Sox2, TLX, and FoxOs and epigenetic factors like Tet2, as well as changes to the neurogenic niche and systemic environment through manipulations such as exercise and exposure to young blood (Favaro et al., 2009; Gontier et al., 2018; Paik et al., 2009; van Praag et al., 1999; Qu et al., 2010; Villeda et al., 2014). Importantly, neurogenesis can be regulated by immune system components such as cytokines, toll-like receptors, and T cells (Borsini et al., 2015; Rolls et al., 2007; Ziv et al., 2006). One such factor, β2-microglobulin, which is the non-covalently bound light chain of MHC I, has been implicated as a systemic factor inhibiting neurogenesis (Smith et al., 2015). However, the role of MHC I molecules themselves in regulating neural stem and progenitor cell (NSPC) function have never been studied. Given the roles of MHC I molecules in suppressing neural processes and the susceptibility of NSPCs to immune influence, I sought to investigate the roles of H2-K^b and H2-D^b in NSPC function.

Using a combination of *in vivo* genetic mouse models and *in vitro* NSPC functional assays, I specifically identify H2-K^b as a unique negative regulator of NSPC proliferation and adult hippocampal neurogenesis. Additionally, I used transcriptomic and biochemical analysis to provide evidence that loss of H2-K^b in NSPCs enhances proliferation through fibroblast growth factor receptor 1 signaling pathways. Together, these data demonstrate a novel, non-canonical role for a specific MHC I molecule, H2-K^b, in NSPCs. As psychiatric disorders, neurodegenerative diseases, stroke, and aging can impact the process of neurogenesis (Kaneko and Sawamoto, 2009; Kuhn et al.,

1996), a better understanding of specific molecules regulating NSPC function may allow us to harness the innate regenerative capacity of the CNS to combat these brain insults.

Results

Absence of H2-K^b and H2-D^b increases neurogenesis in the adult hippocampus

I first sought to investigate the role of H2-K^b and H2-D^b in negatively regulating adult neurogenesis in the hippocampal dentate gyrus (DG) *in vivo*. Taking advantage that *H2-K1* (encoding H2-K^b) and *H2-D1* (encoding H2-D^b) genes are both on chromosome 17 of the mouse genome, I made use of a genetic knockout mouse model in which excision of both genes co-segregate on the same chromosome in a single mutant mouse (KD$^{-/-}$). Neurogenesis was assessed in the hippocampus of adult (3 months) KD$^{-/-}$ mice and age-matched wild type (WT) controls by immunohistochemical analysis. Cell proliferation was assessed by short-term 5-bromo-2'-deoxyuridine (BrdU)-labeling of dividing cells. Absence of H2-K^b and H2-D^b resulted in significantly more BrdU-positive proliferating cells (**Figure 1B**) and Doublecortin (Dcx)-positive neuroblasts in the DG of KD$^{-/-}$ mice compared to WT controls (**Figure 1C**). Interestingly, the number of Nestin-positive radial glial-like stem cells was not different between KD$^{-/-}$ and WT animals (**Figure 1A**), pointing to a regulatory role for H2-K^b and H2-D^b during proliferative phases of adult neurogenesis. Additionally, I assessed neuronal differentiation and survival in a second cohort of mice using a long-term BrdU incorporation paradigm, in which post-migratory differentiated neurons co-express both BrdU and the mature neuronal marker NeuN. The number of BrdU/NeuN double-positive differentiated mature neurons was increased in KD$^{-/-}$ compared WT mice

(**Figure 1D**). Given H2-K^b and H2-D^b molecules are critical for immune cell development and function (Vugmeyster et al., 1998), I assessed microglia activation and detected no differences in the co-expression of ionized calcium-binding adapter molecule 1 (Iba1) and cluster of differentiation 68 (CD68) between dentate gyri of $KD^{-/-}$ and WT mice (**Figure S1A**). Altogether, these data indicate that classical MHC I molecules, H2-K^b and H2-D^b, negatively regulate cell proliferation and neurogenesis in the DG of the adult hippocampus.

Regulation of NSPC proliferation by classical MHC I molecules

Having observed the involvement of H2-K^b and H2-D^b during the proliferative phases of neurogenesis *in vivo*, I next investigated the role of classical MHC I molecules in NSPC proliferation *in vitro*. I subdissected hippocampi from $KD^{-/-}$ and WT mice and cultured primary progenitor cells following an established protocol (Babu et al., 2011). I characterized expression of H2-K^b and H2-D^b mRNA by qPCR and H2-K^b protein by Western blot analysis in primary WT NSPCs, and confirmed absence of H2-K^b and H2-D^b expression in primary $KD^{-/-}$ NSPCs (**Figure 1E**; **Figure S1B**). Primary $KD^{-/-}$ and WT NSPCs were then cultured with growth factors EGF and FGF (self-renewal conditions) and treated with the BrdU analog, 5-ethynyl-2´-deoxyuridine (EdU). I observed a significant increase in the percentage of EdU-positive proliferating cells in $KD^{-/-}$ compared to WT NSPCs (**Figure 1F)**. To corroborate my findings in knockout NSPCs, I used a viral-mediated RNA interference (RNAi) approach. I generated lentiviral constructs encoding either short hairpin RNA (shRNA) sequences concomitantly targeting H2-K^b and H2-D^b (sh-KD) or targeting luciferase as a control

(sh-control). Knockdown of H2-K^b and H2-D^b expression was confirmed by Western blot and qPCR analysis in WT NSPCs expressing shRNA targeting H2-K^b and H2-D^b compared to control shRNA (**Figure 1G**; **Figure S1C**). Consistent with knockout studies, acute abrogation of H2-K^b and H2-D^b resulted in an increase in the percentage of EdU-positive proliferating cells compared to control conditions (**Figure 1H**). These *in vitro* results indicate that H2-K^b and H2-D^b molecules inhibit NSPC proliferation.

H2-K^b uniquely identified as a negative regulator of neurogenesis in the adult hippocampus *in vivo* and of NSPC proliferation *in vitro*

Previous studies have ascribed H2-K^b and H2-D^b with predominantly analogous functions in the brain (Shatz, 2009). Given the diversity of this family of molecules, I wanted to explore potential differential roles each may play in NSPC function. To test this possibility, I first analyzed a publicly available dataset describing the transcriptomic dynamics of early phase adult hippocampal neurogenesis at the single-cell level (Shin et al., 2015). Single-cell gene expression profiles were generated for Nestin-positive cells and their immediate progeny, with each cell bioinformatically assigned along a continuous trajectory from quiescent NSC activation to initiation of neurogenesis. Specifically, I divided the neurogenic lineage into an early time point (T1) where NSCs express glial fibrillary acidic protein (GFAP), and a later time point (T2) where differentiating intermediate neural progenitors begin to express T-box brain protein 2 (Tbr2) (**Figure 2A; Figure S2**). Trendlines of *H2-K1*, *H2-D1*, and the proliferation marker *Ki67* expression were fitted along this neurogenic trajectory (**Figure 2A**). Interestingly, as *Ki67* expression increased *H2-K1* expression decreased, while *H2-D1*

expression remained static (**Figure 2A,B**). Correspondingly, the frequency of cells expressing *H2-K1* was significantly lower during T2 compared to T1, while no changes were observed for *H2-D1,* consistent with the housekeeping gene *Gapdh* (**Figure 2B**). These bioinformatics data suggest a potential preferential role for H2-K^b in regulating NSPC function during proliferative phase of adult neurogenesis.

To investigate whether the exclusivity of H2-K^b, and not H2-D^b, in regulating hippocampal neurogenesis can be verified *in vivo,* I utilized genetic mouse models in which *H2-K1* ($K^{-/-}$) or *H2-D1* ($D^{-/-}$) are individually excised. Neurogenesis was examined by immunohistochemistry in adult $K^{-/-}$, $D^{-/-}$, and WT mice. I observed a significantly higher number of Dcx-positive neuroblasts in the DG of $K^{-/-}$, but not $D^{-/-}$ mice, compared to WT controls (**Figure 2C,D**). $K^{-/-}$ mice also displayed an increase in the number of long-term BrdU-retaining, neuronal nuclei (NeuN)-double positive mature differentiated neurons (**Figure 2C,D**). Nestin-positive radial glial-like stem cell numbers were not different between groups (**Figure 2C,D**), suggesting the influence of H2-K^b molecules is exerted during proliferative stages of neurogenesis rather than the stem cell pool.

I then sought to delineate the individual functions of H2-K^b and H2-D^b in regulating NSPC proliferation *in vitro*. Primary hippocampal NSPCs were isolated from $K^{-/-}$, $D^{-/-}$, and WT mice, then cultured under self-renewal conditions and treated with EdU. No differences in cell proliferation were observed in $D^{-/-}$ compared to WT NSPCs (**Figure 2E).** However, I detected a significant increase in the percentage of EdU-positive proliferating cells in $K^{-/-}$ compared to WT NSPCs (**Figure 2E**), consistent with the bioinformatics analysis and *in vivo* genetic knockout model described above. Interestingly, under differentiation conditions *in vitro*, no differences in the percentage of

neurons and astrocytes were detected between genotypes (**Figure S3A,B**), suggesting a role for H2-K^b in specifically regulating the proliferative process in NSPCs *in vitro*.

To complement the genetic mutant model, and circumvent potential developmental effects of a constitutive knockout, I next used an *in vivo* viral-mediated RNAi approach. I generated lentiviral constructs encoding either shRNA sequences targeting H2-K^b mRNA (sh-K) or targeting luciferase as a control (sh-control) (**Figure 3A**). Adult WT mice were stereotaxically injected into the right DG with sh-K and into the left contralateral DG with sh-control (**Figure 3A**). Cell proliferation was assessed by short-term EdU-labeling and neuronal differentiation was assessed by long-term Brdu-labeling. Local and temporally controlled knockdown of H2-K^b in the adult DG resulted in an increase in the number of EdU-positive proliferating cells, Dcx-positive neuroblasts, and BrdU/NeuN double-positive mature differentiated neurons (**Figure 3B,C**) compared to the contralateral control within each animal. These results were supported by *in vitro* studies in cultured WT NSPCs, where I observed that acute abrogation of H2-K^b expression by sh-K resulted in a significant increase in EdU-positive proliferating cells compared to control conditions (**Figure 3D**). Together, these data identify H2-K^b individually- out of as many as 50 MHC I subtypes - as a negative regulator of cell proliferation and neurogenesis in the hippocampus.

Absence of H2-K^b may increase proliferation by altering cell cycle dynamics of NSPCs

To gain insight into how H2-K^b influences NSPC proliferation, I again made use of the publicly available dataset described above (**Figure 2A,B**). I scored each single

cell analyzed for expression of signatures indicative of the G1/S or G2/M phases of the cell cycle (as described in Tirosh et al., 2016), and subsequently identified each cell as either positive or negative for *H2-K1* expression (threshold: 5 CPM). I found that the percentage of cycling cells was significantly lower in *H2-K1*-positive cells (5%) versus *H2-K1*-negative cells (28.8%; p=0.0374, Fisher's exact test), indicating NSCs and their immediate progeny are more likely to be non-cycling when enriched for *H2-K1* (**Figure 4A,B**). These bioinformatics data provides evidence that in an intact hippocampus, H2-K^b regulates the proliferative potential of NSPCs possibly in a cell-intrinsic manner.

Increased hippocampal NSPC proliferation is a phenomenon known to occur under certain stimuli; in the case of seizures, a shortened cell cycle length is the molecular mechanism underlying the enhancements in cell proliferation (Varodayan et al., 2009). I examined if similar changes occurred in cell-cycle dynamics of NSPCs lacking H2-K^b. Primary hippocampal NSPCs isolated from $K^{-/-}$ and WT mice were cultured under self-renewal conditions, and were subsequently treated with EdU at 2 hour intervals over a 24-hour period. Absence of H2-K^b resulted in an increase in the ratio of proliferating cells to the total number of cells in the population, corresponding to the growth fraction (GF; **Figure 4D**). Furthermore, I observed a decrease in both the length of the S-phase (T_s) and length of the cell cycle (T_c; **Figure 4D**), calculated as described in Ponti et al., 2013. These *in vitro* data indicate that NSPCs lacking H2-K^b may show increased proliferation due to an acceleration of cell cycle progression.

Transcriptome analysis identifies growth factor receptor-mediated signaling downstream of H2-K^b in NSPCs

To gain mechanistic insight into how MHC I molecules regulate NSPC proliferation, I profiled gene expression in primary NSPCs derived from $K^{-/-}$, $D^{-/-}$, $KD^{-/-}$, and WT mice using RNA-sequencing. Abrogation of *H2-K1* expression was corroborated in $K^{-/-}$ and $KD^{-/-}$ NSPCs (**Figure S4A,B**), in line with excision of Exon 2 and Exon 3 previously reported in these genetic models (Pérarnau et al., 1999). Hierarchical clustering revealed high similarity between biological replicates within individual genotypes (average Pearson Correlation Coefficient; PCC>0.99). Interestingly, replicates from $K^{-/-}$ NSPCs were segregated from all other genotypes (**Figure 5A**); further corroborated by differential expression analysis (DESeq2 adjusted $p<0.05$) between WT and individual knockout NSPCs (**Figure 5C**). As expected, we found a larger number of differentially expressed genes (844 genes) in $K^{-/-}$ NSPCs compared to $D^{-/-}$ (118 genes) or $KD^{-/-}$ (398 genes) NSPCs, with very few genes (13 genes) common between genotypes (**Figure 5B**). Taken together with the functional studies above, these transcriptional data further support H2-K^b as a distinct regulator of NSPC function.

Next, I focused on genes whose expression was significantly different between WT and $K^{-/-}$ NSPCs. Among the most differentially upregulated genes detected in $K^{-/-}$ NSPCs, I found several growth factor and extracellular matrix protein encoding genes (**Figure 5D**); many of which are associated with increased NSPC proliferation (Chen et al., 2013; Lee et al., 2009; Rabenstein et al., 2015). Accordingly, I found "cell proliferation" among the most significantly enriched gene ontology (GO) terms. To elucidate pathways that might underlie increased proliferation observed in $K^{-/-}$ NSPCs, I performed pathway enrichment analysis (**Figure 5E**). Genes upregulated in $K^{-/-}$ NSPCs comprised several growth factor receptor-associated pathways, among these epidermal

growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) signaling (**Figure 5E**). Apoptosis pathway-associated genes did not differ between groups, whereas p53 tumor suppressor pathway genes were downregulated in $K^{-/-}$ NSPCs (**Figure 5E**).

Western blot analysis confirmed that known receptor tyrosine kinase signaling pathways were more highly activated in $K^{-/-}$ compared to WT NSPCs. I assessed Akt and Erk pathways in WT and $K^{-/-}$ NSPCs grown under absent (0 ng/mL), low (5 ng/mL), or regular (20 ng/mL) growth factor conditions. Western blot analysis showed that enhanced phosphorylation of Erk (Thr202/Tyr204), but not Akt (Tyr450), occurred in $K^{-/-}$ NSPCs in low, non-saturating growth factor conditions (**Figure 6A**). Ribosomal protein S6, a downstream target of receptor tyrosine kinase signaling involved in cell proliferation, is also more highly phosphorylated (Ser235/236) in $K^{-/-}$ NSPCs than WT NSPCs. Thus far, the results suggest that H2-K^b molecules negatively regulate cell proliferation through inhibition of receptor tyrosine kinase signaling pathways.

H2-K^b forms macromolecular complex with specific growth factor receptors

Intriguingly, receptors (such as insulin receptors and EGFRs) and other proteins have been shown to associate in *cis* with MHC I molecules, forming macromolecular complexes on the same cell surface (Arosa et al., 2007; Dixon-Salazar et al., 2014; Fehlmann et al., 1985; Schreiber et al., 1984). Given that these interactions can affect receptor signaling and internalization, I wanted to determine whether H2-K^b molecules could physically associate with the receptors revealed by transcriptomic analysis. Coimmunoprecipitation experiments were performed in mouse Neuro-2a (N2a) cells

exogenously expressing a FLAG-tagged H2-K^b and receptors that are highly expressed in NSPCs: EGFR, FGFR1, and FGFR3 (**Figure 6B**). Transfection of N2a cells with H2-K^b and receptors, as well as antibody specificity, were confirmed by Western blot analysis of whole cell lysates (input lanes, **Figure 6C-E**). Anti-FLAG magnetic beads were used to immunoprecipitate H2-K^b along with its associated proteins. Immunoprecipitation of H2-K^b selectively coprecipitated FGFR1, but not FGFR3, EGFR, or the constitutively expressed protein GAPDH (IP lanes, **Figure 6C-E**). Thus, H2-K^b molecules are able to form macromolecular complexes with specific growth factor receptors known to be expressed on NSPCs.

Correspondingly, I next investigated the functional role of FGFR signaling pathways in mediating the enhancement in proliferation previously observed in $K^{-/-}$ NSPCs. Primary NSPCs were isolated from hippocampi of $K^{-/-}$ and WT mice and cultured under self-renewal conditions in the presence or absence of a FGFR pharmacological inhibitor, PD161570. $K^{-/-}$ and WT NSPCs were then exposed to EdU and assessed for proliferation. While no differences in cell proliferation were observed in WT NSPCs at concentrations used for FGFR inhibitor treatment (**Figure 6F**), I detected a decrease in the percentage of EdU-positive proliferating cells in $K^{-/-}$ NSPCs exposed to PD161570 (**Figure 6F**). These data indicate that H2-K^b molecules negatively regulate NSPC proliferation by inhibiting FGFR-mediated signaling, potentially through a physical interaction with these receptors.

Discussion

In this study, I identify classical MHC I molecule, H2-K^b, as a negative regulator of hippocampal neurogenesis. Using genetic mouse models as well as primary NSPC cultures, I show that deletion or knockdown of H2-K^b molecules causes increased proliferation and neurogenesis in the DG *in vivo* as well as in progenitor cells cultured *in vitro*. Subsequent transcriptome analysis of WT and $K^{-/-}$ NSPCs revealed that this enhanced proliferative potential is due to an enrichment of several growth factor receptor signaling pathways. I show also that H2-K^b is able to physically interact with a specific growth factor receptor, FGFR1, putatively modulating its signaling. My findings attribute H2-K^b a novel inhibitory function in NSPCs, describing for the first time a role for MHC I molecules in modulating cellular functions in a non-neuronal CNS cell type.

To date, the inhibitory effects of MHC I have been described in neural processes such as neurite outgrowth, synaptogenesis, hippocampal and cerebellar synaptic plasticity, and activity-dependent refinement of developing visual system neurons (Glynn et al., 2011; Goddard et al., 2007; Huh et al., 2000; McConnell et al., 2009b). We have also previously shown that exogenous addition of β2-microglobulin, the non-covalently associated subunit of the MHC I complex, can decrease the number of Dcx-expressing neuroblasts in the adult hippocampus and negatively affect cognitive function (Smith et al., 2015). However, when β2-microglobulin was administered to mice lacking transporter associated with antigen processing 1 (Tap1), in which MHC I molecules cannot be trafficked to the cell membrane, the negative effects of β2-microglobulin were mitigated. This suggested direct involvement of MHC I molecules in regulating hippocampal neurogenesis but the mechanisms remain unknown. These previous studies all investigated classical MHC I molecules in the CNS using broad

genetic manipulations, where β2-microglobulin, Tap1, or both were constitutively knocked out in the whole body. These models targeted MHC I as a whole class of molecules, as they decreased global cell surface expression of most if not all murine MHC I proteins indiscriminately. Because of the available genetic models, there has been a general assumption that classical MHC I subtypes performed analogous functions in the brain. However, as MHC I molecules comprise of a highly polymorphic gene family with over 50 sequences described, it is critical to begin to elucidate specific roles and cell-type significance for individual MHC I molecules in the CNS. Recently, it was shown that reintroduction of neuronal H2-D^b alone could reverse synaptic deficits in $KD^{-/-}$ mice, suggesting that a single MHC I molecule was sufficient for functional synapse elimination and refinement in the visual system (Lee et al., 2014). Correspondingly, I examined involvement of H2-K^b and H2-D^b separately in single knockout models and primary NSPCs derived from them. It should be noted that isolation of hippocampi from postnatal $K^{-/-}$ and $D^{-/-}$ mice may contain NSPCs from both the SGZ and SVZ, nor can oligodendrocyte precursors be excluded; thus, the proliferative effects observed may apply globally to other dividing cell populations in addition to hippocampal NSPCs. These data reveal a distinct role for H2-K^b in directing cell proliferation *in vitro* and hippocampal neurogenesis *in vivo*. Additionally in this study, spatially- and temporally-controlled knockdown using RNAi in WT mice allowed me to separate the general effects of systemic immune compromise in genetic knockout models from the absence of H2-K^b specifically in the hippocampus. I found that while H2-K^b inhibits NSPC proliferation, H2-D^b does not have an analogous role in NSPC function with few differences detected by *in vivo* histological analysis and NSPC RNA

sequencing of $D^{-/-}$ compared to WT animals. This functional difference may stem from disparities in structure, spatiotemporal distribution, or binding partners between H2-K^b and H2-D^b, and further studies are required for detailed comparative characterization.

My study also provides the first unbiased transcriptomic insight into pathways that may lie downstream of MHC I in the CNS. RNA sequencing analysis revealed enrichment of several growth factor receptor signaling pathways in $K^{-/-}$ NSPCs, identifying functional roles for FGFR, EGFR, PDGFR, and NGF signaling in mediating enhancements in proliferation. To date, many studies have shown that a number of soluble growth factors, such as insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), brain derived neurotrophic factor (BDNF), FGF2, and EGF regulate adult neurogenesis (Kuhn et al., 1997; Zigova et al., 1998; Lichtenwalner et al., 2001; Cao et al., 2004). It has been further demonstrated that restoring growth factor signaling by increasing FGF2 or EGF in the neurogenic niche or activating FGFR in adult NSPCs is sufficient to enhance adult neurogenesis in rodents where aging or neurodegenerative disease has caused functional decline of stem cell function (Jin et al., 2003; Kang and Hebert, 2015; Woodbury and Ikezu, 2014). It is promising that despite pathological neurogenic decline, the remaining endogenous NSPCs are amenable to rejuvenation through activation of growth factor signaling. Further insights into precisely how physical interactions between H2-K^b and growth factor receptors affect their trafficking dynamics, internalization, and signaling specifically on NSPCs will first be required to better understand how H2-K^b can be therapeutically targeted.

Under physiological conditions, neurogenesis has been implicated in spatial learning and memory, pattern separation, and affective behaviors (Ming and Song,

2011). Although tightly controlled, this process is highly vulnerable under pathological conditions: in mammalian models NSPC loss of function occurs in aging and neurodegenerative disorders while aberrant gain of NSPC proliferation can occur in epilepsy and schizophrenia (Haughey et al., 2002; Kuhn et al., 1996; Parent et al., 1997; Toro and Deakin, 2007; van Wijngaarden and Franklin, 2013). Preventing NSPC dysfunctions may be essential for restoring brain functions under pathological conditions, and identification of specific molecules and mechanisms controlling neurogenesis will inform translational efforts. H2-K^b is an attractive therapeutic target as MHC I molecules have been implicated in several CNS and systemic diseases —many of which present with NSPC dysfunction— by human genome-wide association studies (GWAS). Single nucleotide polymorphisms on the MHC complex region on chromosome 6 have been linked with highly significant disease risk in schizophrenia and age-associated disorders (International Schizophrenia Consortium et al., 2009; Jeck et al., 2012; Shi et al., 2009; Stefansson et al., 2009). In addition to a genetic association to disease, MHC I expression has directly been shown to be dynamic in certain pathophysiological states: MHCI molecules increase in rodent hippocampal neurons and neuromuscular junctions with age, interfering with synaptic homeostasis (Starkey et al., 2012; Tetruashvily et al., 2016), and an upregulation of neuronal H2-K^b, H2-D^b, and MHC I receptor, PirB, is observed in stroke (Adelson et al., 2012). As animals lacking H2-K^b and H2-D^b are protected from tissue damage and exhibit enhanced motor recover after stroke (Adelson et al., 2012), it remains for further studies to test whether targeting H2-K^b molecules in the aging hippocampus could prevent or reverse age-related neurogenic decline.

In summary, these studies have identified classical MHC I molecule H2-K^b as an inhibitor of NSPC proliferation and hippocampal neurogenesis. Adult animals with a genetic deletion or acute knockdown of H2-K^b in the DG exhibit enhanced cell division and increased adult-born neurons in the hippocampus. A hyper-proliferative phenotype is also observed in cells isolated from the postnatal $K^{-/-}$ brain and cultured *in vitro*, devoid of influences from the neurogenic niche or systemic environment. RNA sequencing of WT and $K^{-/-}$ NSPCs further revealed that H2-K^b reduces proliferation by dampening growth factor receptor signaling pathways. As many CNS disorders with neurogenic dysfunction also co-exhibit changes in MHC I expression, these results revealing the direct functional contribution of H2-K^b in NSPC function may help in illuminating new pathways to target in their treatment.

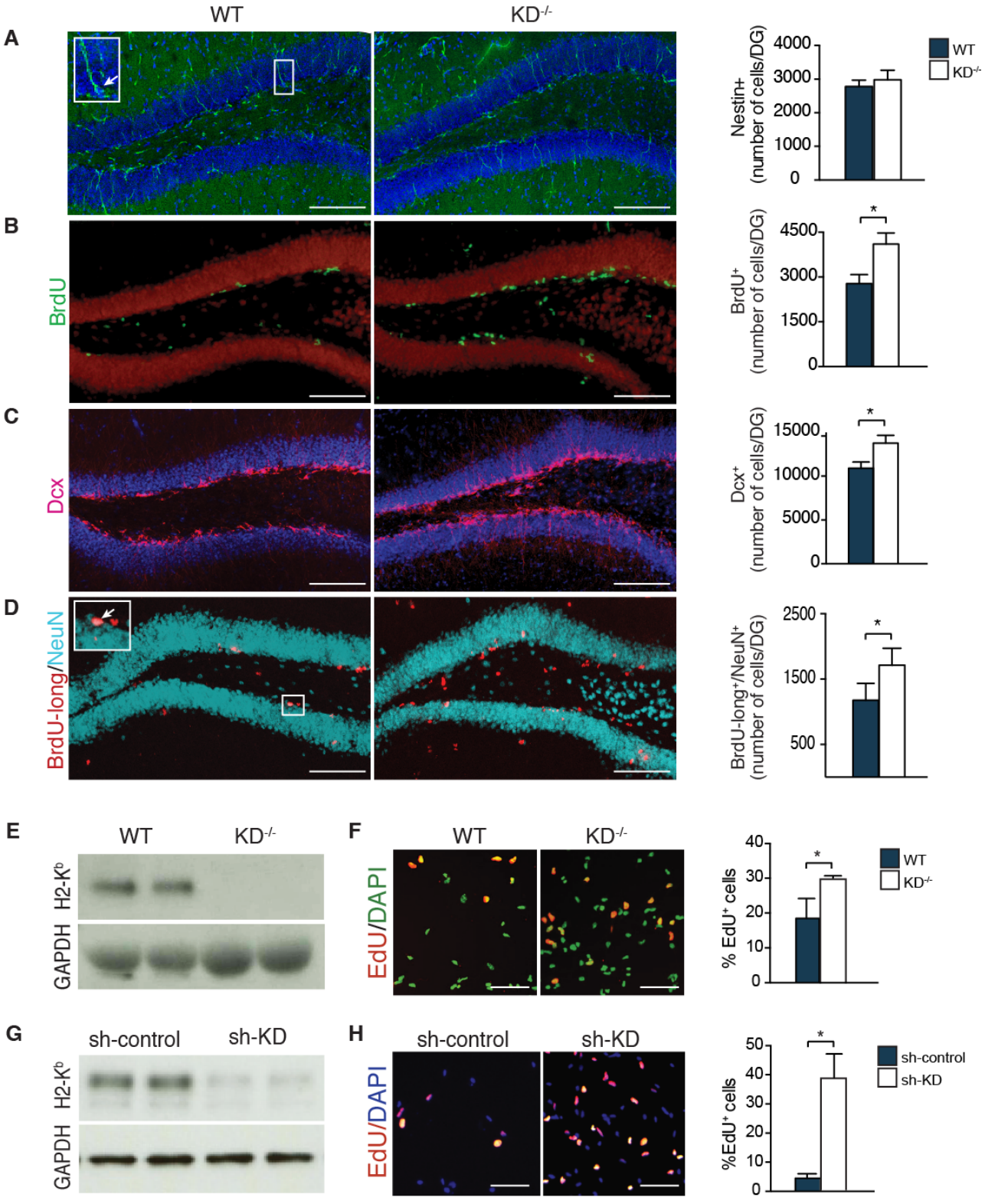
A
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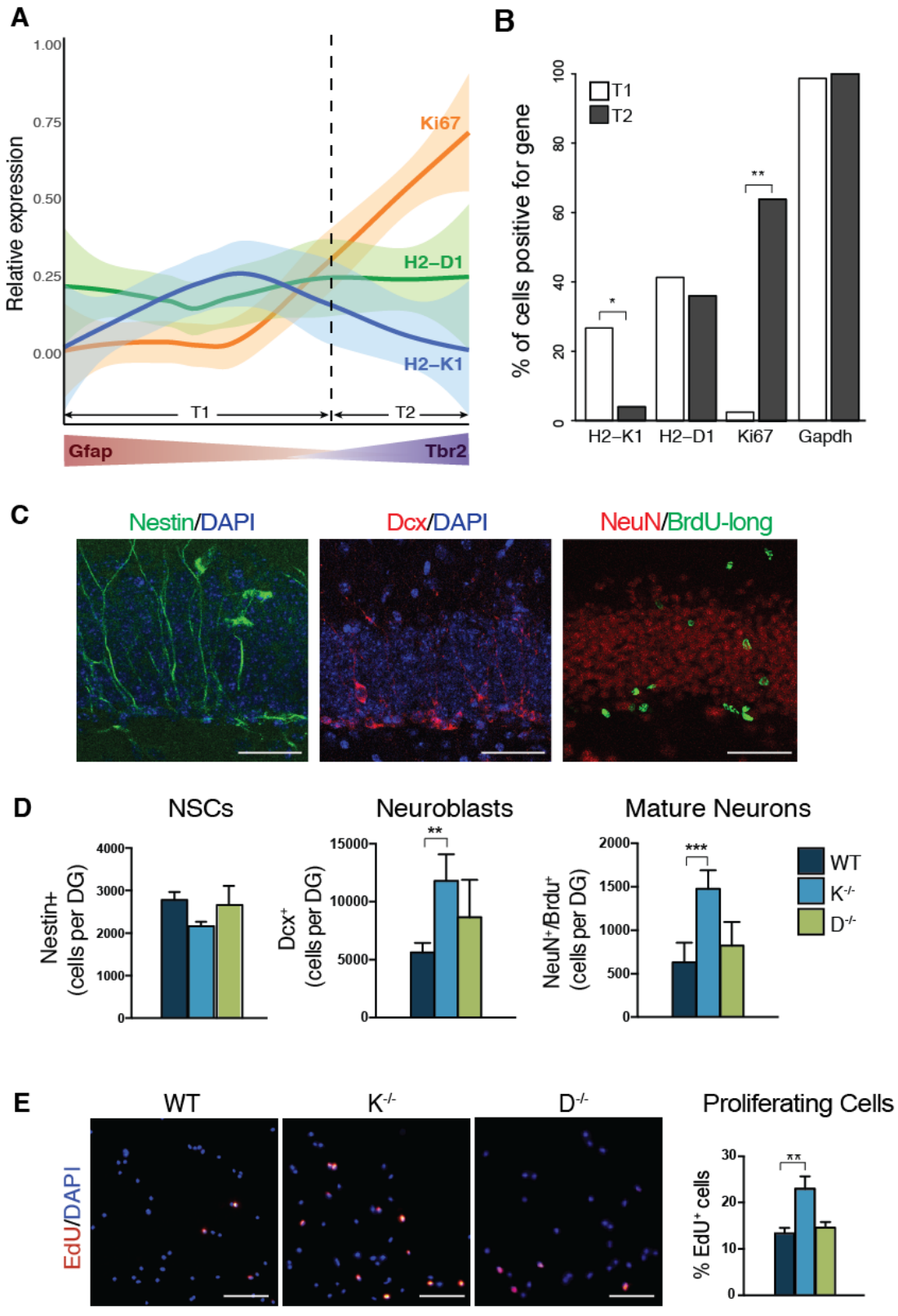
Figure 1. Deletion of classical MHC I molecules, H2-K^b and H2-D^b, increases hippocampal neurogenesis and NSPC proliferation. Representative field and quantification of Nestin$^+$ neural stem cells (NSCs) in the dentate gyrus (DG) of adult (3 months) WT and H2-K^b/H2-D^b double knockout (KD$^{-/-}$) mice (A). Representative field and quantification of BrdU$^+$ dividing cells (B) and Dcx$^+$ neuroblasts (C) in the DG of adult WT and KD$^{-/-}$ mice. A second cohort of adult WT and KD$^{-/-}$ mice were injected with 6 days of BrdU and differentiated cells co-expressing long-term retained BrdU (BrdU-long) and the mature neuronal marker NeuN in the DG were visualized and quantified 4 weeks later. Inset shows higher magnification of a double-labeled cell, indicated by arrow (D). Primary hippocampal neural stem and progenitor cells (NSPCs) were isolated from WT and KD$^{-/-}$ post-natal mice. Confirmation of knockout and specificity of our anti-H2-K^b antibody was assessed by Western blot analysis of WT and KD$^{-/-}$ NSPC lysates (E). See also Fig. S1B for H2-K^b and H2-D^b mRNA expression. Primary hippocampal WT and KD$^{-/-}$ NSPCs were cultured under self-renewal conditions and treated with 5-ethynyl-2´-deoxyuridine (EdU) for 6 hours. Representative field and percentage of EdU$^+$ dividing cells are shown (F). WT NSPCs were infected with lentiviruses encoding shRNA concomitantly targeting H2-K^b and H2-D^b (sh-KD) or targeting luciferase as a control (sh-control). Knockdown was confirmed by Western blot analysis of sh-KD and sh-control NSPC lysates probed with anti-H2-K^b and anti-GAPDH antibodies. See also Fig. S1C for H2-K^b and H2-D^b mRNA expression. WT NSPCs infected with lentiviruses encoding sh-KD and sh-control were cultured under self-renewal conditions and treated with EdU for 6 hours. Representative field and percentage of EdU$^+$ dividing cells are shown (H). Scale bars, 100μm (A-C) and 50μm (E,G). WT n = 5, KD$^{-/-}$ n=4-5 for histological analysis; n=3 replicates per group for *in vitro* experiments. All data are represented as mean ± SEM; *p<0.05. Student's t-test (A-G).


A
Relative expression
1.00
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B
% of cells positive for gene
T1
T2
H2–K1
H2–D1
Ki67
Gapdh
C
Nestin/DAPI
Dcx/DAPI
NeuN/BrdU-long
D
NSCs
Neuroblasts
Mature Neurons
Nestin+ (cells per DG)
Dcx+ (cells per DG)
NeuN+/Brdu+ (cells per DG)
WT
K-/-
D-/-
E
WT
K-/-
D-/-
EdU/DAPI
Proliferating Cells
% EdU+ cells

Figure 2. H2-K^b, but not H2-D^b, negatively regulates hippocampal neurogenesis and NSPC proliferation. Relative expression of *H2-K1*, *H2-D1* and *Mki67* along a bioinformatically assigned trajectory of adult neurogenesis. Single adult hippocampal NSCs were ordered according to pseudotime (x-axis) estimates as given in (Shin et al., 2015). Relative gene expression (y-axis, log2(CPM+1)/max(log2(CPM+1))) was fitted with local polynomial regression fitting (bold lines) with 95% confidence interval (light colored area). The cutoff point between time points T1 and T2 was determined by the time of increasing Tbr2 expression (last stationary point of the T*br2* regression line; see Fig. S2) (A). Number of cells positive (CPM>5) for *H2-K1, H2-D1, Ki67* and *Gapdh* expression (y-axis) between T1 and T2 (B). Representative field (C) and quantifications (D) of Nestin$^+$ NSCs, Dcx$^+$ neuroblasts and adult-born neurons co-expressing BrdU-long and NeuN in the DG of 3-month-old WT, H2-K^b knockout (K$^{-/-}$), and H2-D^b knockout (D$^{-/-}$) mice. Primary hippocampal WT, K$^{-/-}$, and D$^{-/-}$ NSPCs cultured under self-renewal conditions were treated with EdU for 6 hours. Representative field shown and mean percentage of EdU$^+$ dividing cells were quantified (E). Scale bars, 50μm (C) and 100μm (E). WT, n=5; K$^{-/-}$, n=5; D$^{-/-}$, n=5 for histological analysis; n=3 replicates per group for *in vitro* experiments. Data are represented as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001. Fisher's exact test (B); ANOVA with Dunnett's post hoc test (D, E).

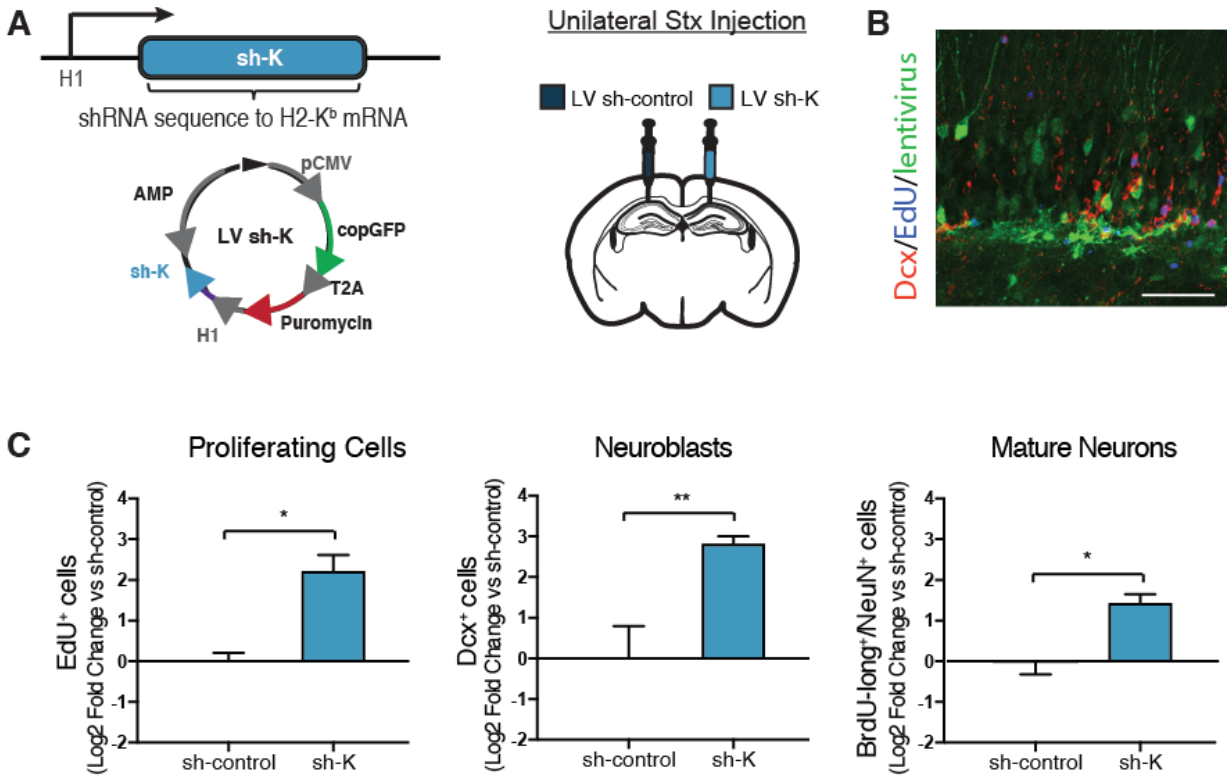
A
sh-K
H1
shRNA sequence to H2-Kᵇ mRNA
pCMV
AMP
LV sh-K
copGFP
sh-K
T2A
Puromycin
H1
Unilateral Stx Injection
LV sh-control
LV sh-K
B
Dcx/EdU/lentivirus
C
Proliferating Cells
EdU⁺ cells
(Log2 Fold Change vs sh-control)
sh-control
sh-K
Neuroblasts
Dcx⁺ cells
(Log2 Fold Change vs sh-control)
sh-control
sh-K
Mature Neurons
BrdU-long⁺/NeuN⁺ cells
(Log2 Fold Change vs sh-control)
sh-control
sh-K
D
sh-control
sh-K
EdU/DAPI
Proliferating Cells
EdU⁺ cells
sh-control
sh-K

Figure 3. Acute abrogation of H2-K^b increases adult hippocampal neurogenesis *in vivo* and NSPC proliferation *in vitro*. Schematic illustrating vector map of lentivirus encoding an H1 promoter-driven short hairpin RNA (shRNA) targeting H2-K^b (sh-K) or luciferase as a control (sh-control) in tandem with a GFP reporter. Adult (3 months) WT mice were stereotaxically injected with sh-K in one DG and sh-control in the contralateral DG (A). Representative field of Dcx$^+$ neuroblasts, EdU$^+$ dividing cells, and cells infected with the lentivirus, as visualized by the GFP reporter (B). Quantification of EdU$^+$ proliferating cells, Dcx$^+$ neuroblasts, and BrdU-long/NeuN double-positive mature neurons in sh-K injected DG compared to the contralateral control-injected DG. Data represented as mean Log2 fold change over control ± SEM (C). WT NSPCs infected with lentiviruses encoding sh-K and sh-control were cultured under self-renewal conditions and treated with EdU for 6 hours. Representative field and percentage of EdU$^+$ dividing cells are shown. Data represented as mean number of cells per field ± SEM (D). Scale bars, 50μm (B), 100μm (D). n=6 mice for histological analysis of stereotaxically-injected mice; n=3 replicates per group for *in vitro* experiments. *p<0.05, **p<0.01; Student's t-test (C, D).

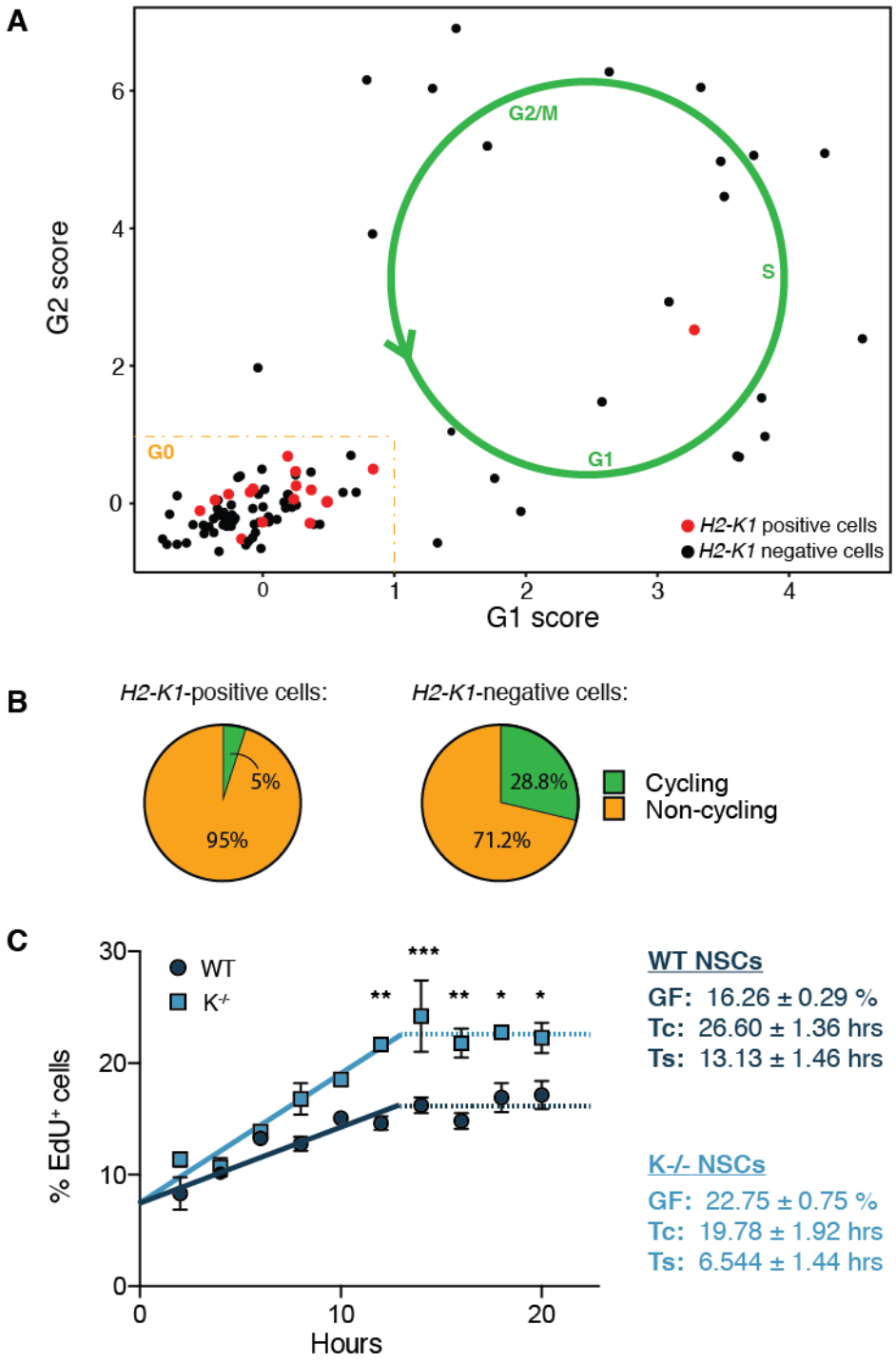
A
G2 score
G1 score
G2/M
S
G1
G0
H2-K1 positive cells
H2-K1 negative cells
B
H2-K1-positive cells:
H2-K1-negative cells:
5%
95%
28.8%
71.2%
Cycling
Non-cycling
C
% EdU+ cells
Hours
WT
K-/-
WT NSCs
GF: 16.26 ± 0.29 %
Tc: 26.60 ± 1.36 hrs
Ts: 13.13 ± 1.46 hrs
K-/- NSCs
GF: 22.75 ± 0.75 %
Tc: 19.78 ± 1.92 hrs
Ts: 6.544 ± 1.44 hrs

Figure 4. H2-K^{b} regulates cell cycle dynamics at the cell autonomous level. A publicly available dataset of single adult hippocampal NSCs (Shin et al., 2015) was sorted by expression of G1 (x-axis) and G2 (y-axis) cell cycle scores as described in Tirosh et al. 2016. Cells positive for H2-K1 expression (>5 CPM) are indicated in red (A). The percentage of cycling cells was found to be significantly lower in *H2-K1*-positive cells (5%) versus *H2-K1*-negative cells (28.8%); p=0.0374, Fisher's exact test (B). Primary hippocampal WT and K$^{-/-}$ NSPCs cultured under self-renewal conditions were treated with EdU at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 hours (hrs) prior to fixation, and percentage of EdU^{+} cells was determined by flow cytometry. Percent of cells incorporating EdU reached a plateau at 13.47 hrs for WT NSPCs and 13.24 hrs for K$^{-/-}$ NSPCs (Tc-Ts) when the cycling population (GF) entered the S phase. Linear regression for increasing phase of WT NSPCs: $y = 0.6133 x + 7.996$; R^2=0.829 and K$^{-/-}$ NSPCs: $y = 1.165 x + 7.406$; R^2= 0.889. Values for cell cycle parameters calculated from cumulative labeling are shown in the right panel. GF, growth fraction; Tc, length of cell cycle; Ts, length of S phase (C). n=2 replicates. Data represented as mean ± SEM; *p<0.05, **p<0.01, ***p<0.001. Two-way repeated measures ANOVA with Sidak's post hoc test (C).

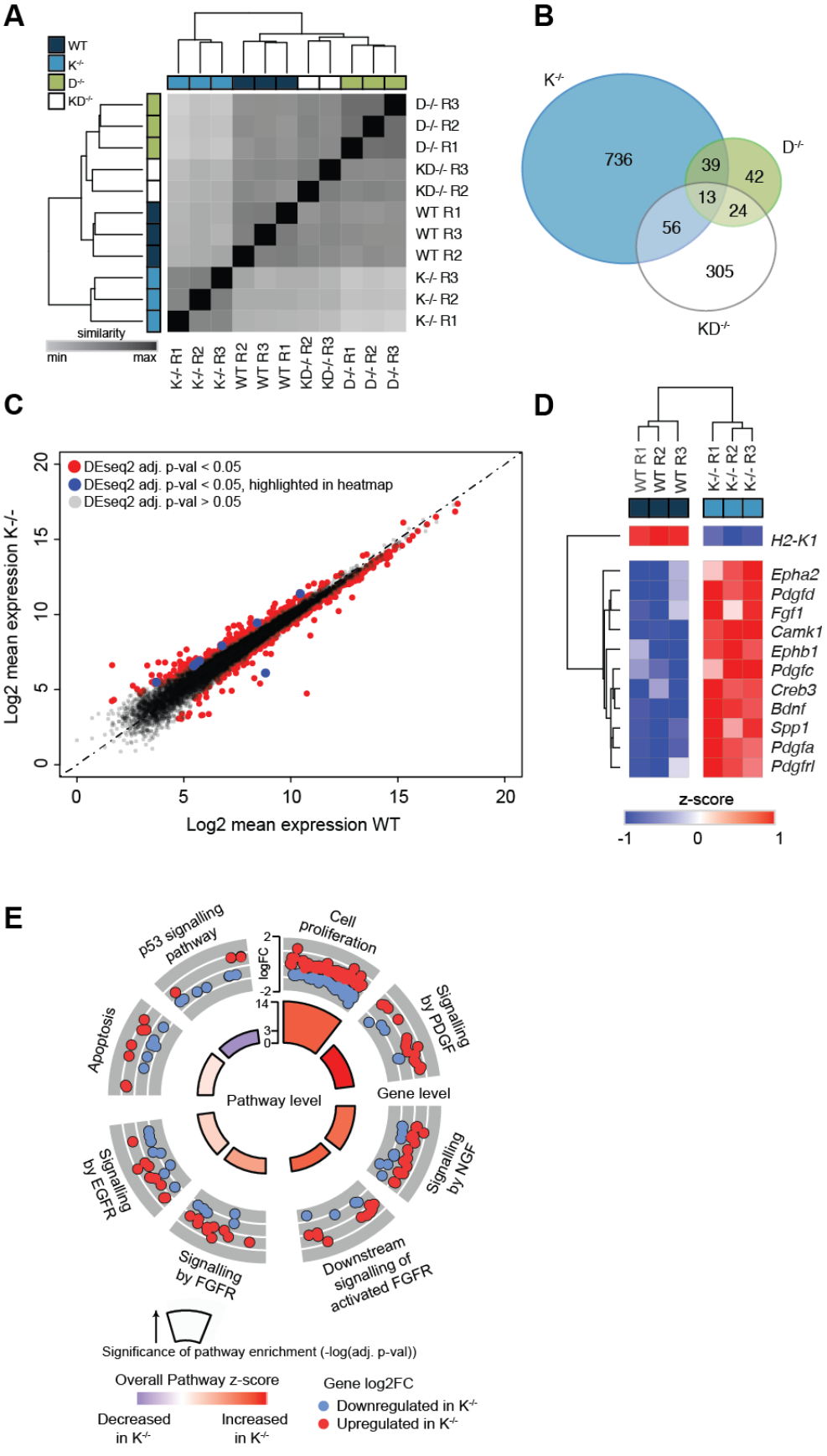
A
WT
K-/-
D-/-
KD-/-
D-/- R3
D-/- R2
D-/- R1
KD-/- R3
KD-/- R2
WT R1
WT R3
WT R2
K-/- R3
K-/- R2
K-/- R1
similarity
min
max
K-/- R1
K-/- R2
K-/- R3
WT R2
WT R3
WT R1
KD-/- R2
KD-/- R3
D-/- R1
D-/- R2
D-/- R3
B
K-/-
D-/-
KD-/-
736
39
42
13
24
56
305
C
DEseq2 adj. p-val < 0.05
DEseq2 adj. p-val < 0.05, highlighted in heatmap
DEseq2 adj. p-val > 0.05
Log2 mean expression K-/-
Log2 mean expression WT
0
5
10
15
20
D
WT R1
WT R2
WT R3
K-/- R1
K-/- R2
K-/- R3
H2-K1
Epha2
Pdgfd
Fgf1
Camk1
Ephb1
Pdgfc
Creb3
Bdnf
Spp1
Pdgfa
Pdgfrl
z-score
-1
0
1
E
p53 signalling pathway
Cell proliferation
Signalling by PDGF
Signalling by NGF
Downstream signalling of activated FGFR
Signalling by FGFR
Signalling by EGFR
Apoptosis
logFC
2
-2
14
3
0
Pathway level
Gene level
Significance of pathway enrichment (-log(adj. p-val))
Overall Pathway z-score
Decreased in K-/-
Increased in K-/-
Gene log2FC
Downregulated in K-/-
Upregulated in K-/-

Figure 5. RNA-sequencing identifies growth factor receptor-mediated signaling downstream of H2-K^{b} in NSPCs. Pairwise distances (Euclidean distance) between RNA-sequencing libraries prepared from WT, KD$^{-/-}$, K$^{-/-}$, and D$^{-/-}$ primary hippocampal NSPCs. Dendrogram was calculated by unsupervised hierarchical clustering (average linkage). (WT, n=3 replicates [denoted R1-R3]; KD$^{-/-}$, n = 2 replicates; K$^{-/-}$, n=3 replicates; and D$^{-/-}$, n=3 replicates) (A). Venn diagram illustrating genes differentially expressed (DEGSeq2 adj. $p<0.05$) between WT, KD$^{-/-}$, K$^{-/-}$, and D$^{-/-}$ NSPCs (B). Scatterplot of the average gene expression in WT (x-axis) and K$^{-/-}$ (y-axis) NSPCs (C). Differentially expressed genes (DEGSeq2 adj. $p<0.05$, 432 down and 408 upregulated) are indicated in red. Genes shown in heatmap (D) are colored in blue. Unsupervised hierarchical clustering of RNA-seq libraries (columns) in the space of genes indicated in (C) (z-score normalized, rows) (D). Cellular and signaling pathways identified by pathway enrichment analysis (ConsensusPathDB) of genes differentially expressed between WT and K$^{-/-}$ NSPCs are subdivided in gray. Outer ring illustrates expression of altered genes (log2 fc K$^{-/-}$ vs WT) within each pathway. Inner ring illustrates significance (-log10 adj. p-val) and overall expression levels of genes within each pathway (z-score) (E).

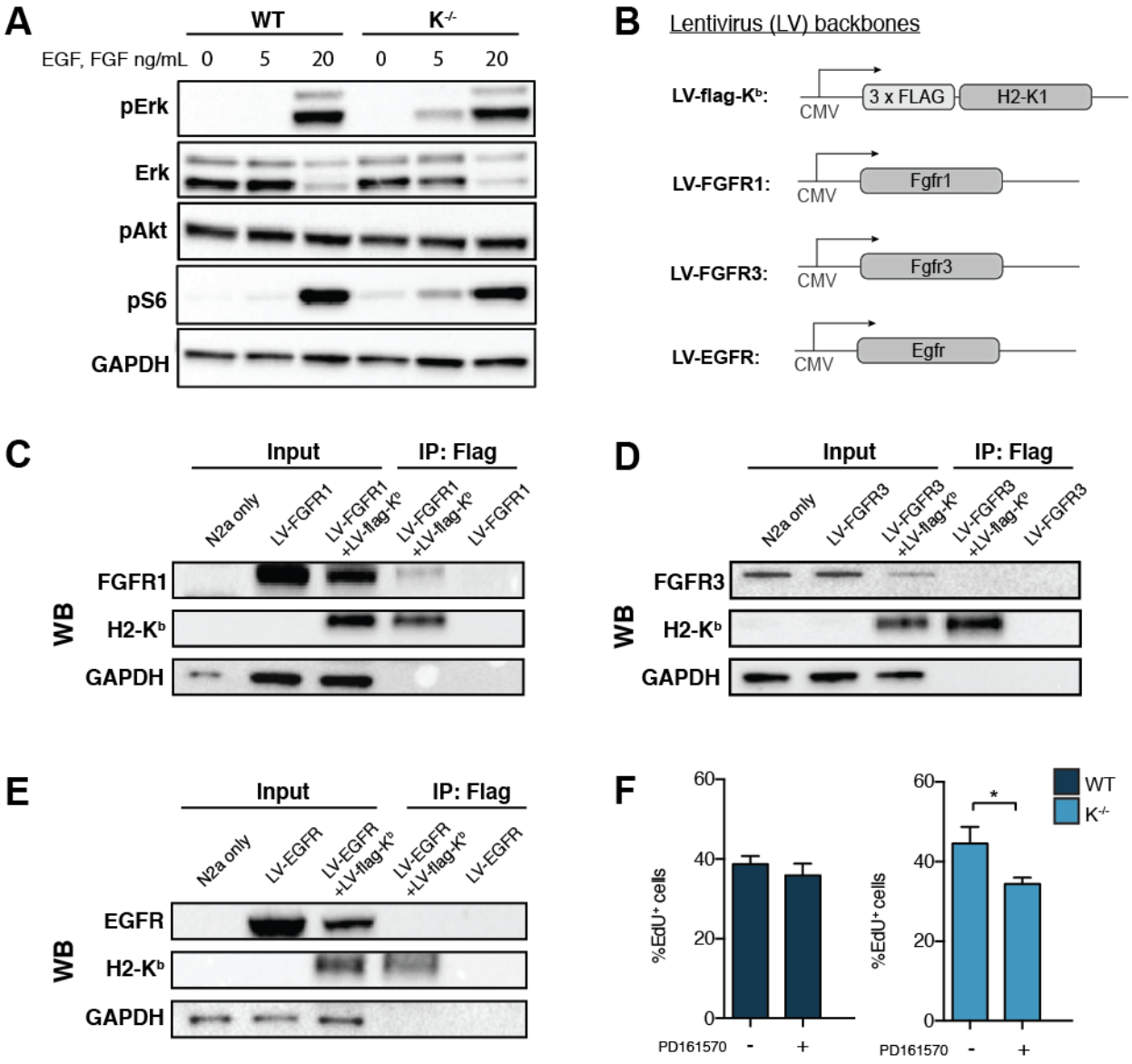
A
WT
K-/-
EGF, FGF ng/mL 0 5 20 0 5 20
pErk
Erk
pAkt
pS6
GAPDH
B
Lentivirus (LV) backbones
LV-flag-Kb:
CMV
3 x FLAG
H2-K1
LV-FGFR1:
Fgfr1
LV-FGFR3:
Fgfr3
LV-EGFR:
Egfr
C
Input
IP: Flag
N2a only
LV-FGFR1
LV-FGFR1 +LV-flag-Kb
LV-FGFR1 +LV-flag-Kb
LV-FGFR1
WB
FGFR1
H2-Kb
GAPDH
D
Input
IP: Flag
N2a only
LV-FGFR3
LV-FGFR3 +LV-flag-Kb
LV-FGFR3 +LV-flag-Kb
LV-FGFR3
WB
FGFR3
H2-Kb
GAPDH
E
Input
IP: Flag
N2a only
LV-EGFR
LV-EGFR +LV-flag-Kb
LV-EGFR +LV-flag-Kb
LV-EGFR
WB
EGFR
H2-Kb
GAPDH
F
%EdU+ cells
60
40
20
0
PD161570 - +
*
WT
K-/-

Figure 6. H2-K^{b} inhibits growth factor receptor signaling pathways in NSPCs through the formation of macromolecular complexes with receptors. Western blot analysis of activated Akt, Erk, and ribosomal proteign S6 in WT and K$^{-/-}$ NSPCs grown under absent (0 ng/mL), low (5 ng/mL), or regular (20 ng/mL) growth factor conditions (A). Schematics of lentiviral vectors generated for N2a cell transfection in co-immunoprecipitation experiments (D-E): CMV promoter-driven 3x-FLAG-tagged H2-K1, Fgfr1, Fgfr3, and Egfr (B). N2a cells were co-transfected with 3xFLAG H2-K1 and Fgfr1 (C), Fgfr3 (D), or Egfr1. Anti-FLAG magnetic bead selectively co-precipitate FGFR1, but not FGFR3, EGFR, or intracellular proteins (GAPDH). Primary hippocampal WT (left panel) and K$^{-/-}$ (right panel) NSPCs were exposed to fibroblast growth factor receptor inhibitor PD 161570 (PD, 0.1uM or phosphate-buffered saline control for one hour and subsequently treated with EdU for 6 hours to test for proliferation (F). Data represent as mean percentage of EdU^{+} cells per field ± SEM. n=3 replicates per group for *in vitro* experiments; *p<0.05. ANOVA with Dunnett's post hoc test (F).

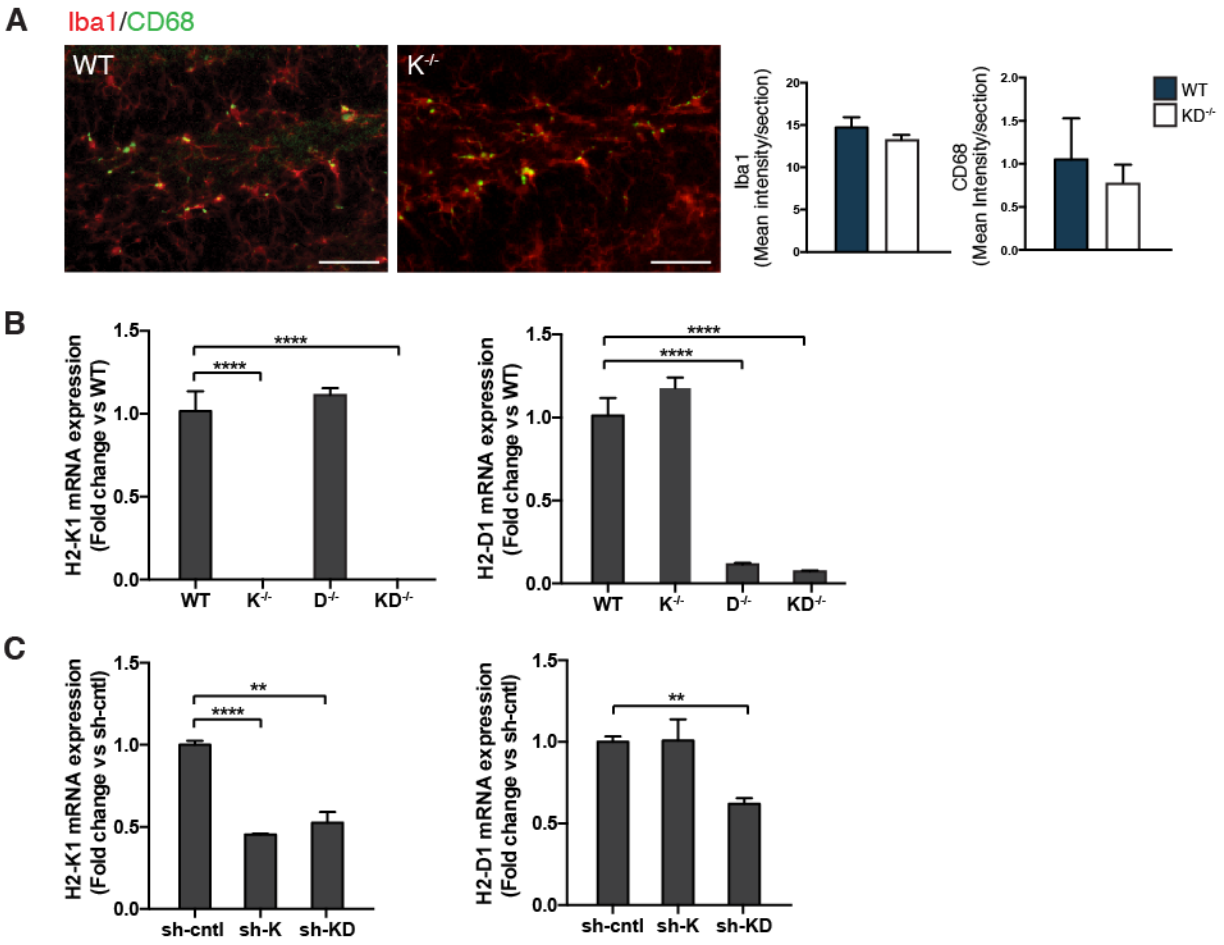

Figure S1. Characterization and validation of MHC I knockout and knockdown *in vivo* and *in vitro* models. Representative field and quantification of ionized calcium-binding adapter molecule 1 (Iba1) and cluster of differentiation 68 (CD68) mean intensity in the DG of adult WT and $KD^{-/-}$ mice (A). Quantitative RT-PCR of H2-K^b (left panel) and H2-D^b (right panel) mRNA from primary NSPCs isolated from WT, $KD^{-/-}$, $K^{-/-}$, and $D^{-/-}$ mice (B). Quantitative RT-PCR of H2-K^b (left panel) and H2-D^b (right panel) mRNA from primary WT NSPCs infected with lentiviruses encoding shRNA targeting H2-K^b and H2-D^b (sh-KD), H2-K^b (sh-K), or luciferase as a control (sh-cntl). Scale bars, 50µm (A). WT n=5, $KD^{-/-}$ n=4 for histological analysis; n=3 replicates per group for *in vitro* experiments. All data represented as mean ± SEM; **p<0.01, ****p<0.0001. Student's t test (A), ANOVA with Dunnett's post hoc test (B, C).

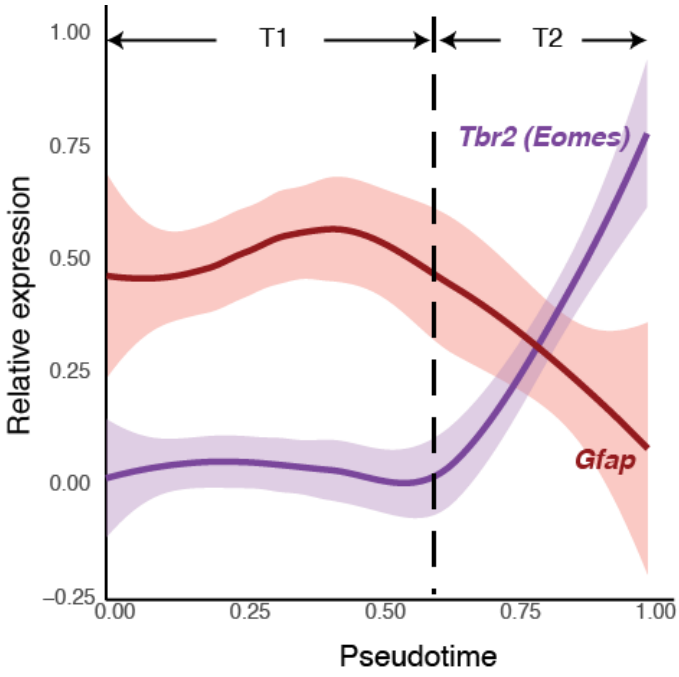

Figure S2, related to Figure 2. Single-cell transcriptomic characterization of H2-K1 expression during neurogenesis. Relative expression of *Tbr2* along a trajectory of adult neurogenesis, as described in Figure 2A. The cutoff point between time points T1 and T2 was determined by the time of increasing Tbr2 expression (last stationary point of the Tbr2 regression line).

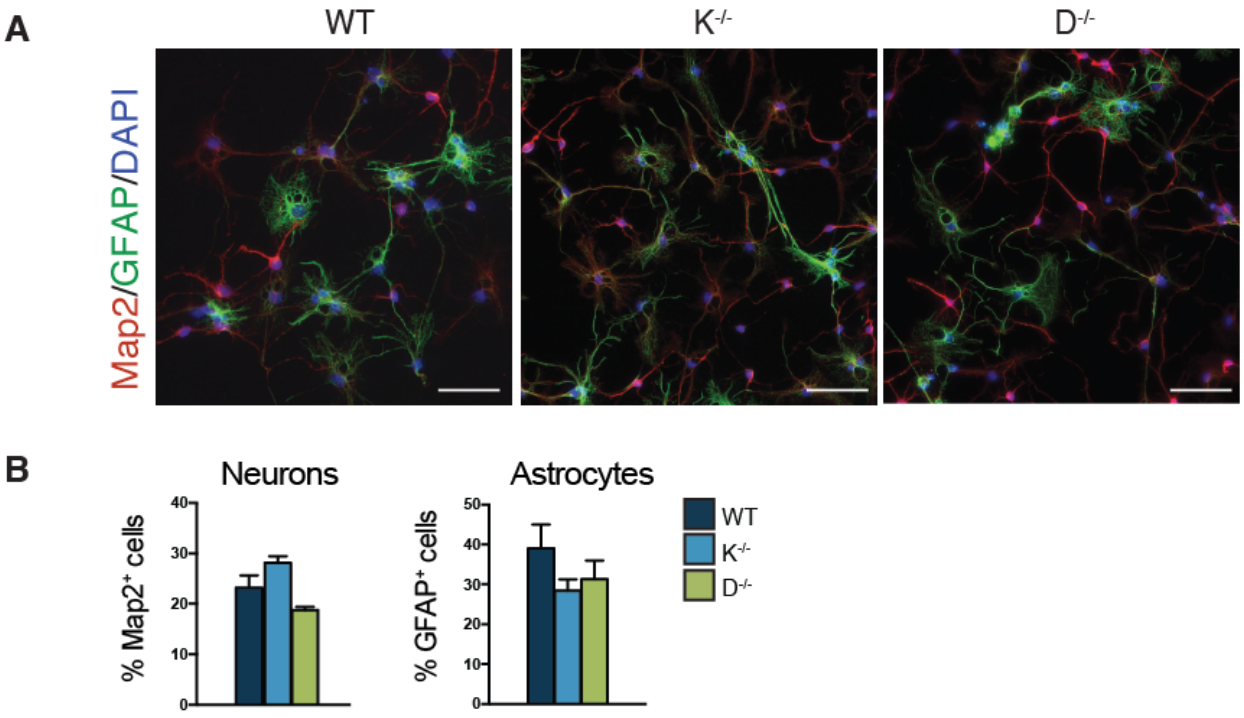

Figure S3. No apparent alteration in neuronal differentiation potential in NSPCs lacking H2-K^{b} or H2-D^{b}. WT, K$^{-/-}$, and D$^{-/-}$ NSPCs were cultured under growth factor-free conditions and allowed to differentiate for 6 days. Representative field (A) and quantification (B) of microtubule-associated protein 2-positive (Map2^{+}) neurons and glial fibrillary acidic protein-positive (GFAP^{+}) astrocytes. Data represented as mean percentages ± SEM; n=3 replicates per group. ANOVA, with Dunnett's post hoc test (B).

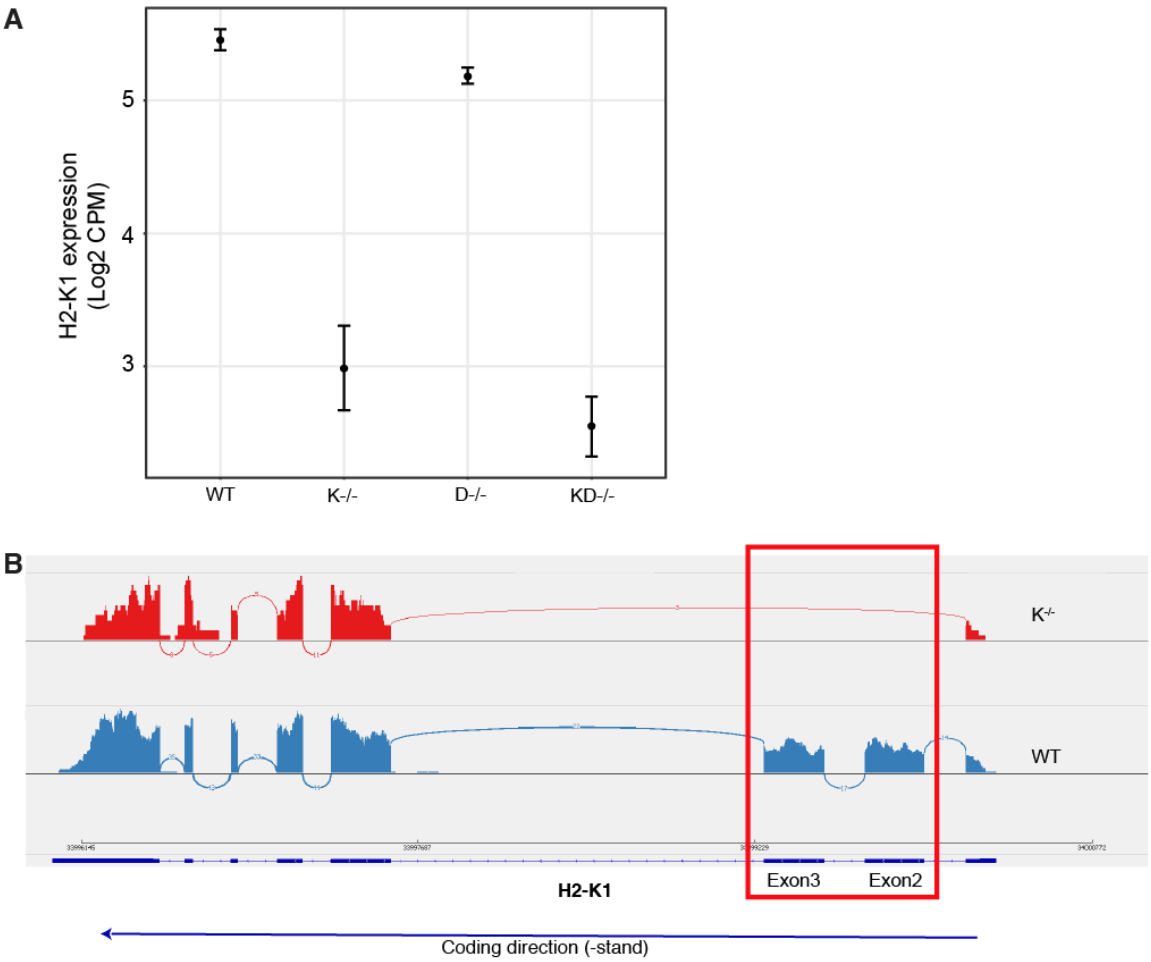

Figure S4, related to Figure 5. Validation of H2-K^{b} knockout in primary NSPCs by RNA-sequencing analysis. Normalized (Log2(CPM=1)) RNA-sequencing expression of *H2-K1* (y-axis) in WT (n=3 replicates), KD $^{-/-}$ (n=2), K$^{-/-}$ (n=3), and D$^{-/-}$ (n=3) primary hippocampal NSPCs (A). Interactive Genome Viewer (Broad Institute) Sashimi plot visualizing RNA-sequencing read density (colored areas) and junction-spanning reads (lines) in WT (bottom) and K$^{-/-}$ NSPCs (B). K$^{-/-}$ NSPCs are devoid of reads mapping to exons 2 or 3. Junction spanning reads map from exon 1 to exon 4.

Chapter 4

Role of Classical MHC I Molecules in Regulating Cognitive Function

In order to translate experience into behavioral change and memory, neural plasticity resulting in the selective strengthening and weakening of appropriate synapses must occur. A surprising regulator of synaptic plasticity in the hippocampus are major histocompatibility complex class I (MHC I) molecules, as animals deficient for cell surface expression of these proteins display enhanced long-term potentiation (LTP) and absent long-term depression (LTD). LTP and LTD mechanisms comprise the cellular basis of experience-dependent development, learning and memory, and their impairments are prominent in neurological disorders. To date, it remains to be elucidated how individual MHC I molecules (of the more than 50 encoded in the mouse genome) function to regulate learning and memory. Here I test mice genetically lacking each of the classical MHC I molecules, H2-K^b or H2-D^b, individually ($K^{-/-}$ and $D^{-/-}$ mice, respectively) in a battery of behavioral tests. This study demonstrates that $D^{-/-}$ mice exhibit impairments in locomotion, anxiety-related behavior, and hippocampus-dependent spatial and contextual memory. Remarkably, I show that acute ablation of H2-D^b in the adult hippocampus, using an RNA interference approach, can improve hippocampus-dependent memory. My results establish the developmental necessity of one specific MHC I molecule, H2-D^b, in maintaining intact cognition and show a potential for the therapeutic targeting of this molecule in CNS disorders that present with hippocampal dysfunction.

Introduction

Pleiotropy—where one protein can drive multiple, apparently unrelated phenotypic features in different tissues or cell types— is a feature prominent in a growing number of molecules first characterized in the immune system. Many proteins first discovered to have immune roles have since been detected in the healthy developing and adult central nervous system (CNS), and additionally demonstrated to have non-canonical roles in regulating nervous system development, refinement, and plasticity (Boulanger, 2009). One particular class of molecules, major histocompatibility complex class I (MHC I), play a role not only in the early establishment of appropriate connectivity and synaptic properties of developing neurons (Bilousova et al., 2012; Datwani et al., 2009; Glynn et al., 2011; Huh et al., 2000), but also influence the later structural and functional synaptic plasticity of the CNS that is malleable throughout life (Fourgeaud et al., 2010; Goddard et al., 2007; Huh et al., 2000; McConnell et al., 2009b).

Plasticity at the synapse, in the form of long-term potentiation (LTP) or long-term depression (LTD), is thought to underlie behavioral change through the selective strengthening or weakening of pertinent synapses (Bliss and Collingridge, 1993; Kessels and Malinow, 2009). Adult hippocampal plasticity occurs when repetitive, coincident depolarization of a set of synapses causes activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors, resulting in subsequent changes to the trafficking and function of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors. A large magnitude, transient activation of NMDA receptors leads to LTP via AMPA receptor insertion, whereas weak, persistent

activation of NMDA receptors induces removal of AMPA receptors resulting in LTD. The result is a long-lasting modification of synaptic strength between two or more synapses involved in the circuit. There is an abundance of data supporting the notion that intact synaptic plasticity is necessary for learning and memory (see Martin et al., 2000 for an extensive review): impairments to hippocampus-dependent spatial and associative memory were observed in animals with genetic or pharmacological disruptions in LTP (Mayford et al., 1996; Morris et al., 1986) and conversely, manipulations that facilitate the induction of LTP have been shown to enhance memory formation (Lynch, 1998; Seabrook et al., 1997). Synaptic plasticity mechanisms are also critical during early activity-dependent pruning and elimination of neural circuitry and impairments in synaptic plasticity mechanisms are a hallmark of many neuropsychiatric disorders as well as in the aging brain (Barnes, 2003; Kolb and Gibb, 2011; Lau and Zukin, 2007). Identifying novel molecular mechanisms that control synaptic plasticity will help further elucidate the neural basis of normal cognition and reveal potential targets in pathological brain function.

MHC I molecules first appeared as unexpected regulators of CNS function in an unbiased screen for genes involved in activity-dependent synaptic remodeling of the mammalian visual system (Corriveau et al., 1998). MHC I mRNA is highly expressed in brain regions exhibiting continuous plasticity, such as the adult hippocampus, and its expression levels are dynamically regulated by the activity state of the neuron (Corriveau et al., 1998; Huh et al., 2000); these attributes make them prime candidates for the modulation of synaptic transmission between neurons. Indeed, transgenic mice lacking β2-microglobulin (β2m), the non-covalently bound light chain of the MHC I

complex, and the transporter associated with antigen processing 1 (Tap1), which transports peptides across the endoplasmic reticulum membrane, have been studied in this context. MHC class I is a large gene family and this double knockout model ($\beta2m^{-/-}$ $Tap1^{-/-}$) broadly reduces cell-surface expression of the more than 50 MHC I molecules expressed in the murine genome (Ljunggren et al., 1995). $\beta2m^{-/-}Tap1^{-/-}$ mice display enhanced high-frequency stimulation-induced LTP and absent low-frequency stimulation-induced LTD in the CA1 of the hippocampus (Huh et al., 2000; Nelson et al., 2013), mediated by enhanced NMDA receptor-mediated currents and subsequent increase of AMPA receptors at the cell surface (Fourgeaud et al., 2010). As acquisition and consolidation of explicit memories is believed to require NMDA-dependent LTP and LTD in the hippocampus, surface MHC I-deficient mice were tested for hippocampal-dependent learning and memory. Despite increased synaptic potentiation via relief of inhibition by MHC I, $\beta2m^{-/-}Tap1^{-/-}$ mice performed worse than wild-type (WT) littermates in contextual fear conditioning and object or social recognition tests of memory (Nelson et al., 2013). These broad models of surface MHC I abrogation provide convincing, though indirect, evidence of the necessity of these molecules in modulating hippocampal plasticity and associated behaviors. To date, it remains to be elucidated how individual MHC I molecules (of the more than 50 encoded in the mouse genome) function in the CNS. To directly tease apart the roles specific MHC I molecules play in the regulation of hippocampus-dependent cognition, I tested mice genetically lacking each of the classical MHC I molecules, H2-K^b or H2-D^b, individually ($K^{-/-}$ and $D^{-/-}$ mice, respectively) in a battery of behavioral tests. To complement these constitutive genetic knockout models, I also used a lentivirus-mediated short hairpin RNA (shRNA)

approach to knockdown H2-K^b or H2-D^b with spatiotemporal precision in the hippocampus. Together, these results demonstrate the developmental necessity for a single MHC I molecule, H2-D^b, in preserving normal locomotion, anxiety-related behavior, and spatial memory, and furthermore show that its acute ablation in the adult hippocampus can remarkably improve hippocampus-dependent memory.

Results

Animals lacking H2-D^b have abnormal nesting, locomotor, and anxiety phenotypes

To elucidate the roles H2-K^b and H2-D^b may play in regulating cognitive function, I made use of mice genetically lacking H2-K^b ($K^{-/-}$) or H2-D^b ($D^{-/-}$) individually. In order to characterize these mice and to exclude potential developmental or motor deficits that may confound cognitive testing, general behaviors such as home cage nest forming, locomotor activity, and anxiety were tested in 3 month old WT, $K^{-/-}$, and $D^{-/-}$ mice. Nesting scores (on a 4-point scale, **Figure 1A**) were recorded two hours after mice were individually housed and given a fresh nestlet. $D^{-/-}$ mice had significantly lower nesting scores at 2 hours than WT or $K^{-/-}$ mice (**Figure 1B**). However, these differences disappeared after 24 hours as $D^{-/-}$ mice could eventually form nests like the other groups, suggesting delayed but otherwise normal home cage daily living behavior. In the open field test, $D^{-/-}$ mice exhibited increased total locomotor activity but no differences in time spent in the center of the field compared to WT and $K^{-/-}$ mice. In the elevated zero maze, a test specifically for anxiety, $D^{-/-}$ mice spent twice as much time in

the open, exposed arms of the maze as $K^{-/-}$ and control mice did (**Figure 1F**). The increased locomotor phenotype of $D^{-/-}$ mice seen in the open field was recapitulated in the elevated zero maze (**Figure 1E**). These behavioral phenotyping data suggest $D^{-/-}$ mice have heightened locomotor or exploratory activity levels and an attenuated anxiety response, whereas performance in $K^{-/-}$ mice is indistinguishable from WT mice.

Animals lacking H2-D^b have impaired spatial and fear memory

Hippocampal slices from mice lacking surface expression of MHC I molecules ($\beta2m^{-/-}Tap1^{-/-}$) have previously been shown to exhibit enhanced LTP and absent LTD after tetanic stimulation of the Schaffer collaterals (Huh et al., 2000; Nelson et al., 2013). Given that genetic and pharmacological manipulations that affect hippocampal LTP are bidirectionally correlated with changes in spatial memory (Martin et al., 2000), it naturally follows that I tested WT, $K^{-/-}$, and $D^{-/-}$ mice for hippocampal-dependent learning and memory using the radial arm water maze (RAWM) and contextual fear conditioning. 3-month-old WT, $K^{-/-}$, and $D^{-/-}$ mice were tested in a six-arm maze in two-day paradigm, with the first (training) day consisting of 12 alternating visible and hidden trials followed by 3 hidden trials, and the second (testing) day consisting of 15 hidden trials (**Figure 2A,B**). A decrease in errors made is indicative of learning and memory of the task. $K^{-/-}$ mice did not exhibit any differences in number of errors made compared to WT mice on either day (**Figure 1C**). Surprisingly, $D^{-/-}$ mice performed worse, both in short-term learning (last block on Day 1) and memory (Day 2), when compared to WT mice (**Figure 2D**).

An associative learning task, fear conditioning, was also used to assess hippocampus-dependent and independent memory in $K^{-/-}$, $D^{-/-}$, and WT mice. In this two-day paradigm, mice were exposed to two pairings of an unconditioned stimulus (foot shock) with conditioned stimuli (tone and context) on the first day, and tested for contextual and cued memory manifested as freezing behavior on the second day (**Figure 3A**). At baseline $D^{-/-}$ mice froze less during the first two minutes of exposure to the chamber (prior to presentation of tone and shock) on the training day (**Figure 3B**), reiterating the hyperactive phenotype seen in the open field and elevated zero maze task (**Figure 1C,E**). To account for baseline differences, net freezing during the contextual and cued tasks was calculated by subtraction of baseline freezing from freezing during each test on Day 2. Impaired hippocampus-dependent contextual memory— in which mice were placed in the same chamber as during training— was observed in both $K^{-/-}$ and $D^{-/-}$ mice (**Figure 3C**). Mice were then tested for cued auditory memory, which is not hippocampus-dependent but rather contingent on intact amygdala function, after the conditioned tone is played in a chamber with different walls, floor, and scent. There is a direction of change towards less freezing for the auditory conditioned stimulus for both $K^{-/-}$ and $D^{-/-}$ mice, though not statically significant (**Figure 3D**). Intact cued conditioning in $K^{-/-}$ and $D^{-/-}$ mice suggests that their impaired contextual memory was not due to an inability to freeze or a problem with the amygdala, but specifically implicated hippocampal involvement. These results, combined with the phenotypes observed in RAWM, begin to discern the individual roles H2-K^b and H2-D^b might play in regulating specific types of behavior and memory. In additional to locomotor and anxiety phenotypes, mice lacking H2-D^b appear to be more severely impaired in both

hippocampal-dependent spatial and associative memory tasks tested compared to WT mice, whereas $K^{-/-}$ mice are only impaired in the contextual fear conditioning task.

Acute abrogation of H2-D^b improves spatial learning and memory

Targeted deletion of specific genes offers an invaluable tool for studying the roles of those molecules in a biological pathway or system. However, as genetic $K^{-/-}$ and $D^{-/-}$ mouse models are constitutive, whole-body knockouts, potential for developmental compensation or contribution of other cell types should not be overlooked and can be addressed by using complementary methods to probe the roles of H2-K^b and H2-D^b in the brain. To this end, I next used an *in vivo* viral-mediated RNA interference approach to gain spatial and temporal control over the abrogation of H2-K^b or H2-D^b. I generated lentiviral constructs encoding either shRNA sequences targeting H2-K^b mRNA (sh-K), H2-D^b mRNA (sh-D) or targeting luciferase as a control (sh-control) (**Figure 4A**). 2 to 2.5 month old WT mice were bilaterally stereotaxically injected into the hippocampal dentate gyrus (DG) with sh-control, sh-K, or sh-D (**Figure 4B**) and behavioral testing was started when animals were between 3 to 3.5 months of age. Spatial distribution of the lentivirus in the hippocampus was verified by lentivirally-encoded GFP (LV-GFP) localization in fixed brain tissue (**Figure 4C**). Efficacy of shRNA knockdown was validated in mouse neuro2A cells: Western blot analysis confirmed knockdown of H2-K^b protein (**Figure 4D,E**) versus control shRNA, and due to lack of a specific antibody, H2-D^b knockdown was confirmed by qPCR (**Figure 4F**).

Local and temporally controlled knockdown of neither H2-K^b nor H2-D^b in the adult DG caused differences in open field behavior, with all groups displaying similar

total distance travelled and center time (**Figure 5A,B**). In the elevated zero maze test for anxiety, sh-D-injected mice appeared to spend more time in the open arm, though the variance was high and this result was not statistically significant (**Figure 5C**). With similar baseline measures of activity confirmed, I proceeded to test if acute knockdown of H2-K^b or H2-D^b in the DG would specifically affect hippocampal-dependent learning and memory in the RAWM and contextual fear conditioning paradigms.

Adult mice with a spatiotemporally specific knockdown of H2-K^b in the DG did not exhibit differences in errors made compared to control-injected animals in either learning or testing days of the RAWM (**Figure 6A**), suggesting hippocampal H2-K^b is not necessary for intact spatial memory. In contrast to H2-D^b knockout mice, which had severely compromised spatial memory, temporally controlled knockdown of H2-D^b in the DG caused significant improvements in the RAWM, with a notable decrease in errors occurring during testing on Day 2 (**Figure 6B**). These results highlight the ability of one molecule, H2-D^b, to be detrimental for learning and memory when completely abrogated throughout development but to become beneficial when partially knocked down acutely in an adult mouse, implying that timing or degree of the manipulation is important. Despite robust differences in RAWM, neither sh-K nor sh-D-injected mice exhibited differences in baseline, contextual, or cued freezing behaviors compared to control-injected mice in fear conditioning (**Figure 7A-C**).

Altogether, these results clearly reveal a role for H2-D^b in the regulation of locomotion, anxiety behavior, and hippocampus-dependent memory. Interestingly, while complete loss of H2-D^b during development is detrimental for spatial learning and memory, an acute knockdown specifically in the DG of an otherwise healthy, young

mouse considerably improves its hippocampal-dependent learning. Contrarily, H2-K^b appears not to be necessary for normal locomotion, anxiety behavior, or spatial learning in the RAWM as $K^{-/-}$ mice behaved similarly to WT mice in all tasks except associative fear conditioning. Acute knockdown of H2-K^b elicited no differences in spatial or contextual association memory. Whether or not these differences imply differential distributions, mechanisms, or cell types involved between H2-K^b and H2-D^b remain to be further elucidated.

Although spatiotemporal resolution was gained by using an acute RNA interference approach, obtaining cell-type specificity of knockdown was not achievable with a lentiviral-mediated shRNA approach. To complement the genetic and lentiviral approaches, we collaborated with Applied StemCell, Inc. to generate conditional H2-D^b knockout mice in order to study how manipulation of the molecule on specific brain cell types will alter cognitive behaviors in future studies.

Discussion

Here I demonstrate that the presence of H2-D^b throughout development is required for normal locomotor activity, normal anxiety-related behavior, and spatial and contextual memory. These findings recapitulate the results of a previous study (Nelson et al., 2013) revealing hippocampus-dependent memory impairments in $\beta2m^{-/-}Tap1^{-/-}$ mice, but importantly, further reveal the specificity of one individual MHC I molecule in regulating hippocampal cognition. Intriguingly, though full-body, constitutive $D^{-/-}$ mice display marked behavioral abnormalities, acute knockdown of H2-D^b localized to the

hippocampus results in improved spatial learning and memory compared to WT baseline levels.

Previously, the neuroimmune field has made use of broad genetic models such as the $\beta2m^{-/-}$ $Tap1^{-/-}$ double knockout mouse to study the role of MHC I molecules as a family in CNS function. However, the conclusions drawn about the necessity of MHC I molecules in maintaining synaptic development and plasticity are indirect because β2m and Tap1 are needed only for stable expression of MHC I at the cell surface. This model also indiscriminately decreases surface expression of the up to 50 MHC I proteins found in the murine genome. To provide insights into specificity, subsequent studies have utilized mice with genetic deletions of just the two classical MHC I molecules, $H2\text{-}K^b$ and $H2\text{-}D^b$ ($KD^{-/-}$). These studies observed plasticity abnormalities in the form of expanded retinogeniculate projections, increased ocular dominance plasticity, extensive cortical connectivity, increased synapse density, and lack of LTD in $KD^{-/-}$ mice (Adelson et al., 2016; Datwani et al., 2009; Dixon-Salazar et al., 2014; Lee et al., 2014). *In situ* hybridization experiments have shown that both $H2\text{-}K^b$ and $H2\text{-}D^b$ transcripts are present in many regions of the brain and that their spatial expression patterns parallel each other, though the signal of $H2\text{-}K^b$ mRNA is much lower (Huh et al., 2000). This has led to a general assumption that $H2\text{-}K^b$ and $H2\text{-}D^b$ perform analogous functions in the CNS. Recently, Lee *et al*. showed that the neuronal reintroduction of just $H2\text{-}D^b$ in $KD^{-/-}$ mice was sufficient to restore synapse elimination and LTD deficits at retinogeniculate synapses back to WT levels (Lee et al., 2014). This result was the first time the role of an individual MHC I protein in the CNS was discerned, though the sufficiency of $H2\text{-}K^b$

or other MHC I molecules were not tested, nor was the role of neuronal H2-D^b in cognitive function.

The presence of H2-D^b, through its modulation of the calcium-permeability of AMPA receptors, appears to be necessary for maintaining LTD at synapses in the retinogeniculate system (Lee et al., 2014). H2-D^b is also highly expressed in the hippocampus, a brain structure of immense neuroplastic potential. Taken along with the findings showing increased hippocampal LTP and absent LTD in $\beta2m^{-/-}Tap1^{-/-}$ mice, it stands to reason that H2-D^b molecules may similarly regulate LTP and LTD mechanisms with consequences in behavioral output. Synaptic plasticity in the hippocampus is widely thought to be a candidate mechanism for certain types of long-term memory, including those of spatial and contextual nature. My study elucidates for the first time a developmental necessity of specifically H2-D^b in not only hippocampus-dependent learning and memory but also in locomotor and anxiety-related behaviors. Because the general discourse of the literature suggests that plastic changes in the brain support improved cognitive functions, one might expect MHC I-deficient mice, which show augmented hippocampal LTP, to exhibit enhancements in relevant learning and memory tasks. Additionally, there is direct evidence that mice lacking both H2-K^b and H2-D^b have enhanced motor learning on the rotarod due to altered cerebellar plasticity (McConnell et al., 2009b). I found instead that $D^{-/-}$ mice were impaired in both hippocampus-dependent memory tasks (consistent with Nelson et al., 2013), suggesting that presence of H2-D^b during development is necessary in preserving hippocampal cognition. MHC I molecules are detected in the prenatal and postnatal developing nervous system and play critical roles in the initial pruning and wiring of the

brain (Chacon and Boulanger, 2013; Glynn et al., 2011). Disruptions in the precise regulation of synapse elimination are a hallmark of many neurodevelopmental disorders such as autism spectrum disorders, Fragile X syndrome, and epilepsy (Chu et al., 2010; Comery et al., 1997; Hutsler and Zhang, 2010). Brain tissue from human patients or mouse models of these pathologies display increased synaptic density and impaired pruning, and interestingly, there is a high rate of co-diagnosis of these disorders with cognitive dysfunction (van Rijckevorsel, 2006; Zoghbi and Bear, 2012). My studies reveal that the presence of H2-D^b is required for cognitive function, possibly in part due to its mediation of activity-dependent neural wiring in early brain development. Without guidance of post-natal synaptic pruning by MHC I, exceedingly dense hippocampal circuits may lead to lifelong impairments in learning and memory.

It is also plausible that increased LTP and nonexistent LTD may lead to a saturation in hippocampal circuits, occluding further plastic changes required to facilitate learning or the retrieval of memories. It has been shown that animals receiving high-frequency stimulation in the perforant pathway of the hippocampus failed to learn a spatial water maze task if no further LTP could be induced in the pathway (Moser, 1998). Indeed, there are several examples where mice with genetic mutations (deletion of post-synaptic density 95 or Fragile mental retardation 2) that cause enhancements in LTP show learning deficits in the water maze and fear conditioning tasks (Gu et al., 2002; Migaud et al., 1998). It is conceivable that in $D^{-/-}$ mice, behavioral training can cause too many synapses within the network to become strongly potentiated. With LTD absent, not enough synapses become depressed which manifests as compromised information storage and recall capacity.

Though I believe the cognitive phenotypes are likely a direct consequence of lack of H2-D^b in the CNS, there is the possibility that they are an indirect result of a compromised immune system in the genetic knockout models. Initial characterizations found peripheral $CD8^+$ T cells to be substantially reduced in $K^{-/-}$ mice (30-50%) and only slightly limited in $D^{-/-}$ mice (10-20%) (Perarnau et al., 1999). That fact that $K^{-/-}$ mice exhibit near-wild type levels of cognition despite a more altered peripheral immune profile lends support to the idea that CNS expression of MHC I accounts for the changes in cognition. Secondarily, Wolf et al. have also addressed the peripheral immune contribution to spatial learning; it was found that systemic depletion of $CD4^+$ T cells impaired memory in the water maze, whereas systemic depletion of $CD8^+$ T cells did not affect this ability (Wolf et al., 2009). $K^{-/-}$ and $D^{-/-}$ mice do not have impairments in $CD4^+$ T cell numbers; rather, they are increased in compensation for the loss of $CD8^+$ cells (Perarnau et al., 1999). Given these considerations, peripheral immunological deficits are unlikely to account for the impaired cognition seen in H2-D^b-deficient mice.

To directly address hippocampal contribution of H2-K^b and H2-D^b in cognition without effects of developmental compensation or systemic contribution in a constitutive knockout model, I made use of an *in vivo* RNAi strategy. Adult WT mice with a lentivirus-encoded shRNA targeting H2-D^b injected bilaterally into the hippocampi surprisingly displayed cognitive phenotypes opposite of $D^{-/-}$ mice: acute knockdown of H2-D^b in the DG improved learning and memory in the RAWM substantially over control-injected mice. These disparate results may result from the extent of H2-D^b protein abrogation. The shRNA did not completely deplete H2-D^b in the brain as is the case in a genetic mutant model; it is possible that the enhancement of LTP in this case

would not reach saturating levels, allowing for further LTP induction to improve performance during cognitive testing. Temporal control over the knockdown may also be critical. As these are WT mice with an acute injection, there would be no pruning or connectivity deficits from developmental periods that would affect cognitive testing later in life. This demonstration that targeting one molecule for abrogation during adulthood can improve cognitive function has exciting potential for therapeutic implementation in CNS disorders with hippocampal dysfunction. But importantly, the electrophysiological and molecular mechanisms of how acute reduction of H2-D^b alters hippocampal plasticity remain to be elucidated by future studies.

Finally, although major behavioral deficits were observed in $D^{-/-}$ mice, $K^{-/-}$ mice also displayed impairments though only in contextual fear conditioning. Mice lacking H2-K^b have normal locomotor, anxiety, and spatial learning phenotypes despite absence of these molecules throughout development. This may be due to lower levels of CNS expression of H2-K^b or that its localization is not pertinent to the behaviors tested in these studies, or perhaps that H2-K^b individually is not necessary for driving proper synaptic pruning and neurite outgrowth during development. Another intriguing possibility is the ability of H2-K^b to regulate neural stem and progenitor cell (NSPC) function in the hippocampus, a manipulation that has the potential to impact hippocampus-dependent behaviors such as contextual fear conditioning (Saxe et al., 2006). I have discovered that animals lacking H2-K^b show increased NSPC proliferation and neurogenesis in the adult hippocampus (see Chapter 3). Although the majority of studies show that ablation of hippocampal neurogenesis in adult animals impairs subsequent memory formation, a new line of evidence suggests that adult-born neurons

can additionally mediate forgetting by the retrograde disruption of memory circuits (Akers et al., 2014; Frankland et al., 2013). This hypothesis proposes that because immature neurons are more electrically excitable than mature ones (Ge et al., 2007), network homeostasis in the hippocampus may silence some previously existing circuits to compensate for the increased activity of dividing neuronal precursors, leading to degradation of information stored in them. Thus, the increase in hyperactive neuronal precursors observed in $K^{-/-}$ mice throughout adulthood could explain the impairment in contextual association memory.

The disparities in cognitive and neurogenic phenotypes in $K^{-/-}$ and $D^{-/-}$ mice highlight for the first time distinct roles for each classical MHC I molecule in regulating separate CNS processes. Their expression and functions may, importantly, also differ between cell types of the brain. The generation of conditional knockout mice for specific MHC I proteins is our first step towards directly elucidating their roles in neurons, NSPCs, glial, and other cell types in a cell- autonomous manner.

In summary, my results demonstrate the developmental necessity for a single MHC I molecule, H2-D^b, in preserving normal locomotion, anxiety-related behavior, and spatial and contextual memory. Moreover, I show that its acute downregulation in the adult hippocampus can remarkably improve hippocampus-dependent memory.

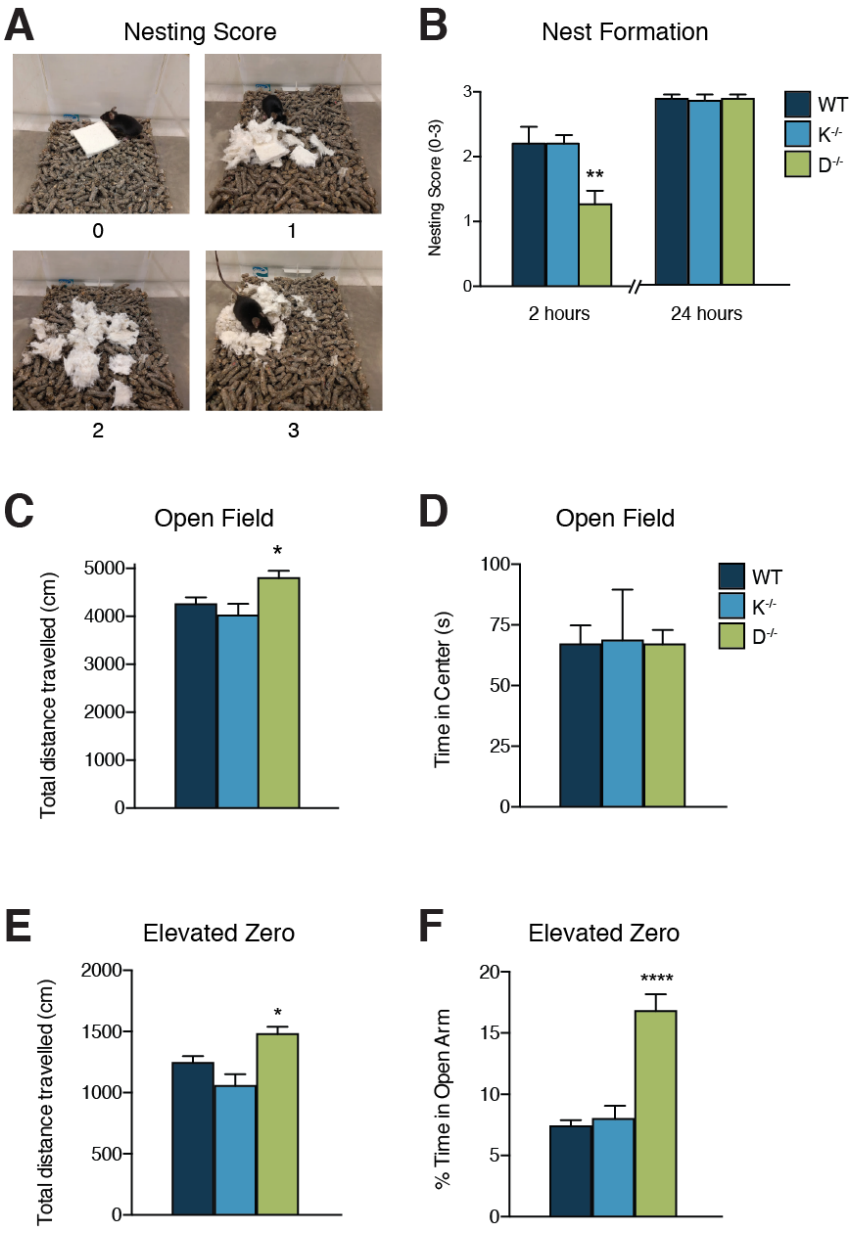

Figure 1. Animals constitutively lacking H2-D^b exhibit deficits in locomotion and anxiety. A four-point scale (A) was used to score 3-month-old mice lacking H2-K^b (K$^{-/-}$), H2-D^b (D$^{-/-}$), or WT mice for nest formation 2 and 24 hours after single housing (B). Locomotor activity (C), as measured by total distance traveled, and time spent in center (D) were measured in WT, K$^{-/-}$, and D$^{-/-}$ mice allowed to freely explore an open field for 10 minutes. Overall activity level (E), measured by total distance travelled, as well as percent of time spent in open arms (F) during 10 minutes on the elevated zero maze were tested in WT, K$^{-/-}$, and D$^{-/-}$ mice**.** WT n= 15, K$^{-/-}$ n= 10, D$^{-/-}$ n= 15. All data are represented as mean ± SEM; *p<0.05, **p<0.01, ****p<0.0001. One-way ANOVA with Dunnett's *post hoc* test.

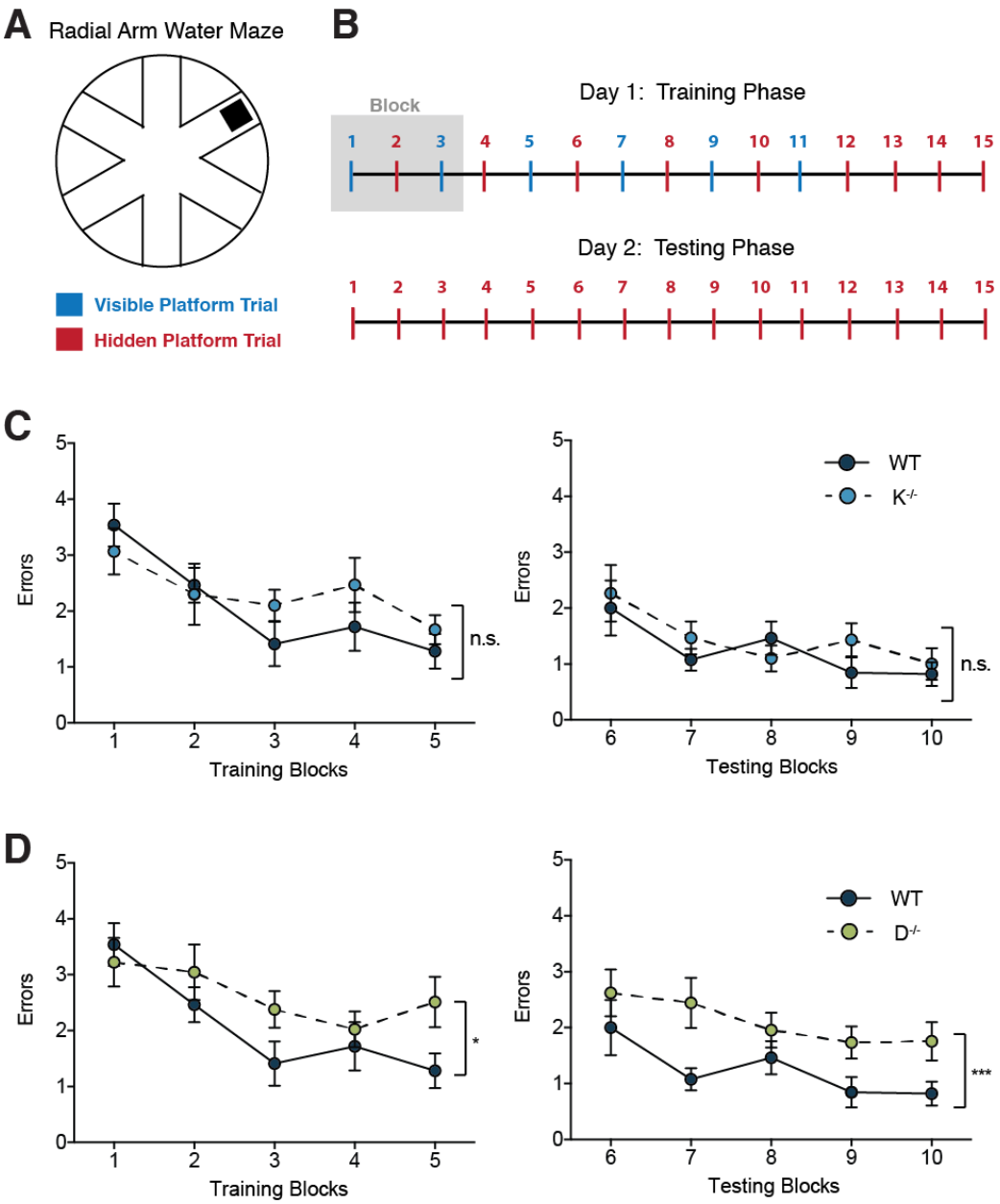

Figure 2. $D^{-/-}$ mice, but not $K^{-/-}$ mice, exhibit deficits in spatial learning and memory. 3-month-old WT, $K^{-/-}$, and $D^{-/-}$ mice were tested for learning and memory in a two-day paradigm using a radial arm water maze with six arms (A). Day 1 consists of 12 training trials where a visible and hidden escape platform is alternated, followed by 3 hidden platform trials. Day 2 consists of 15 testing trials in which only a hidden platform is used (B). For each mouse, errors made in three consecutive trials were averaged (3 trials = 1 block). $K^{-/-}$ mice exhibited no differences compared with WT mice in learning during training blocks or memory during testing blocks (C), whereas $D^{-/-}$ mice were impaired both in learning and memory of the task (D). WT n= 15, $K^{-/-}$ n= 10, $D^{-/-}$ n= 15. All data are represented as mean ± SEM; *p<0.05, ***p<0.0001; n.s., not significant. Two-way repeated measures ANOVA.

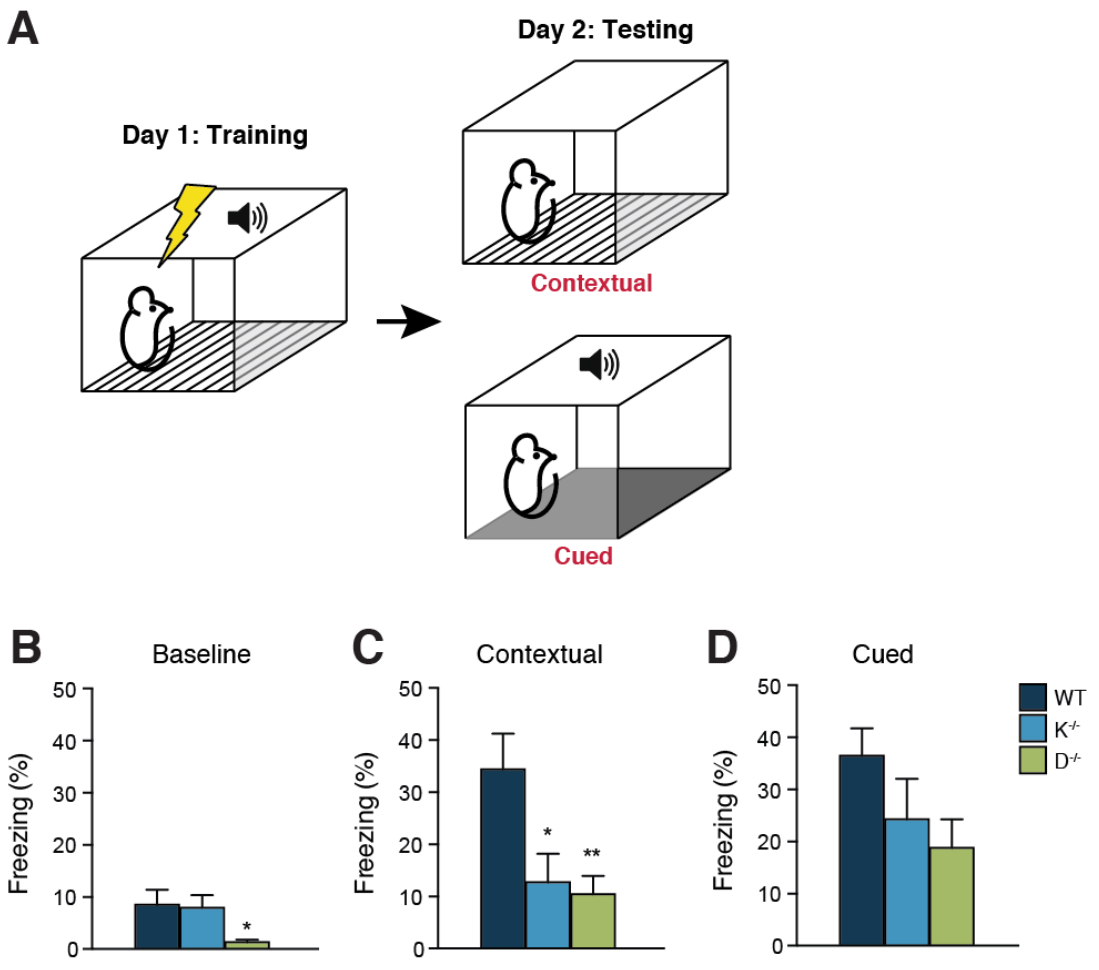

Figure 3. $K^{-/-}$ and $D^{-/-}$ mice have impaired associative memory in contextual, but not cued, fear conditioning. A two-day fear conditioning protocol was used to test 3-month-old WT, $K^{-/-}$, and $D^{-/-}$ mice in hippocampal-dependent (contextual) and independent (cued) forms of associative memory. On Day 1, each mouse was placed in a fear-conditioning chamber and allowed to explore for 2 min (baseline) before receiving two pairings of a tone and foot shock. On Day 2, each mouse was first placed in the same chamber (with no administration of a tone or foot shock) and percent time spent freezing during the first 30 seconds represents contextual memory. One hour later, mice were placed in a new context containing a different odor, floor, and walls. Animals were allowed to explore for 2 min before being re-exposed to the tone; percent time spent freezing during the subsequent 30 seconds represents cued memory (A). Baseline freezing (B), net contextual freezing (contextual minus baseline)(C), and net cued freezing (cued minus baseline)(D) were measured for WT, $K^{-/-}$, and $D^{-/-}$ mice. WT n= 15, $K^{-/-}$ n= 10, $D^{-/-}$ n= 15. All data are represented as mean ± SEM; *p<0.05, **p<0.01. One-way ANOVA with Dunnett's *post hoc* test.

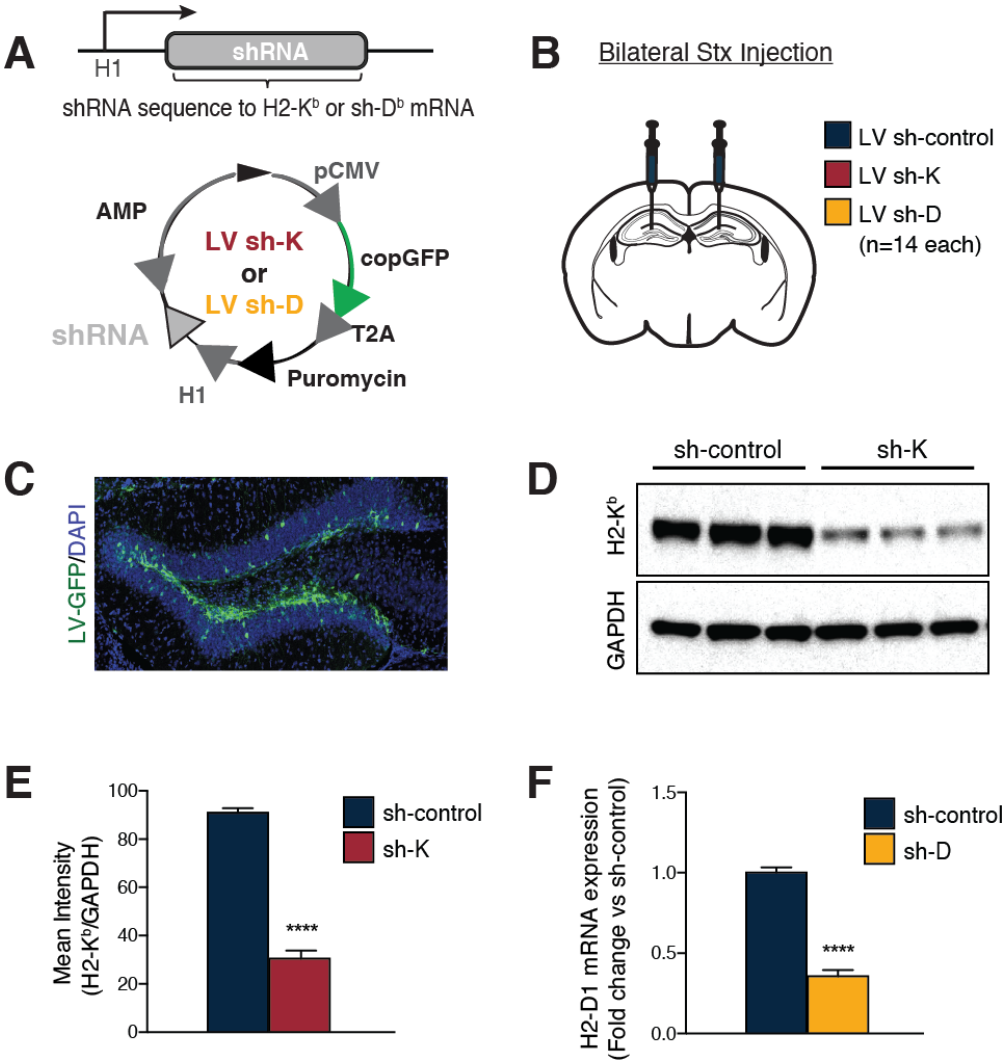

Figure 4. Design and validation of lentivirus-mediated shRNA strategy targeting H2-K^b and H2-D^b *in vivo*. Schematic illustrating vector map of lentiviruses encoding H1 promoter- driven short hairpin RNAs (shRNAs) targeting H2-K^b (sh-K), H2-D^b (sh-D), or luciferase as a control (sh-control)(A). Bilateral stereotaxic injections into the dentate gyrus of the hippocampus were carried out in 2-month-old WT mice (B). Lentivirus-encoded GFP (LV-GFP) could be visualized to confirm infection with the virus in the hippocampus six weeks after injection (C). A mouse neuro2a cell-line was used to confirm knockdown of H2-K^b and H2-D^b via transfection with the respective lentiviral vectors. H2-K^b protein knockdown was confirmed by Western blot analysis (D-E) and H2-D^b mRNA knockdown was confirmed by real-time PCR (F). *In vitro* experiments were carried out in triplicate, and all data are represented as mean ± SEM; ****p<0.0001. Student's t-test.

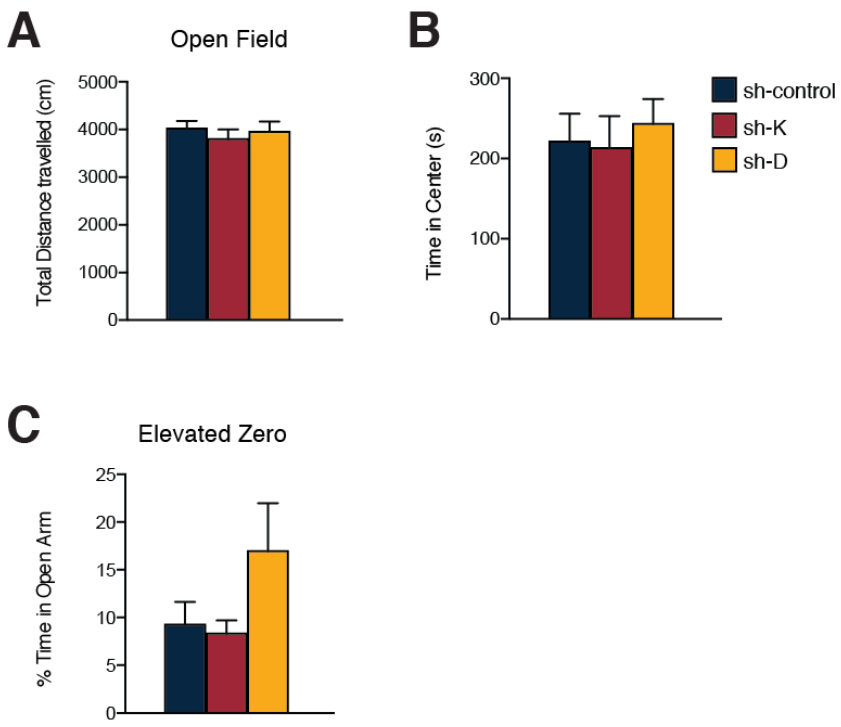

Figure 5. Acute knockdown of H2-K^b or H2-D^b in the hippocampal dentate gyrus do not cause changes in locomotion or anxiety behaviors. Overall activity level (A), measured by total distance travelled, as well as percent of time spent in center (B) during 10 minutes in the open field were tested in 3-month-old WT mice bilaterally injected in the dentate gyrus with shRNA targeting H2-K^b (sh-K), H2-D^b (sh-D) or luciferase (sh-control) as a control**.** n=14 per group and all data are represented as mean ± SEM. No significant differences, one-way ANOVA with Dunnett's *post hoc* test.

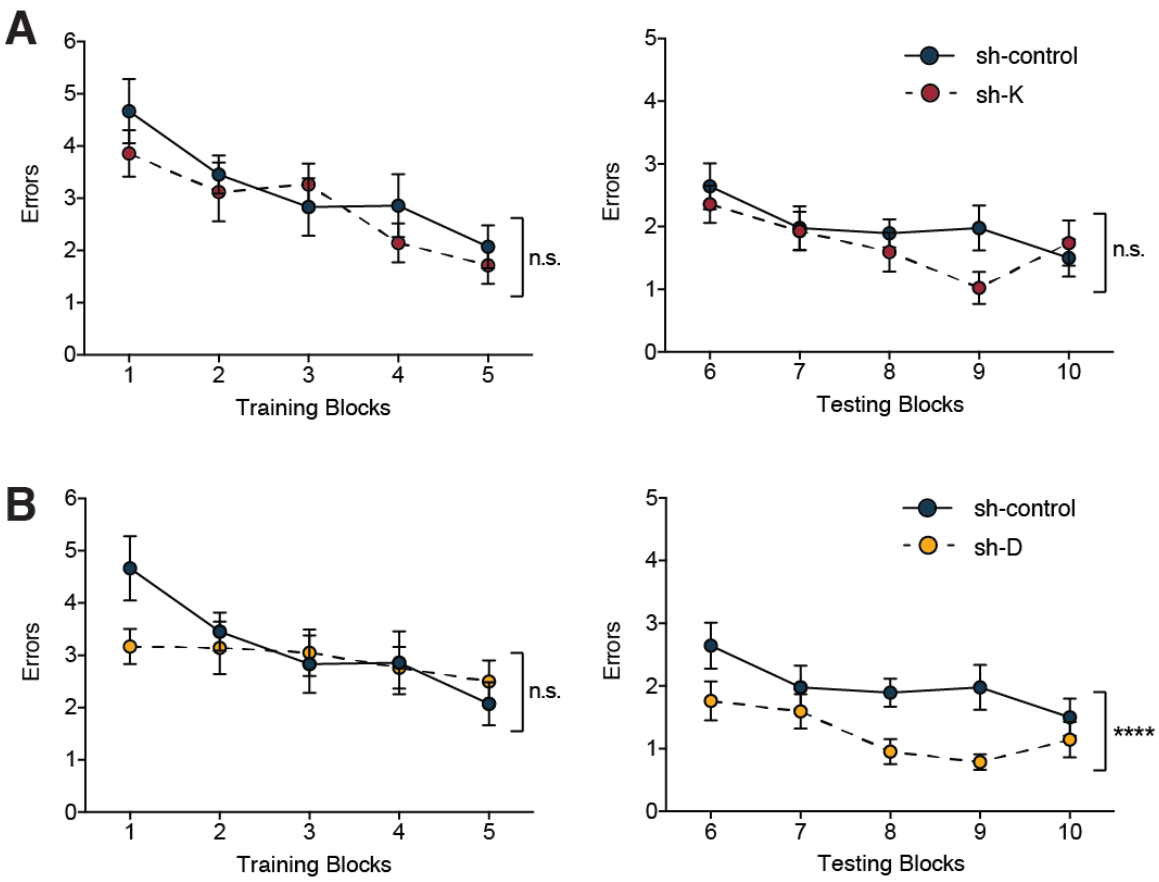

Figure 6. Acute knockdown of H2-D^b in the hippocampal dentate gyrus improves spatial memory. 3-month-old WT mice bilaterally injected with sh-K into the dentate gyrus exhibited no differences compared with sh-control-injected mice in learning during training blocks or memory during testing blocks (A). sh-D-injected mice exhibited improved spatial memory during the testing blocks (B). n= 14 per group, and all data are represented as mean ± SEM. ****p<0.0001; n.s., not significant. Two-way repeated measures ANOVA.

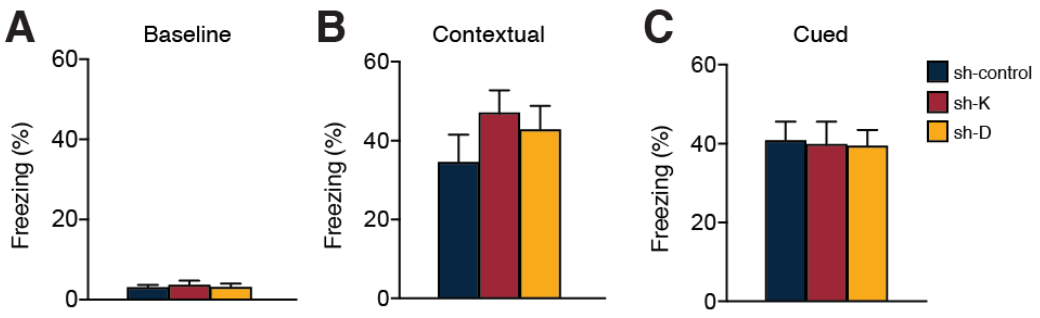

Figure 7. Acute knockdown of H2-K^b or H2-D^b in the hippocampal dentate gyrus do not cause changes in contextual or cued fear conditioning. No differences were detected in baseline freezing (A), net contextual freezing (B), or net cued freezing (C) in 3-month-old WT mice bilaterally injected in the dentate gyrus with sh-K, sh-D, or sh-control**.** n=14 per group and all data are represented as mean ± SEM. No significant differences, one-way ANOVA with Dunnett's *post hoc* test.

Chapter 5

Concluding Remarks and Future Directions

This chapter summarizes my findings on the newly uncovered roles of two specific MHC I molecules, H2-K^b and H2-D^b, and the distinct function each plays in the central nervous system (CNS). In this dissertation, I show that H2-K^b molecules are involved in the regulation of neural stem and progenitor cell (NSPC) function and hippocampal neurogenesis, and identify the developmental necessity of H2-D^b molecules in maintaining cognitive function throughout life. Additionally, I show that acute abrogation of H2-K^b and H2-D^b can increase neurogenesis and improve learning and memory, respectively, in adult animals. Combined with the future investigations I will raise in this chapter, these results may have potential therapeutic applications in regenerative medicine and the treatment of neurological disorders that present with CNS dysfunction. In the following sections, I will present additional preliminary data that apply my findings towards combatting the neurogenic decline seen in aging, as well as address the broader implications of my research in other pathological conditions.

Classical MHC I molecule, H2-K^b, is a negative regulator of NSPC proliferation and hippocampal neurogenesis

Previous studies have described the inhibitory effects that MHC I molecules have on regulating neuronal processes such as neurite outgrowth, synaptogenesis, hippocampal and cerebellar synaptic plasticity, and activity-dependent refinement of developing visual system neurons (Glynn et al., 2011; Goddard et al., 2007; Huh et al., 2000; McConnell et al., 2009b). My study provides the first evidence that one specific

MHC I molecule, H2-K^b, is critical in the regulation of non-neuronal cell types in the brain: NSPCs in the hippocampus. Using genetic mouse models as well as primary NSPC cultures, I show that deletion of H2-K^b (but not H2-D^b) molecules causes increased proliferation and neurogenesis in the DG *in vivo* as well as increased cell division in NSPCs cultured *in vitro*. As primary NSPC cultures are devoid of any influence from other cell types of the niche or systemic environment, the inhibitory effects of endogenous H2-K^b molecules appear to be at the progenitor level. Another line of support comes from my analysis of a publically available dataset describing the transcriptomic dynamics of early phase adult hippocampal neurogenesis at the single-cell level (Shin et al., 2015). In this early neurogenic trajectory where quiescent stem cells transition into activated progenitors, the H2-K^b expression trend line appears dynamic, decreasing as the cells become more proliferative; by contrast, H2-D^b expression remains static. Using the same data set, I also found that NSCs and their immediate progeny are more likely to be non-cycling when enriched for *H2-K1* (the gene encoding H2-K^b), reiterating the inhibitory influence of endogenous H2-K^b.

I then recapitulated these results with an acute knockdown of H2-K^b specifically in the DG of the hippocampus where NSPCs reside. Lentivirus-mediated knockdown of H2-K^b enhanced hippocampal neurogenesis in the adult mouse in a spatially and temporally specific manner. However, to directly investigate the cell-autonomous effects of H2-K^b on NSPCs, the field would benefit from the generation of a conditional knockout model. Crossing these mice with NSPC-specific promoters will allow us to distinguish stem cell effects from contribution of nearby cells *in vivo*.

Although the exact contribution of adult hippocampal neurogenesis to cognitive function remains elusive, a number of studies have consistently shown the causal relationship between neurogenesis and hippocampus-dependent types of learning memory (reviewed in Costa et al., 2015 and Oomen et al., 2014). Future studies testing mice with H2-K^b conditionally deleted from NSPCs in cognitive tasks will help determine the functional implications of a knockout-induced increase in newborn neurons. As hippocampal neurogenesis in animal models is impaired in physiological aging as well as disease conditions associated with cognitive impairment, depression, and anxiety, elucidating novel targets that govern the neurogenic process carries intriguing therapeutic potential.

H2-K^b regulates NSPC proliferation through modulation of growth factor receptor signaling

To gain mechanistic insight into how MHC I molecules regulate NSPC proliferation, I profiled gene expression in primary NSPCs derived from $K^{-/-}$ and WT mice using RNA-sequencing. Prior to this, there has been no unbiased transcriptomic analysis of any CNS cell type from MHC I-deficient mice. I performed pathway enrichment analysis for genes differentially regulated in $K^{-/-}$ NSPCs, and found an upregulation of several growth factor receptor-associated pathways, among these epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR) signaling. Interestingly, receptors (such as insulin receptors and EGFRs) and other proteins have been shown to associate in *cis* with MHC I molecules, forming macromolecular complexes on the same cell surface (Arosa et al., 2007; Dixon-Salazar

et al., 2014; Fehlmann et al., 1985; Schreiber et al., 1984). Given that these interactions can affect receptor signaling and internalization, I hypothesized that H2-K^b may be binding to receptors identified by our RNA sequencing experiment and potentially affecting their function. Coimmunoprecipitation experiments indeed showed that H2-K^b binds to specific FGFRs. Further experiments are required to establish how the formation of macromolecular complexes with H2-K^b affects the trafficking dynamics, internalization, and signaling capacity of growth factor receptors on NSPCs. More broadly, techniques such as protein mass spectrometry can be used to conduct an unbiased search for H2-K^b binding partners on neural or other stem cell types. Since all nucleated cells express MHC I molecules, these findings demonstrating their ability to modulate receptor signaling to affect proliferation may have biological implications for any healthy or pathological cell type with the ability to divide.

Spatial and temporally-specific abrogation of classical MHC I molecule, H2-D^b, can improve hippocampal-dependent cognitive function

While H2-D^b molecules appear to play little role in NSPCs, the second body of work in this dissertation illuminates their function in hippocampus-dependent behavior. Previous studies have discovered MHC I molecules to be unexpected regulators of synaptic plasticity in the hippocampus, as animals deficient for cell surface expression of these proteins display enhanced long-term potentiation (LTP) and absent long-term depression (LTD) (Fourgeaud et al., 2010; Huh et al., 2000). LTP and LTD are two mechanisms that underlie experience-dependent development, learning and memory, and their impairments are prominent in neurological disorders. Using H2-K^b ($K^{-/-}$) and

H2-D^{b} (D$^{-/-}$) genetic knockout mice, I found that the presence of H2-D^{b} throughout development is required for mediating normal locomotor activity, anxiety-related behavior, and spatial and contextual memory. These findings reveal the specificity of one individual MHC I molecule H2-D^{b} (of the more than 50 in the mouse genome) in regulating cognition, with the effects of the knockout potentially attributable to changes within and outside of the hippocampus. Intriguingly, though full-body, constitutive D$^{-/-}$ mice display marked behavioral abnormalities, the second main result of my studies demonstrates that acute knockdown of H2-D^{b} localized by virally encoded shRNA injection to the hippocampus can elicit striking enhancements in spatial learning and memory in adult mice.

The neurogenic phenotypes observed in K$^{-/-}$ mice and cognitive phenotypes observed in D$^{-/-}$ mice highlight for the first time distinct roles for each classical MHC I molecule in regulating separate CNS processes. One cause of these differences could be that their expression levels, binding or signaling partners, or even functions may differ between cell types of the brain. The generation of conditional knockout mice for specific MHC I proteins is our first step towards directly elucidating their roles in neurons, NSPCs, glial, and other cell types in a cell- autonomous manner. Future studies will make use of the conditional H2-D^{b} knockout mouse we have generated to pinpoint whether the cognitive differences I observed were due only to neuronal H2-D^{b} expression, or also contribution by other cell types. Forthcoming RNA sequencing of the hippocampi of D$^{-/-}$ mice as well as mice with an acute knockdown of H2-D^{b} can complement my functional experiments by providing mechanistic insights into how this

molecule might regulate genes and pathways involved in hippocampal plasticity and associated cognitive function.

MHC I as a potential therapeutic target in the aging CNS

In the adult brain, NSCs in the dentate gyrus (DG) region of the hippocampus have the capacity for proliferation and differentiation into excitatory granule cells that integrate into hippocampal circuitry (Christian et al., 2014). Though ongoing, the process of neurogenesis exhibits significant age-related decline across species (Boldrini et al., 2018; Gould et al., 1999; Kuhn et al., 1996; Maslov et al., 2004; Sorrells et al., 2018; Spalding et al., 2013), and stems from cell-intrinsic alterations, as well as changes to the neurogenic niche and systemic environment (Fan et al., 2017; Faigle and Song, 2013). While mechanisms underlying this age-related decline remain to be unraveled, identification of specific molecules regulating NSC function with age may allow us to develop means to maintain innate regenerative capacity well beyond developmental periods.

My discovery that MHC I molecules negatively regulate hippocampal neurogenesis and cognitive function is particularly relevant because MHC I pathways and components, such as β2-microglobulin, have been shown to increase in the aging brain (Smith et al., 2015; Starkey et al., 2012). I built upon these findings by characterizing expression of classical MHCI molecules specifically, H2-K^b and H2-D^b, in the hippocampus of adult and aged WT mice by quantitative real time PCR (qPCR) and Western blot analysis. I observed an increase in H2-K^b and H2-D^b mRNA and H2-K^b protein in aged (18 months) compared to adult (3 months) mice (**Figure 1A,B**). As my

results described in Chapter 3 and Chapter 4 implicate MHC I molecules in adult neurogenesis and hippocampal-dependent cognitive processes, this observation posits these proteins as targets for abrogation in old age.

To date, I have explored the potential benefit of decreasing surface expression of global MHC I molecules on age-related regenerative decline during normal physiological aging. Transporter associated with antigen processing 1 (Tap1) is critical for trafficking of MHCI molecules, with absence of Tap1 resulting in reduced stable expression of classical MHCI molecules at the cell surface (Kaer et al., 1992). I examined changes in adult hippocampal neurogenesis in young adult and aged Tap1 knockout ($Tap1^{-/-}$) and WT control mice by immunohistochemical analysis. Cell proliferation was assessed by short-term BrdU-labeling of dividing cells. In young adult animals, reduced MHC I surface expression had no effect on the number of BrdU-positive proliferating cells or adult Dcx-positive neuroblasts in the DG (data not shown), consistent with previous reports (Laguna Goya et al., 2010; Smith et al., 2015). However, aged $Tap1^{-/-}$ mice had higher numbers of BrdU-positive and Dcx-positive cells compared to WT controls (**Figure 1C,D**). These preliminary data indicate that reduced expression of surface MHC I molecules has the potential to offset age-related regenerative decline. More specific studies abrogating H2-K^b specifically or localizing knockdown solely to NSCs in aged animals will be needed to directly explore this possibility.

Broad implications of MHC I in disease

Schizophrenia

As we continue to unravel the immune contributions to CNS function, researchers are exploring neuroimmune-based mechanisms in the etiology, pathophysiology, and treatment of complex brain disorders. One such example is the increasing evidence pointing towards immune dysregulation in schizophrenia, a disabling disorder affecting 1% of the worldwide population and characterized by dissociation with reality and emotional and cognitive problems. Indeed, genetic aberrations on chromosome 6—which is densely comprised of immune genes— have been consistently linked with schizophrenia. More specifically, the MHC I has been independently identified as a region of association in three large-scale genome-wide association studies in schizophrenia (International Schizophrenia Consortium et al., 2009; Shi et al., 2009; Stefansson et al., 2009). These types of studies showing that single nucleotide polymorphisms (SNPs) of schizophrenia tend to span the MHC region are correlational; hence, more research is needed to examine the functional outcomes of these SNPs, and specific manipulations of MHC I in models of schizophrenia would likewise be illuminating.

Reviewed in Chapter 1, there has been ample evidence for the direct role MHC I molecules play in the shaping and refinement of the developing CNS as well as synaptic plasticity in the adult hippocampus and cerebellum. Interestingly, the phenotypes observed in mice deficient of MHC I molecules are reminiscent of the neural deregulations of patients diagnosed with schizophrenia, suggesting their involvement in the etiology or pathophysiology of the disease. MHC I molecules inhibit synaptic density in the cortex throughout development; likewise in schizophrenia postmortem tissue, a decrease in synapse density and markers for dendritic spines are decreased (Stanley et

al., 1995). Another function MHC I has in the brain is the regulation of synaptic transmission and plasticity. MHC I molecules repress NMDA-type glutamate receptors, resulting in limited AMPA receptor trafficking and impaired activity-dependent changes in synaptic strength (Fourgeaud et al., 2010). NMDA receptor hypofunction is observed in schizophrenia and thought to cause impairments in synaptic plasticity (Gonzalez-Burgos and Lewis, 2012; Snyder and Gao, 2013); could these deficits be cause, in part, by abnormal MHC I expression in neurons of susceptible individuals?

I show that, in addition to their involvement in CNS structure and connectivity, a specific MHC I molecule H2-D^b can also influence hippocampal-dependent cognitive function. Although the structural and electrophysiology roles of MHC I developmentally may be more likely contributors to disease etiology, these findings relating to their role in cognition may be important in providing symptomatic relief, as impaired episodic memory has been directly associated to schizophrenia-related SNPs in MHC I (Walters et al., 2013).

The roles of MHC I in synaptic pruning, cognitive function, and even neurogenesis, could underlie some of the symptoms seen in neuropsychiatric disorders such as schizophrenia. It remains for future studies to use inducible, conditional transgenic mice to gain a more comprehensive understanding of the function of MHC I in various cell types and time points, and how this relates to the pathophysiology of disease.

Cancer

Immunotherapy efforts, such as adoptive T-cell therapy, depend on MHC I-mediated recognition of tumor-associated antigens to stimulate tumor rejection. However, downregulation of MHC I molecules is observed in 40-90% of all human tumors and a hallmark across a variety of adult and pediatric cancers (Haworth et al., 2015). In many cases spanning neuroblastomas, Ewing scarcomas, colorectal, endometrial, and lung carcinomas, low MHC I expression predicts poor patient prognosis (Facoetti et al., 2005; Yeung et al., 2013). Classical cancer immunology attributes this unfavorable survival to the capability of tumors to evade both endogenous and therapeutic immune surveillance, as malignant cells can no longer be recognized by cytotoxic T lymphocytes (CTLs). However, novel evidence suggests that in addition to mediating cell-to-cell (or *trans)* interactions with T-cell receptors on immune cells, surface MHC I molecules also have the capability to interact in *cis* with other receptors on its own cell surface (Arosa et al., 2007). Specifically, MHC I molecules have been shown to form *cis*-associations with molecules such as EGFRs and insulin receptors (Dixon-Salazar et al., 2014; Schreiber et al., 1984), and has been proposed to inhibit signaling and modulate internalization. Thus, the loss of MHC I may exacerbate cancer progression by relieving *cis*-inhibitory-interactions with growth factor receptors on the surface of cancer cells, thereby promoting cell proliferation.

Chapter 3 presents data supporting this hypothesis, using a cell type normally protected from peripheral immune influence. Transcriptomic profiling of primary hippocampal NSCs lacking the classical MHC I molecule H2-K^b revealed a significant increase in the expression of genes associated with several growth factor receptor-associated pathways, such as PDGF, EGFR, and FGFR signaling. Of note,

overexpression or hyperactivity of these growth factor pathways have been clearly implicated in driving malignant growth (Heldin, 2013; Katoh and Nakagama, 2014; Normanno et al., 2006). Accordingly, $K^{-/-}$ NSCs exhibited increased proliferation when cultured in a cell-type specific environment *in vitro.* These data suggest that MHC I inhibits proliferation in a cell-intrinsic manner, possibly by inhibiting growth factor receptor signaling pathways, independent of its canonical immune function. The cell-autonomous effects of MHC I loss on cancer cell proliferation remain to be studied and may be therapeutically co-opted as an additional means of tumor suppression.

In summary, my studies have uncovered the distinct roles of two MHC I molecules which have until now been ascribed analogous functions in the brain. I have demonstrated that H2-K^b negatively regulates hippocampal NSC proliferation, likely through *cis*-inhibitory interactions with growth factor receptors. The discovery of a novel cellular regulator of a fundamental process such as proliferation may have relevance in both healthy and pathologically dividing cells, inside and outside of the brain. I also demonstrate the developmental necessity for H2-D^b in preserving normal locomotion, anxiety-related behavior, and spatial and contextual memory. Excitingly, its acute downregulation in the adult DG can remarkably improve hippocampus-dependent memory. I hope that this work may provide foundations for future advances in the understanding of immune molecules and the non-canonical roles they play in shaping our brains, both in health and in disease.

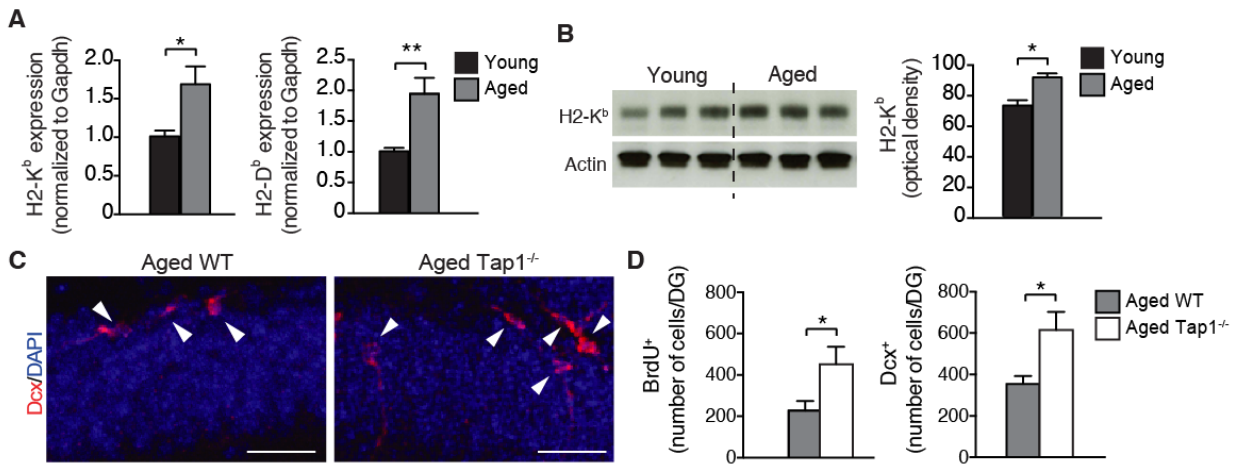

Figure 1. **Reduction in surface expression of global classical MHC I molecules offsets age-related neurogenic decline.** Quantitative RT-PCR of H2-D^b and H2-K^b mRNA from hippocampal lysates of adult (3 months) and aged (18 months) wild type (WT) mice (A). Representative Western blot and quantification of hippocampal lysates probed with anti-H2-K^b and anti-Actin antibodies from young and aged WT mice (B). Representative field of Dcx^+ neuroblasts in the dentate gyrus (DG) of aged (18 months) WT and transporter associated with antigen processing 1 knockout ($Tap1^{-/-}$) mice (C). Quantification $BrdU^+$ proliferating cells and Dcx^+ neuroblasts in the DG of aged WT and $Tap1^{-/-}$ mice (D). Scale bars, 50μm (C). WT n=5, $Tap1^{-/-}$ n=5 for histological analysis; n=3 replicates per group for RT-PCR and Western blot analysis. All data are represented as mean ± SEM; *p<0.05, **p<0.01. Student's t-test (A,B,D).

Chapter 6

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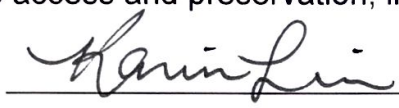
Author Signature  Date 6/11/2018