Regulation of Adult Neurogenesis by the ERK5 MAPK Signaling Pathway and its Functional Implications in Learning and Memory as well as Olfaction

Yung-Wei Pan

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2012

Reading Committee:

Zhengui Xia, Chair

Daniel R. Storm

Horacio de la Iglesia

Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

Regulation of Adult Neurogenesis by the ERK5 MAPK Signaling Pathway and its Functional Implications in Learning and Memory as well as Olfaction

Yung-Wei Pan

Chair of Supervisory Committee:
Professor Zhengui Xia
Departments of Environmental and Occupational Health Sciences and
Molecular and Cellular Biology

Adult neurogenesis is a life-long developmental process occurring in two discrete regions in the adult mammalian brain, the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) along the lateral ventricles. External stimuli including environmental enrichment activities and olfactory stimulation induce adult neurogenesis. Thus, adult neurogenesis may play an important role in cognition and olfaction. Furthermore, increased neurogenesis has been observed after brain injuries such as stroke and DNA damage, promoting the idea that it may be advantageous to recruit endogenous neural stem cells to treat a variety of neurodegenerative diseases. However, despite the capability of the adult brain to continuously produce new neurons, signaling mechanisms regulating this process have not been fully elucidated. Intrinsic differences between the two neurogenic regions including the microenvironment lend additional motivation to identify similarities and differences in regulatory mechanisms of this process. In addition, the functional significance of newborn neurons in the SGZ and SVZ is still unclear.

Signaling molecules currently implicated in regulating adult neurogenesis are expressed in either or both neurogenic regions but are not solely restricted to the SGZ or SVZ. Furthermore, the expression pattern of many of these proteins are found in cells other than adult

neural stem/progenitor cells of the neurogenic regions, implicating a less specific effect on adult neurogenesis per se. We identified ERK5 MAP Kinase as a novel protein specifically expressed along both neurogenic regions in the Type 1 neural stem/progenitor and Type 2 transiently amplifying progenitor or newborn neuron cell populations. This result posits the involvement of the ERK5 signaling pathway in regulating adult neurogenesis. Indeed, we present evidence that ERK5 regulates the terminal differentiation phase of adult-born neurons in the SGZ. Using ERK5 inducible-conditional knockout mice, we also demonstrate the functional significance of adult-born neurons in many hippocampus-dependent learning and memory assays as well as olfactory behavior assays. Together, this dissertation presents a novel body of work identifying the ERK5 signaling pathway as a novel regulatory mechanism of adult neurogenesis; and provides the intriguing possibility of the therapeutic potential of adult-born neurons to combat aging- or neurodegenerative disease-related olfactory and learning and memory deficits.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I am particularly grateful to Zhengui Xia for being an extraordinary mentor taking a chance on a graduate student with no former training or experience in neuroscience. Zhengui's optimism about research and the boundless possibilities that science and discovery affords drives her passion as a professor, scholar, and colleague. Thank you for allowing me to learn and grow in your lab en route to becoming a better scientist able to think holistically about scientific questions and to never shy away from asking the burning questions. Thank you for building my confidence in myself both professionally and personally; I feel extremely lucky to have had you as a mentor during my very impressionable graduate school years.

I wish to thank my committee members, Daniel R. Storm, Robert Hevner, Jonathan Cooper, Horacio de la Iglesia, and Bradley Preston for providing advice and support as I trudged along my thesis work. Thank you for focusing me when I had abstract ideas that I wanted to pursue. The feedback you have provided through the years on my thesis project and professional development has been extensive yet appreciated. I am indebted to Zhengui, Dan, and Horacio for serving as members on my reading committee, and in particular Zhengui and Dan for reading countless drafts of my manuscripts as I progressed through my writing pains as a graduate student.

The many past and present members of the Xia Lab have made the years an experience to remember. Paige Cundiff, Beibei Cai, and Heather Klintworth were my big sisters who fostered and guided me during the early years in lab. Noah Sorscher has been a unique graduate student colleague through the years. Glen Abel has always been the constant voice of guidance in the lab and works tirelessly to ensure things run smoothly. Anna Engstrom, our newest graduate student is one of the most energetic, optimistic, and inquisitive graduate

students I have come across and sincerely hope that I was able to impart some form of knowledge upon her during our short overlap together in the lab. Tan Li, a visiting graduate student from China is a master at diving in head first into new questions and is unafraid of unfamiliar things. Always eager to try something new, he has single-handedly helped me finish an ongoing project in the lab. Former post-docs Lidong Liu and Won-Seok Choi welcomed me into the lab and served as invaluable sources of knowledge. Wenbin Wang, June Zou, and Peter Kim are current post-docs in the lab who have provided valuable advice through the years and truly helped me become a better scientist.

A big thank you to the MCB program for admitting me and giving me a shot at graduate school. The women in the MCB office, Terry Duffey, MaryEllin Robinson, Diane Darling, and Milli Morris, have all been there for me whenever I needed anything.

I have established many new friendships in Seattle from the MCB program, the MBA program, various living situations, and people who I play ball with. Also, my friends from high school and undergrad have all been there for me when I needed them most. There are too many of you to list, but I want to thank you all sincerely for helping me stay sane and for reminding me that there's more to life than the lab.

Lastly, I want to thank my family for supporting me in more ways than they can imagine, always pushing me to be better in all aspects of life. My mom and my sisters, Diana and Chrissy, have all been part of this entire experience. My wife, Hanh, has been the most consistent source of support all these years, and I am truly indebted to her for helping me stay focused when things seemed most gloom.

Dedication

To my wife Hanh, who if not for her pushing me to stay in grad school when I wanted to quit after the first quarter, none of this would be possible. You are often times more invested in my successes than I am, and for that I want to thank you for seeing something in my abilities that I often fail to recognize.

Chapter 1

Introduction

Adult Neurogenesis

Neurogenesis occurs both during the development of the mammalian central nervous system (CNS) and throughout adulthood in discrete regions of the brain (Ming and Song, 2005; Taupin, 2006; Ming and Song, 2011). The genesis of adult-born neurons was not fully accepted despite the first reported evidence in the SGZ of adult rats (Altman and Das, 1965). In fact, it was many years later following the seminal paper on songbird adult neurogenesis that the neurogenesis field came to accept mammalian adult neurogenesis as a physiological phenomenon (Alvarez-Buylla et al., 1988).

Discoveries in the past 10–20 years led to the identification of neural stem cells capable of generating adult-born neurons under normal physiological conditions in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) along the lateral ventricles (Alvarez-Buylla and Lois, 1995; Alvarez-Buylla and Garcia-Verdugo, 2002; Taupin, 2006; Imayoshi et al., 2008; Imayoshi et al., 2009; Ming and Song, 2011). Neurons born from the SVZ migrate along the rostral migratory stream and integrate into the olfactory bulb of rodents, while neurons born in the SGZ integrate locally and establish themselves into the hippocampal circuitry (Gage et al., 1998; Doetsch et al., 1999; Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Deng et al., 2010; Aimone et al., 2011; Ming and Song, 2011). The integration of adult-born neurons in the olfactory bulb suggests a novel role in olfactory behavior (Carleton et al., 2002; Petreanu and Alvarez-Buylla, 2002; Doetsch and Hen, 2005; Whitman and Greer, 2009; Bardy and Pallotto, 2010; Lazarini and Lledo, 2011; Kageyama et al., 2012). Similarly, since adult neurogenesis occurs within the dentate gyrus of the hippocampal formation, this suggests a functional role in learning and memory (Gould et al., 1999; Carlen et al., 2002; Liu et

al., 2003b; Leuner et al., 2004; Meshi et al., 2006; Farioli-Vecchioli et al., 2008; Deng et al., 2010; Erickson et al., 2011).

The production of adult-born neurons has even been proposed to counteract the deleterious effects of aging-related deficits in sensory mechanisms and memory. Furthermore, increased neurogenesis has been observed after brain injuries such as stroke and DNA damage, promoting the idea that it may be advantageous to recruit endogenous neural stem cells to treat a variety of neurodegenerative diseases. However, despite the promising therapeutic potential of adult-born neurons, many questions remain unanswered. Cellular and molecular mechanisms regulating this process are unclear, how adult neurogenesis responds to environmental changes is still being investigated, and the exact functional significance of adult-born neurons in these discrete regions is an area of much debate. Additional evidence suggesting external stimuli including environmental enrichment activities and olfactory stimulation induce adult neurogenesis has been reported (van Praag et al., 1999; Taupin, 2006; Mak et al., 2007; Shapiro et al., 2007). Thus, supporting the hypothesis that adult neurogenesis may play an important role in cognition and olfaction.

Environmental factors influencing adult neurogenesis

As the field of adult neurogenesis has taken shape in recent years, many studies identified increased neurogenesis along either the SVZ or SGZ or both after exposing rodents to enriched environments, permitting free access to running wheels, or exposing rodents to olfactory stimulation (Kempermann et al., 1997a; Nilsson et al., 1999; van Praag et al., 1999; Taupin, 2006; Mak et al., 2007; Shapiro et al., 2007; Hodge et al., 2008). Furthermore, a significant increase in proliferating adult neural stem cells was detected along the SVZ and the SGZ of female mice after exposure to dominant male pheromones, suggesting a role of adult-born neurons in mate selection (Mak et al., 2007). These studies used the thymidine analog, 5-

bromo-2-deoxyuridine (BrdU), to label dividing cells and tracked their existence along the SVZ or SGZ to determine the amount of proliferating or surviving newborn neurons. Aside from stimuli-induced adult neurogenesis in the rodent brain, inflammation due to induced ischemic injury or epileptic seizure have also been shown to play a role promoting adult neurogenesis in rodent models, which suggests a neuronal replacement role of newborn neurons (Ekdahl et al., 2009). Independently, environmental enrichment, running, or olfactory stimulation resulted in significant increases in proliferating cells and newborn neurons; however, controversy in the field exists as to whether these newborn neurons provide a functional role in the adult rodent brain.

Adult neurogenesis has even been implicated in maternal behavior and mate preference in female mice (Mak et al., 2007; Larsen et al., 2008), which has been linked to the increased production of hormones (Shingo et al., 2003; Mak et al., 2007; Larsen et al., 2008). Prolactin levels were increased through gestation day 7 when female mice were mated with fertile or sterile males, which correlated strongly with increased proliferating cells along the SVZ and surviving neurons found in the olfactory bulb (OB) (Shingo et al., 2003). Additionally, presentation of male pheromones led to prolactin-mediated neurogenesis and these results have been recapitulated by administration of exogenous prolactin (Larsen et al., 2008). Lastly, prolactin-mediated neurogenesis has been implicated in female mate preference. Exposure of females to dominant male pheromones resulted in increased neurogenesis, while the effect was not observed when females were exposed to subordinate male pheromones (Mak et al., 2007). Collectively, these results suggest that hormones are involved in stimulating adult neurogenesis and newborn adult neurons play a role in maternal behavior and mate selection.

Adult neurogenesis generally declines with aging; and irregular adult neurogenesis has been suggested to contribute to disease pathologies. The reduction in neurogenesis in aged

animals can be partially counteracted by enrichment activities or voluntary exercise, however, whether the increase in neurogenesis sufficiently improves learning and memory or olfaction in aged mice is unclear (Klempin and Kempermann, 2007). Irregular adult neurogenesis has been linked to depression, anxiety-related behaviors, diabetes, stress, and schizophrenia (Duan et al., 2007; Aonurm-Helm et al., 2008; Kronenberg et al., 2008; Stranahan et al., 2008; Balu and Lucki, 2009; Lee and Son, 2009; Perez et al., 2009; Revest et al., 2009). These data suggest that basal levels, although low, of adult neurogenesis may be important to avoid, minimize, or reduce the risk of disease pathogenesis. However, additional research in the field is necessary to clarify whether a reduction in adult neurogenesis is the cause or the result of such diseases and whether neurodegenerative diseases can be linked to irregular adult neurogenesis.

The idea that neurogenesis can be stimulated in adult animals is an area of intense interest because of the therapeutic potential of using adult neural stem cells to treat a variety of neurodegenerative diseases, aging-related decline of sensory mechanisms, and other neurological diseases such as depression. Recent studies have demonstrated that perhaps the small percentage of newborn neurons in adult animals is important for mate selection, maternal behavior, reducing neuronal disease pathologies, and minimizing aging-related decline in neurogenesis. Since environmental factors are able to promote neurogenesis in adult animals and tantalizing evidence now shows that adult-born neurons may provide a functional benefit, it is extremely crucial to the field to fully understand the regulatory mechanisms mediating this process.

Molecular mechanisms implicated in adult neurogenesis

Recently, expression of the pro-neural transcription factor Neurog2 has been identified in the developing DG (Galichet et al., 2008). Using conditional transgenic mice, the authors discovered that without functional Neurog2, there was a significant decrease in the size of the

upper blade of the DG, which suggests an essential role of Neurog2 in DG development and thus implying a similar role in adult neurogenesis (Ozen et al., 2007; Galichet et al., 2008). In another study, isolated E14 neural stem cells (NSC) retrovirally transduced to overexpress Neurog2 displayed increased cell cycle exit and neuronal differentiation *in vitro* (Yi et al., 2008). Additionally, other intrinsic factors identified to play a role in regulating adult neurogenesis in the SVZ or the SGZ or both in adult rodents are: Pax6, T-domain transcription factor 2 (Tbr2), Mash1/Ascl1, Sox2, NeuroD, and REST (Hsieh et al., 2004; Komitova and Eriksson, 2004; Englund et al., 2005; Kim et al., 2007; Suh et al., 2007; Hodge et al., 2008; Osumi et al., 2008; Gao et al., 2009; Kuwabara et al., 2009; Hsieh and Eisch, 2010; Gao et al., 2011). Many recent discoveries of intrinsic properties regulating adult neurogenesis *in vitro* or *in vivo* parallel those previously identified as regulating embryonic neurogenesis (Qian et al., 1997; Qian et al., 2000; Ross et al., 2003); these findings may provide further clues to help characterize the molecular mechanisms regulating adult neurogenesis.

Characterization of stem cell maintenance and cell fate specification has revealed a vascular nature of the SVZ niche (Merkle et al., 2007; Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008). These studies identify the SVZ as a heterogenous region containing Type B, radial-glia like stem cells, Type C, transiently amplifying progenitor cells and Type A, migrating neuroblasts. Type B cells may transport key extracellular factors to the SVZ niche to influence neurogenesis by directly contacting blood vessels. For example, the increased levels of prolactin and luteinizing hormone in female mice exposed to dominant male pheromones resulted in significant increases in BrdU-positive, as well as immature neuron specific marker Doublecortin (DCX) along the SVZ (Mak et al., 2007). Estrogen has also been implicated in the promotion of NSC proliferation *in vitro* when applied to E15 rat NSC cultures, however its role on adult NSCs remains unknown (Okada et al., 2008). By contrast, in the hippocampus,

increased glucocorticoid levels due to sleep deprivation in adult rats led to a significant reduction in the number of BrdU-positive cells (Mirescu et al., 2006).

Numerous signaling pathways have been implicated in post-natal or adult rodent brain neurogenesis. Post-natal development encompasses post-natal day 0 (P0) to P21 while young adult or adult neurogenesis consists of animals older than 6 weeks of age. Sonic Hedgehog, Notch, and Wnt/β-catenin signaling pathways have been suggested to play a role in post-natal or adult neurogenesis or both (Lie et al., 2005; Balordi and Fishell, 2007b, a; Breunig et al., 2007; Breunig et al., 2008; Crews et al., 2008; Han et al., 2008). Additionally, receptor tyrosine kinase family (TrkB/C) has been implicated in modulating the neurogenic effect observed in the SGZ of adult mice (Ke et al., 2007; Rolls et al., 2007; Li et al., 2008; Yi et al., 2008).

It is possible that the signaling pathways mediating embryonic neurogenesis may also play a role in adult neurogenesis. For example, a recent study of ERK2 function during embryonic neurogenesis shows that deletion of ERK2 resulted in impaired cortical layering, cortical thickness, and associative learning (Samuels et al., 2008). In another study, neuropeptide Y (NPY) was found to promote neurogenesis along the SVZ postnatally; acute exposure to NPY increased the phosphorylated form of ERK1/2, and this suggests a proliferative response triggered by ERK1/2 activation (Agasse et al., 2008). Moreover, it has been recently shown that NT3, a member of the neurotrophin family, is expressed in proliferating cells residing in the SVZ/VZ of the neocortex (Ohtsuka et al., 2008). NT3 has been implicated in Neurog2-mediated neurogenesis and survival of transplanted E14 rat progenitor cells (Yi et al., 2008). In cortical slices treated with NT3, a significant increase in both phosphorylated ERK1/2 and ERK5 (Ohtsuka et al., 2008) suggests the coupling of extrinsic factors with intrinsic properties through cellular signaling pathways. Although signaling pathways

regulating embryonic neurogenesis may also be involved in adult neurogenesis, the molecular mechanisms regulating neurogenesis in adult rodents are currently unclear.

Extracellular signal-Regulated Kinase 5 (ERK5) signaling pathway

ERK5 is a member of the Mitogen-Activated Protein Kinase (MAPK) family and is activated in response to growth factors and stress. Its kinase domain and TEY dual phosphorylation motif shares high sequence homology with ERK1/2. ERK5 contains a unique loop-12 linker between kinase sub-domains VII and VIII and an extended C-terminus making it distinct from other members of the MAPK family. The unique C-terminus contains a nuclear-localization signal (NLS), transcriptional activation domain, and a proline-rich region. ERK5 is normally localized in the cytoplasm, but upon stimulation by extrinsic factors, it translocates to the nucleus via the NLS sequence (English et al., 1995; Lee et al., 1995; Zhou et al., 1995; Hayashi et al., 2004). Additionally, ERK5 is capable of auto-phosphorylation through its large C-terminus, the region that is also required for the activation of nuclear substrates (Kasler et al., 2000; Morimoto et al., 2007). ERK5 is activated specifically by the upstream kinase MEK5 by dual phosphorylation of the ERK5 TEY motif.

The ERK5 pathway has been implicated in proliferation, differentiation, and survival of many cell types (Hayashi and Lee, 2004). Deregulation of the ERK5 pathway has been observed in a number of malignancies including prostate cancer, bone metastasis and mammary carcinoma. A study in our lab demonstrated that ERK5 is required for G2/M entry (Cude et al., 2007), and ERK5 activation has been shown to be important for cell survival during mitosis (Girio et al., 2007). Additionally, transgenic ERK5 knock-out (KO) animal studies have demonstrated that ERK5 is necessary for proper endothelial cell development and/or function where its loss may contribute to cardiovascular defects in both *erk5* and *mek5* KO animals (Hayashi et al., 2004; Hayashi and Lee, 2004; Wang et al., 2005). Furthermore, ERK5 has been

shown to prevent serum withdrawal-induced apoptosis in cortical neurons (Cavanaugh et al., 2001) and a dominant-negative form of ERK5 has been shown to block brain derived neurotrophic factor (BDNF)-promoted neuronal survival during embryonic cortical neurogenesis (Liu et al., 2003a).

Both our lab and others have demonstrated that neurotrophin-induced activation of ERK5 promotes the survival of newborn neurons during embryonic development (Cavanaugh et al., 2001; Watson et al., 2001; Liu et al., 2003a; Shalizi et al., 2003; Wang et al., 2006b; Finegan et al., 2009). ERK5 also specifies cortical stem/progenitor cells toward a neuronal lineage during development by phosphorylating and modulating the activity of neurogenin (Neurog 1) (Liu et al., 2006; Cundiff et al., 2009). Neuronal cell fate specification in cortical progenitor cells derived from embryonic day 13 (E13) rats progresses by specific inhibition of gliogenesis while sparing effects on cellular proliferation (Liu et al., 2006). ERK5 expression in the brain is high during early embryonic development but declines as the brain matures (Liu et al., 2003a). It is generally thought that ERK5 is not expressed in the adult brain (Di Benedetto et al., 2007). However, using a purified affinity antibody to ERK5 and immunohistochemistry, we have discovered discrete ERK5 expression in both the SGZ and SVZ. The finding that ERK5 expression is specific to the adult neurogenic regions directly implicates its unique role in the regulation of adult neurogenesis.

Functional implications of adult-born neurons in the SGZ

Many experimental approaches have been used to ascertain whether increasing or decreasing adult neurogenesis in the dentate gyrus has any profound effects on learning and memory. Methods used to obtain data from one lab are inconsistent from another and claims that a true functional role of adult neurogenesis remain controversial.

A common method to determine if adult-born neurons are functional is by examining the neuronal electrophysiological properties of newborn neurons. Van Praag et al, first demonstrated in 2002 that running increases the number of adult-born neurons along the SGZ; and these adult-born neurons express mature neuron markers and morphologically resemble granule cells of the dentate gyrus (van Praag et al., 2002). By performing electrophysiological recordings of retroviral-labeled GFP^+ adult-born neurons in hippocampal slices, they showed that many neuronal properties were comparable to existing mature dentate granule cells. Additionally, synaptophysin immunohistochemistry was performed and ultrastructural analysis demonstrated the ability of adult-born neurons to receive synaptic inputs (van Praag et al., 2002). These results were the first to suggest that newborn neurons along the adult SGZ are capable of integrating into the neuronal circuitry and are functional.

To determine if newborn neurons along the SGZ are important for learning and memory, many studies have adopted different paradigms to selectively block neurogenesis in rats and mice to assess the consequences on learning and memory. Dupret, et al, found that expression of pro-apoptotic Bax protein in adult neural stem cells along the SGZ reduced the number of newborn neurons, and caused a significant decline in spatial learning and memory performance assessed by the Morris water maze (Dupret et al., 2008). Revest, et al, used a similar paradigm to express Bax protein and found that suppressing neurogenesis increased anxiety, suggesting that basal levels of SGZ adult neurogenesis are important for the maintenance of affective states and might be a target for treatment of anxiety disorders (Revest et al., 2009). Similarly, focal x-irradiation, treatment with temozolomide, or ganciclovir, and expression of diphtherotoxin all suppressed adult neurogenesis along the SGZ and impaired hippocampus-dependent learning and memory (Saxe et al., 2006; Imayoshi et al., 2008; Clelland et al., 2009; Deng et al., 2009; Garthe et al., 2009). Using the Morris water maze assay, mice were not able to locate the escape platform or remember the location of the escape platform when compared with control

mice (Deng et al., 2009; Garthe et al., 2009). Mice with naturally depressed neurogenesis along the SGZ had impaired spatial navigation (Thuret et al., 2009).

The Barnes maze, radial arm maze, and visual discrimination using a touch-screen box are other assays used to assess hippocampus-dependent spatial learning and memory. Expression of diptherotoxin in adult neural stem cells in mice suppressed SGZ neurogenesis and resulted in impairment of mice to learn spatial navigation in the Barnes maze assay (Imayoshi et al., 2008). Suppression of adult neurogenesis resulted in deficits in pattern separation in the radial arm maze assay (Farioli-Vecchioli et al., 2008; Clelland et al., 2009; Creer et al., 2010; Pan et al., 2012a), suggesting that SGZ neurogenesis is important for spatial pattern separation. Additionally, by using the visual touch-screen box, mice with suppressed neurogenesis had more difficulties discriminating between patterns suggesting that adult-born neurons are functionally important for spatial learning and memory (Clelland et al., 2009). Consistent with the idea that SGZ neurogenesis may be important for learning and memory, Creer et al, recently showed that increasing neurogenesis by permitting mice to free-run on running wheels resulted in an increased ability of mice to discriminate between the more challenging, small but not large, spatial pattern differences using the visual touch-screen box (Creer et al., 2010).

To assess other forms of hippocampus-dependent learning and memory, the context- and cued-fear conditioning assays are often used. In these assays, animals are introduced to a context and permitted to explore for a fixed period of time before the presentation of a mild foot shock (context). Alternatively, a tone can be presented before the mild foot shock (cued). Animals are then returned to their home cages following a brief period of memory consolidation to either associate the foot shock with the context (hippocampus-dependent) or with the tone (hippocampus-independent). To test if learning and memory is impaired, animals are placed in

the same context or presented with the same tone later in a novel context and freezing behavior is monitored. By using these assays, it was determined that blocking SGZ neurogenesis reduced the rate of memory transfer from the hippocampus to the cortex and mice had an inability to remember specific contexts but had no deficits in cued learning and memory (Saxe et al., 2006; Imayoshi et al., 2008; Deng et al., 2009; Kitamura et al., 2009).

Other paradigms used to abrogate normal physiological levels of adult neurogenesis now include interfering with intrinsic molecular mechanisms to determine if a learning and memory deficit develops. By using inducible-conditional transgenic mouse models interfering with normal Wnt/β-catenin signaling, pro-differentiation gene PC3 expression, orphan nuclear receptor TLX expression, and fragile X mental retardation protein (FMRP), deficits in many hippocampus-dependent learning and memory tasks were reported (Farioli-Vecchioli et al., 2008; Zhang et al., 2008a; Jessberger et al., 2009; Guo et al., 2011). Anxiety-like behaviors were also assessed using the open field and elevated plus maze assays after conditionally ablating TrkB receptors in adult animals (Bergami et al., 2008). These results suggest that affecting adult-neurogenesis, whether by induced suppression via x-irradiation, drug administration, pro-apoptotic protein expression or interfering with intrinsic molecular mechanisms, may result in hippocampus-dependent learning and memory deficits.

In contrast, other groups have found no deficits in hippocampus-dependent learning and memory. Meshi, et al, used an enrichment paradigm to promote neurogenesis followed by x-irradiation and found no difference in the Morris water maze or novelty suppressed feeding assays between enriched and irradiated mice (Meshi et al., 2006). Similarly, by using only an x-irradiation paradigm without enrichment, Saxe, et al, also did not report any deficits using the Morris water maze assay (Saxe et al., 2006). Additionally, one study found that x-irradiated mice did not exhibit a hippocampus-dependent learning and memory defect in the contextual fear

conditioning assay while rats did show a deficit (Snyder et al., 2009), thereby suggesting a species-specific difference in the requirement of adult-born neurons for learning and memory. Dupret, et al, also found that suppression of SGZ neurogenesis by overexpression of pro-apoptotic Bax did not affect contextual-fear learning and memory in mice suggesting that not all hippocampus-dependent learning and memory is dependent on adult neurogenesis (Dupret et al., 2008). By using rats as experimental subjects and treating them with methylazoxymethanolacetate to block neurogenesis, Shors, et al, found no deficits in the Morris water maze assay or the contextual fear conditioning assay but did find a decrease in the cued fear conditioning assay (Shors et al., 2002).

It is not clear why different studies led to contradicting results regarding the role of adult-born neurons in learning and memory, but could be due to a number of differences including: sex of mice used, mouse strain differences, differences in treatment paradigms to ablate adult neurogenesis, behavioral assay paradigms, species-specific differences between, gene expression differences, or any combination of the above. The field of adult neurogenesis is now becoming increasingly interesting as more evidence points to a functional role of adult-born neurons.

Functional implications of adult-born neurons from the SVZ

Newly generated neuronal precursors in the SVZ migrate along the rostral migratory stream (RMS) to the core of the olfactory bulb (OB) where they begin radial migration and differentiation into inhibitory interneurons (Belvindrah et al., 2009; Whitman and Greer, 2009). Although these neurons have been extensively characterized at the cellular level, their functional impact on olfactory behavior is still an open question (Whitman and Greer, 2009; Bardy and Pallotto, 2010; Lazarini and Lledo, 2011; Breton-Provencher and Saghatelyan, 2012; Kageyama et al., 2012).

Genetic expression of a lethal diphtheria toxin fragment (DTA) in adult-born neurons, which kills these neurons, led to a substantial loss of adult-born granule cells in the OB and a much smaller OB (Imayoshi et al., 2008). Surprisingly, despite these structural changes in the OB, no deficits in olfactory behavior were observed. However, a subsequent study using lateral ventricle infusion of AraC to ablate adult neurogenesis in the SVZ/OB in mice showed that a reduction in OB adult neurogenesis reduced odor detection sensitivity and impaired short-term olfactory memory but did not affect odor discrimination or reward-associated long-term memory (Breton-Provencher et al., 2009). Although results from this study generated much excitement in the field (Arenkiel, 2010; Bardy and Pallotto, 2010; Lazarini and Lledo, 2011), they contradicted results from other reports (Gheusi et al., 2000; Lazarini et al., 2009; Sultan et al., 2010). In addition, NCAM knockout mice have diminished OB neurogenesis, and are deficient in the odor discrimination task while both the detection threshold for odors and short-term olfactory memory are unaltered (Gheusi et al., 2000). Thus, the exact function of adult-born OB neurons is still highly debated.

Several factors may contribute to this controversy including the specificity of methods used to ablate or suppress adult neurogenesis. Although effective at suppressing adult neurogenesis, x-irradiation or anti-mitotic drugs are not specific for adult-born neurons. They target all dividing cells, may alter the neurovascular niche important for adult neurogenesis, and induce neural inflammation. The side effects intrinsic to these methods may be confounding factors contributing to inconsistent behavior results. Studies using traditional knockout of genes important for neurogenesis are also useful, but their interpretations are limited by widespread abnormalities of brain structure or compensatory effects elicited during development. Transgenic expression of a lethal gene, such as diphtheria toxin or thymidine kinase (Imayoshi et al., 2008; Singer et al., 2009), to kill adult-born neurons is more specific to adult neural

stem/progenitor cells. However, large amounts of cell death in the RMS-OB may interfere with normal olfactory function.

Adult neurogenesis has also been implicated in regulating pheromone-based animal behaviors in mice, such as mating, paternal recognition, and male-male aggression (Mak et al., 2007; Mak and Weiss, 2010; Sakamoto et al., 2011). However, it is not known whether adult neurogenesis influences the detection sensitivity of pheromones. Since pheromones are likely present only in low abundance in their normal living environment, adult neurogenesis regulation of pheromone detection may be an underlying factor of pheromone-based animal behaviors in mice.

Many of the discrepancies described above could be attributed to differences in experimental design of the specific behavioral assays, intrinsic differences in animal strain, or methods of altering adult neurogenesis. The possibility that adult neurogenesis along the SVZ contributes to olfactory behavior is intriguing. Anosmia is a common side effect of many neurodegenerative diseases; and with the possibility that adult-born neurons can integrate into the OB circuitry and contribute to olfaction, new therapies utilizing adult neural stem cells could be realized. However, due to the inconsistencies in the literature, the true functional significance of adult-born OB neurons is unclear.

Goals of dissertation research

Despite increased interest in the adult neurogenesis field, a full understanding and appreciation of the cellular and molecular mechanisms regulating this process as well as the functional relevance of adult-born neurons remains unclear. Based upon these unknowns, the goals of this dissertation were: (1) to determine whether ERK5 MAPK played a specific regulatory role in modulating the genesis of adult-born neurons in the SGZ or SVZ or both, and

(2) determine if adult neurogenesis contributed any functional significance in hippocampus-dependent learning and memory or olfaction. Delineating regulatory mechanisms of adult neurogenesis and defining functional roles of adult-born neurons ultimately aid in the overall understanding of the fundamental biology and necessity of continuous neuronal generation long into adulthood. Determining a novel role of the ERK5 signaling pathway in adult neurogenesis will not only provide additional understanding of molecular neurobiology, but also advance the possibility of directed neurogenesis or neuroregeneration using adult neural stem cells toward the treatment of CNS diseases.

Chapter 2

Inducible and Conditional Deletion of Extracellular Signal-Regulated Kinase 5 Disrupts Adult Hippocampal Neurogenesis

INTRODUCTION

Adult neurogenesis occurs in the dentate gyrus of mammalian brains, including the human brain (Altman and Das, 1965; Alvarez-Buylla et al., 1988; Ming and Song, 2005; Deng et al., 2010). Adult-born neurons functionally integrate into the hippocampal circuitry (Song et al., 2002; van Praag et al., 2002; Schmidt-Hieber et al., 2004; Ramirez-Amaya et al., 2006; Ge et al., 2007; Kee et al., 2007; Toni et al., 2007), suggesting that adult neurogenesis may contribute to neuroplasticity. This idea is supported by the observation that hippocampus-dependent, but not hippocampus-independent learning increases the number of adult-born neurons in the dentate gyrus (Gould et al., 1999; Leuner et al., 2004; Epp et al., 2007). Despite the interest in the physiological roles of adult-born neurons, mechanisms regulating adult neurogenesis have not been fully elucidated.

ERK5 is a member of the mitogen-activated protein (MAP) kinase family that includes ERK1/2, p38, and JNK (Lee et al., 1995; Zhou et al., 1995). It is specifically phosphorylated and activated by MEK5 (English et al., 1995; Zhou et al., 1995). MEK5 is specific for ERK5 and does not phosphorylate ERK1/2, JNK or p38 even when overexpressed (English et al., 1995; Zhou et al., 1995). ERK5 is activated by neurotrophins (NT) through MEK5, which promotes the survival of newborn neurons during embryonic development (Cavanaugh et al., 2001; Watson et al., 2001; Liu et al., 2003a; Shalizi et al., 2003; Wang et al., 2006b; Finegan et al., 2009). Furthermore, ERK5 specifies cortical stem/progenitor cells toward a neuronal lineage during development by phosphorylating and modulating the activity of neurogenin (Neurog) 1 (Liu et al., 2006; Cundiff et al., 2009). ERK5 expression in the brain is developmentally regulated; it is

high during early embryonic development but declines postnatally as the brain matures (Liu et al., 2003a). Interestingly, although there is very little ERK5 expression throughout the adult brain (Di Benedetto et al., 2007), upon closer examination we report here that ERK5 is prominently expressed in the two adult neurogenic regions: the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) along the lateral ventricles. This unique pattern of expression suggests a fundamentally important role for ERK5 in regulating adult neurogenesis.

In this study, we have characterized the cell types expressing ERK5 along the SGZ of the dentate gyrus. To investigate a role for ERK5 in the regulation of adult neurogenesis, we utilized RNAi and transgenic mouse technologies to inhibit ERK5 expression as well as retroviral expression of a constitutive active (ca) MEK5 to stimulate ERK5 both *in vitro* and *in vivo*. Our data suggest a critical role for ERK5 in the regulation of adult hippocampal neurogenesis.

MATERIALS AND METHODS

Animals

The generation of Nestin-CreER^{TM} (Kuo et al., 2006) mice, ERK5$^{loxP/loxP}$ (Wang et al., 2005) mice, and Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice (Pan et al., 2012a) have been described. A small cohort of Nestin-CreER^{TM} mice were also bred with *Gt(ROSA)26Sor-YFP* (R26-YFP) mice (Srinivas et al., 2001) to yield Nestin-CreER^{TM}/R26-YFP$^{loxP/loxP}$ mice. All animal experiments were performed with identically treated littermate controls. Animals were housed under standard conditions (12 h light/dark cycle) with food and water provided *ad libitum*. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Reagents

The following plasmids have been described: The NeuroD2-Luc reporter (pCS2-NeuroD2-Luc) was obtained from Dr. Jim Olson (McCormick et al., 1996), and cDNA sequence for mouse Neurog2 in pcDNA3 from Dr. Jane Johnson (University of Texas at Southwestern Medical Center). A Flag sequence was inserted at the N-terminus of the Neurog2 cDNA. The following primary antibodies and dilutions were used for immunohistochemistry: rat monoclonal anti-BrdU (1:500, AbD Serotec); mouse monoclonal antibodies against PCNA (1:500, Millipore), Sox2 (1:200, R&D Systems), GFAP (1:500, Millipore), NCAM (1:200, Developmental Studies Hybridoma Bank), NeuN (1:500, Millipore), Calretinin (1:200, Abcam), and Calbindin (1:200, Abcam); goat polyclonal antibodies against NeuroD (1:200, Santa Cruz Biotech Inc.) and DCX (1:200, Santa Cruz Biotech Inc.); and rabbit polyclonal antibody against GFP (1:500, Invitrogen). Rabbit polyclonal ERK5 antibody (1:500 dilution) was generated previously (Cavanaugh et al., 2001) and affinity purified using recombinant MBP-ERK5 protein. The following primary antibodies and dilutions were used for immunocytochemistry: mouse monoclonal antibodies against GFP (1:5,000, Invitrogen), Nestin (1:500, Developmental Studies Hybridoma Bank), Sox2 (1:500, R&D Systems), and β-III Tubulin (1:500, Promega); rabbit polyclonal antibodies against PCNA (1:500, Millipore) and GFP (1:5,000, Invitrogen). The following primary antibodies and dilutions were used for Western blot analysis: rabbit polyclonal ERK5 antiserum (1:1,000), rabbit polyclonal MEK5 antibody (1:500, Santa Cruz Biotech Inc.), rabbit polyclonal ERK1/2 (1:10,000, Millipore), rabbit polyclonal p-ERK5 antibody (1:1,000, Cell Signaling), and mouse monoclonal β-actin antibody (1:10,000, Sigma). Secondary antibodies were rabbit polyclonal Horse Radish Peroxidase (HRP) antibody (1:10,000, Calbiochem) and mouse monoclonal HRP antibody (1:20,000, Calbiochem).

BrdU and Tamoxifen administration

Mice were treated with 100 mg/kg BrdU (Sigma) by intraperitoneal (IP) injection 5 times (every 2 h for 10 h) in one day followed by sacrifice 4 weeks later to identify BrdU-retaining, adult-born cells. Tamoxifen (Sigma) was made fresh daily and dissolved in 2% glacial acetic acid in corn oil solution (Sigma). To activate Cre-mediated recombination, 5 mg of pre-warmed tamoxifen was administered orally to 10-12 week-old male mice daily for 7 d (Fig. 2.11A, B) or once per day for 4 d in each cycle, for 3 cycles with 2-week inter-cycle intervals (Fig. 2.11C–M).

Immunohistochemistry (IHC)

Brains were post-fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C after standard intracardial perfusion procedures. Brains were then placed in 30% (w/v) sucrose in PBS at 4°C until brains sunk and immediately frozen at -80°C. IHC was performed on 30 µm-thick coronal brain sections using a free-floating antibody staining method as described (Pan et al., 2012a).

Immunocytochemistry

Cells were fixed in PBS containing 4% PFA and 4% sucrose at room temperature for 30 min. Fixed cells were washed 3 x 5 min in PBS, 5 min in 1% SDS, and washed again 3 x 5 min in PBS. Cells were then incubated in blocking buffer consisting of 5% bovine serum albumin (BSA) in PBST (PBS + 0.1% Triton X-100) for 2 h, followed by incubation with primary antibodies overnight at 4°C. Cells were then washed 3 x 10 min in PBST, followed by incubation with secondary antibodies at 1:5,000 dilution (Alexa Fluor-488) or 1:2,000 dilution (Alexa Fluor-594) for 2 h in blocking buffer. Cells were then washed 3 x 10 min in PBST followed by a 10 min incubation in Hoechst 33342 for nuclei visualization and a final wash of 10 min in PBST prior to mounting onto slides using anti-fade Aqua Poly/Mount solution. Unless otherwise stated, all steps were carried out at room temperature.

Confocal imaging and analysis

All images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with numerical aperture (NA) 0.75, 20X lens or NA 1.3, 40X oil immersion lens. Optical Z-sections (0.5–1 μm) were collected and processed using ImageJ software (NIH). Images were uniformly adjusted for color, brightness, and contrast with Adobe Photoshop CS4 (Adobe Systems Inc).

Quantification of immunostained cells

Greater than 100 immunopositive cells per coverslip per experiment were quantified using an inverted fluorescence microscope (Leitz DMIRB, Leica) with a 40X objective (Leica) following immunocytochemistry. A modified optical fractionator method was used as an unbiased stereological method for obtaining an estimation of total cell counts per SGZ following immunohistochemistry (West et al., 1991; Kempermann et al., 1997b; Malberg et al., 2000). The method for *in vivo* cellular quantification and co-localization analysis per SGZ was as described (Pan et al., 2012a).

SGZ-derived adult neural progenitor cell (aNPCs) cultures

Primary cell cultures were prepared as described (Bull and Bartlett, 2005; Rietze and Reynolds, 2006). Briefly, tissue samples containing the dentate gyrus were micro-dissected and enzymatically digested with 0.1% trypsin-EDTA (Gibco) for 7 min at 37°C followed by incubation with equal volume of 0.014% trypsin inhibitor (Gibco). Tissue samples were then spun down and resuspended in culture media consisting of DMEM/F12 (Gibco), 1X N2 supplement (Invitrogen), 1X B27 supplement without retinoic acid (Gibco), 100 U/mL penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), 2 μg/mL heparin (Sigma), 20 ng/mL EGF (EMD Chemicals), and 10 ng/mL bFGF (Millipore). Culture medium for adult neural progenitor cells always contain EGF and bFGF unless otherwise specified. Tissue was mechanically triturated and filtered

through a 40 μm cell sieve and plated in petri dishes and cultured for 10–14 d until neurospheres are formed. Growth factors were replenished every 3 d during this period. Following primary passage, neurospheres were isolated, dissociated into single-cell suspension enzymatically and mechanically, and replated at low density and cultured for secondary neurosphere formation. Spheres collected from secondary passage were dissociated and plated as a monolayer culture on poly-D-lysine/laminin- (BD Biosciences) or poly-L-ornithine/fibronectin- (BD Biosciences) coated aclar coverslips (Electron Microscopy Sciences) for experiments.

Retrovirus construction and production

The ERK5 shRNA and control non-specific shRNA (shNS) retroviruses have been previously described (Cundiff et al., 2009). Briefly, shNS directed towards dsRED sequence (agttccagtacggctccaa), and shERK5 directed towards murine ERK5 sequence (aa 106-111: acacttcaaacacgacaat), were subcloned into pSIE retroviral vector (Duan et al., 2007). cDNA sequences encoding wild-type ERK5 or constitutively active MEK5 (Kato et al., 1997; Liu et al., 2006) were subcloned into the Sal I/Xho I restriction sites within the multiple-cloning site of an oncoretroviral expression vector, which contains an IRES-GFP sequence and was described in (Kim et al., 2009). High-titer VSV-G pseudotyped retroviral stocks were produced as described (Duan et al., 2007).

Characterization of retroviral ERK5 or caMEK5

NIH-3T3 cells were plated in 6-well tissue culture treated plates at 5 x 10^4 cells per well in DMEM (Gibco) containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. Following overnight plating, protamine sulfate (Invitrogen) was added to culture media at a final concentration of 8 μg/mL and 8 μL of 1 x 10^9 infection units per mL (IU/mL) of retroviruses were added to each well and allowed to transduce cells for 24 h. Where co-transduction was

required, a 1:1 ratio of retroviral ERK5 and caMEK5 were added to each well and cells transduced for 24 h. Following 24 h transduction, media was refreshed and cells were cultured for an additional 3 d before processing for Western blot analysis as described (Liu et al., 2003a).

Viral transduction of aNPCs

For Western blot analysis of shERK5 specificity, aNPCs were plated as a monolayer culture on poly-L-ornithine/fibronectin-coated, 12-well tissue culture plates at a density of 3 x 10^5 cells per well in culture media as described above. Twenty-four hours after plating, protamine sulfate was added to culture media at a final concentration of 8 μg/mL, and cells were infected with 20 μL of 1 x 10^9 IU/mL shNS and shERK5 retroviruses. Four days following retrovirus infection, cells were lysed for Western blot analysis as described (Liu et al., 2003a). For immunocytochemistry studies, aNPCs were plated as a monolayer culture on poly-D-lysine/laminin- or poly-L-ornithine/fibronectin-coated aclar coverslips in 24-well plates at a density of 1 x 10^5 cells per well in culture media. Twenty-four hours after plating, protamine sulfate was added to culture media at a final concentration of 8 μg/mL and 6-8 μL of 1 x 10^9 IU/mL retroviruses were added to each well and allowed to transduce cells for 24 h. In cases where co-transduction with retrovirus and lentivirus were needed (Fig. 2.7), aNPCs were first transduced with retrovirus for 10 h followed by transduction with lentivirus for an additional 24 h at a ratio of 3:1, respectively. Following transduction, culture media was changed and cells were cultured for an additional 5 d before being processed for immunocytochemistry.

Neurotrophin treatment

For neurotrophin activation of ERK5, aNPCs were plated as a monolayer on poly-L-ornithine/fibronectin-coated 12-well plates at a density of 7 x 10^5 cells per well. Forty-eight hours after plating, cells were switched into culture medium free of EGF and bFGF overnight before treatment with BDNF (50 ng/mL, Alomone Labs) or NT3 (100 ng/mL, Millipore). For

neurotrophin stimulation of aNPC neuronal differentiation, cells were plated as a monolayer on poly-L-ornithine/fibronectin-coated aclar coverslips at a density of 1 x 10^5 cells per well. One day after plating, cells were transduced with 8 µL of shNS or shERK5 retrovirus in the presence of 8 µg/mL protamine sulfate. Twenty-four hours later, the virus-containing medium was removed and cells were cultured with fresh medium for 3 d to allow retroviral expression. Cells were then incubated for 3 d in fresh medium containing 100 ng/mL NT3, or 1 µg/mL BSA as a control. Finally, EGF and bFGF were removed from the culture medium and cells were incubated for an additional 5 d in the continued presence of NT3 or BSA to allow neuronal differentiation.

To examine the effect of shERK5 on more differentiated aNPCs, cells were plated as a monolayer at a density of 1 x 10^5 cells per well on poly-L-ornithine/fibronectin-coated aclar coverslips in medium containing 50 ng/mL BDNF or 100 ng/mL NT3 for 3 d. EGF and bFGF were then removed from the culture medium and cells were incubated for an additional 3 d in the continued presence of BDNF or NT3. Cells were then transduced with shNS or shERK5 retroviruses as above and incubated for an additional 5 d in culture medium free of EGF and bFGF but in the continued presence of BDNF or NT3.

Lentiviral Neurog2 transfer construct (pRRL-cPPT-CMV-Neurog2-PRE-SIN-IRES-EGFP)

Flag-Neurog2 cDNA sequence was inserted into a multiple cloning site of lentiviral transfer vector pRRL-cPPT-CMV-X-PRE-SIN-IRES-EGFP, described in (Liu et al., 2006), upstream from the internal IRES-directed marker protein eGFP (enhanced green fluorescent protein). High-titer lentiviral stocks were produced as described (Liu et al., 2006).

NeuroD2-Luciferase reporter gene assay

Primary cortical neurons were prepared from embryonic day 15 (E15) Sprague-Dawley rats (Charles River Laboratories) and cultured in petri dishes for 5 h before transfection. Cells

were transiently transfected with Nucleofector® Transfection Reagent (Amaxa Biosystems, Inc.) as previously described (Cundiff et al., 2009). Briefly, E15 cortical neurons were collected and resuspended in Rat Neural Stem Cell Nucleofector® Transfection Reagent at a density of 6 x 10^6 cells / 100 µl. For each transfection, 6 x 10^6 cells were transfected with 5 µg NeuroD2-Luc reporter, 100 ng pRL *renilla*-Luc reporter (Promega), 1 µg Flag-Neurog2 expression construct or pCDNA3 control plasmid, and 4 µg shERK5 retroviral plasmid or shNS control plasmid using the Amaxa Nucleofector® with A31 protocol. Immediately following Nucleofection, cells were resuspended in pre-warmed (37°C) regular culture medium and incubated at 37°C for 20 min. Cells were then resuspended in regular culture medium (Neurobasal Medium (Gibco), 2% B27 without retinoic acid, 10 ng/mL bFGF) and plated onto 12-well plates coated with poly-D-lysine/laminin. After 72 h in culture, cells from each well were lysed with 100 µl passive lysis buffer and 20 µl lysates were applied for dual luciferase assay per manufacturer's protocol (Promega).

Stereotaxic surgery

Stereotaxic procedure was performed on adult C57/BL6 male mice (8-10 weeks old, Charles River Laboratories) as described (Wong et al., 2000; Athos and Storm, 2001). Mice were anaesthetized by IP injection (21–23 µl/g body weight) of ketamine (7.0 mg/mL) and xylazine (0.44 mg/mL) dissolved in 0.9% bacteriostatic saline (Hospira, Inc.). One microliter of retrovirus (10^9-10^{10} IU/mL) was injected at a rate of 0.25 mL/min bilaterally into the dentate gyrus with the following coordinates relative to Bregma: 1.65 mm posterior, ±1.62 mm medial-lateral, 2.30 mm ventral.

Statistical analysis

All of the *in vitro* cell culture data were from at least two independent experiments with duplicates or triplicates each (total n ≥ 5 for each data point). *In vivo* cellular quantification data

were from at least two independent experiments with n ≥ 12 for data in figure 2.10 and n ≥ 6 for data in figure 2.11. Pair-wise comparison of the means was analyzed by Student's *t*-test, two-tailed analysis for data presented in figures 2.4–2.9. One-way ANOVA with *Fisher's LSD* post-hoc analysis was performed to analyze data presented in figures 5 and 8. Data represent mean ± standard error of means (s.e.m.). n.s. not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

RESULTS

ERK5 expression in the adult mouse brain is specific to the neurogenic regions

ERK5 expression in the adult mouse brain was examined by immunohistochemistry using an affinity-purified ERK5-specific antibody directed against the unique C-terminal tail of ERK5 protein (Cavanaugh et al., 2001). We found no ERK5 protein in cornu ammonis (CA) 1 and CA3 regions of the hippocampal formation, or most other areas of adult brain including the cortex and striatum (Fig. 2.1), consistent with other reports (Di Benedetto et al., 2007). However, ERK5 protein was specifically expressed in the SVZ (Fig. 2.1A, B) and along the SGZ of the dentate gyrus in the hippocampal formation (Fig. 2.1C, D). Specifically, ERK5 was expressed in SGZ cells co-labeled with markers for stem/progenitor cells (Sox2, GFAP), proliferation (BrdU, PCNA), transiently amplifying progenitors and/or newborn neurons (PSA-NCAM, DCX, and NeuroD) (Fig. 2.2A–S, and X). Some of the $ERK5^+$ cells were also positive for both GFAP and Sox2, suggesting ERK5 expression in radial glia-like stem cells (Fig. 2.2A–G, and X). However, very few $ERK5^+$ cells co-expressed NeuN, a marker for mature neurons. Of the few $NeuN^+$ cells that were also $ERK5^+$, the NeuN staining intensity was much lower than that in $NeuN^+/ERK5^-$ cells (Fig. 2.2T–W). Thus, these $ERK5^+/NeuN^+$ cells likely represent cells just beginning to express NeuN and are still in the early stage of terminal differentiation. This is consistent with the fact that none of the $ERK5^+$ cells co-express calbindin, a marker for mature granule neurons (Fig. 2.2X). Finally, almost 80% of $ERK5^+$ cells in the SGZ were also PSA-$NCAM^+$ and DCX^+.

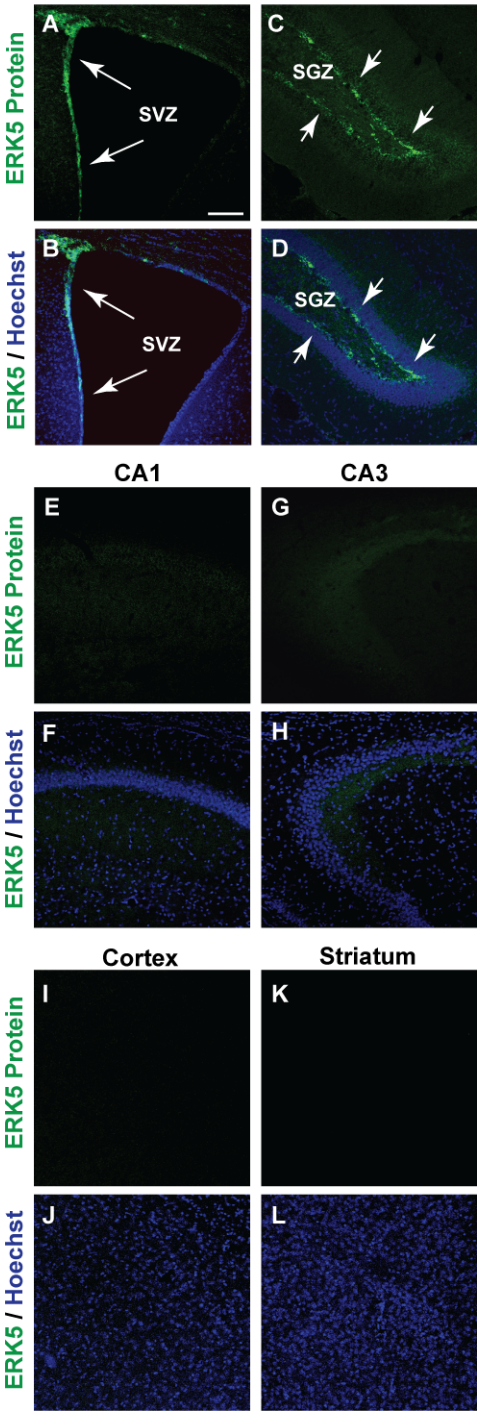


Figure 2.1. ERK5 MAPK expression in the adult mouse brain is restricted to the adult neurogenic regions. Images are representative immunostaining of coronal sections of adult mouse brain tissue showing ERK5 protein expression (green) primarily in the SVZ (A, B) and SGZ (C, D) but not in CA1 or CA3 of the hippocampal formation (E–H), the cortex (I, J), or striatum (K, L). Hoechst staining (blue) was used to identify all cell nuclei (B, D, F, H, J, L). Scale bar in A represents 100 μm and applies to all panels.

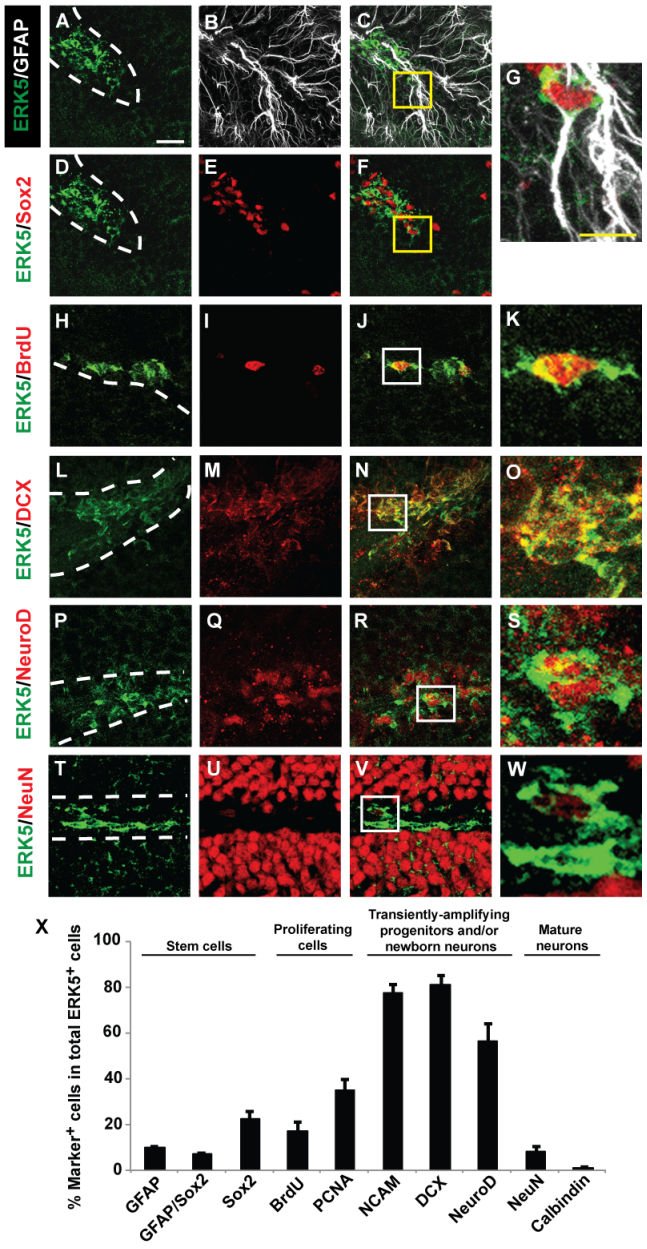


Figure 2.2. ERK5 is primarily expressed in transiently amplifying progenitors and/or newborn neurons in the SGZ. (A–W) Representative confocal images of brain sections immunostained for ERK5 (green), GFAP (A–C, and G) (white), and various other cellular markers (red) including Sox2 (D–G), BrdU (H–K), doublecortin (DCX) (L–O), NeuroD (P–S), and NeuN (T–W). Panel G represents the enlarged boxed areas in panels C and F. Similarly, images in panels K, O, S, and W are enlarged images of their corresponding boxed areas in panels J, N, R, and V. Scale bar in A represents 25 μm and applies to all images except the enlarged ones. Scale bar in G represents 10 μm and applies to K, O, S, and W. Dashed lines outline the SGZ layer of the dentate gyrus. (X) Quantification of marker-positive cells in total ERK5$^+$ cell population along the SGZ. Data represents mean percentage of double- or triple-labeled cells.

The specific expression of ERK5 in adult neurogenic regions is quite unique and interesting, and suggests an important function for ERK5 in regulating adult neurogenesis, particularly for the regulation of the cell fate of transiently amplifying progenitors and/or newborn neuron populations.

ERK5 signaling contributes to neuronal differentiation of SGZ-derived aNPCs in culture

SGZ-derived aNPCs were prepared from the dentate gyrus of 8–10 week-old adult mice as described (Reynolds and Weiss, 1992; Rietze and Reynolds, 2006). Western analysis confirmed ERK5 expression in these cells (Fig. 2.3). When aNPCs were allowed to differentiate in culture by removing bFGF and EGF from the culture media, retroviral infection of ERK5 shRNA, which specifically suppresses the expression of endogenous ERK5 but not the closely related ERK1/2 ((Cundiff et al., 2009) and Fig. 2.3), significantly decreased the number of cells expressing β-III tubulin, a marker for newborn neurons (Fig. 2.4A–E). Concomitantly, shRNA to ERK5 increased the number of cells expressing markers for stem/progenitor cells (Sox2, Nestin) and proliferation (PCNA) (Fig. 2.4F–N). However, it did not promote glial differentiation (33% vs. 26% cells co-expressed glial marker GFAP in shNS or shERK5 infected cells, respectively, $p>0.5$).

To activate endogenous ERK5 signaling, wild type (wt) ERK5 or caMEK5 were subcloned into a retroviral expression vector, upstream from an IRES-directed marker protein eGFP. Retroviral transduction of caMEK5 specifically activated endogenous ERK5 (Fig. 2.5A) and was sufficient to decrease the number of cells positive for Sox2, Nestin, and PCNA, while simultaneously increasing the pool of β-III tubulin$^+$ neurons even in the presence of mitogens bFGF and EGF (Fig. 2.5B–O).

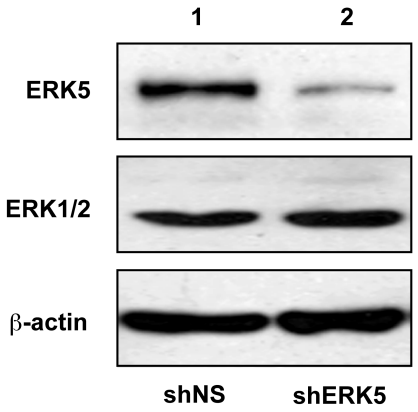


Figure 2.3. shERK5 specifically knocks down the expression of endogenous ERK5 but does not affect closely related ERK1/2. Control (shNS) or shERK5 retroviruses were used to infect SGZ-derived aNPCs. Endogenous ERK5 protein is knocked down in cells infected with shERK5 (lane 1 compared with lane 2). ERK1/2 is unaffected in shERK5-infected cells, thereby demonstrating the specificity of shERK5. β-actin was used as a loading control.

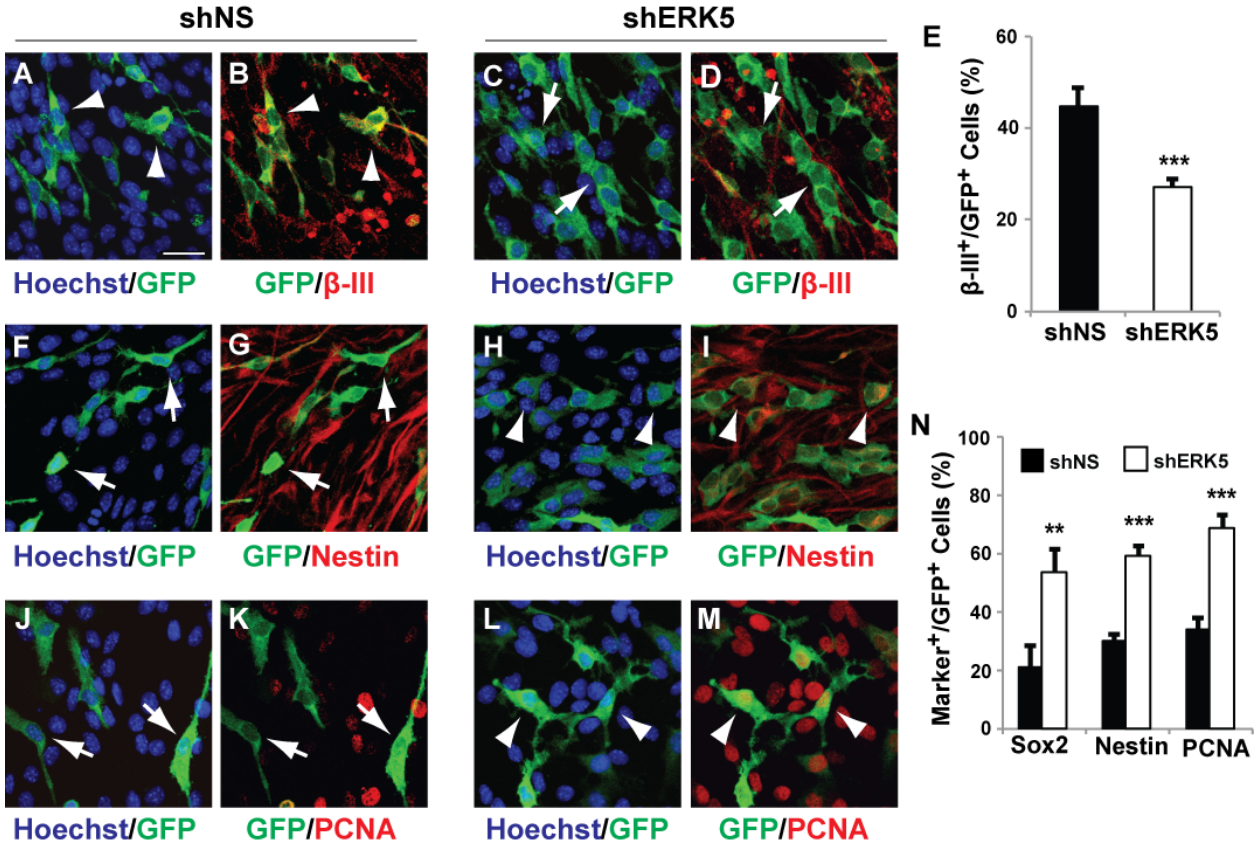


Figure 2.4. ERK5 signaling is necessary for promoting neurogenesis of SGZ-derived aNPCs in culture. aNPCs were infected with non-specific shRNA control retroviral vector (shNS) or shRNA to ERK5 retrovirus as indicated. Both retroviral vectors encode eGFP marker protein under a bicistronic promoter. One day after virus infection, cells were washed and then incubated in culture medium free of EGF and bFGF for 5 d to allow spontaneous differentiation. Cells were then fixed for immunocytochemistry and co-stained for GFP to identify virus-infected cells (green) and various cell-type specific markers (red). (A–D) Co-staining of GFP with β-III Tubulin. (E) Quantification of the percentage of GFP^+ cells that are also β-III $Tubulin^+$. (F–I) Immunostaining of GFP^+ infected cells and $Nestin^+$ neural stem cells. (J–M) Immunostaining of GFP^+ infected cells and $PCNA^+$ proliferating cells. (N) Quantification of the percentage of GFP^+ cells that also express Sox2, Nestin, or PCNA. Scale bar in A represents 25 μm and applies to all images. Arrowheads point to GFP^+ cells co-labeled with various cell markers, while arrows point to GFP^+ cells that are marker negative.

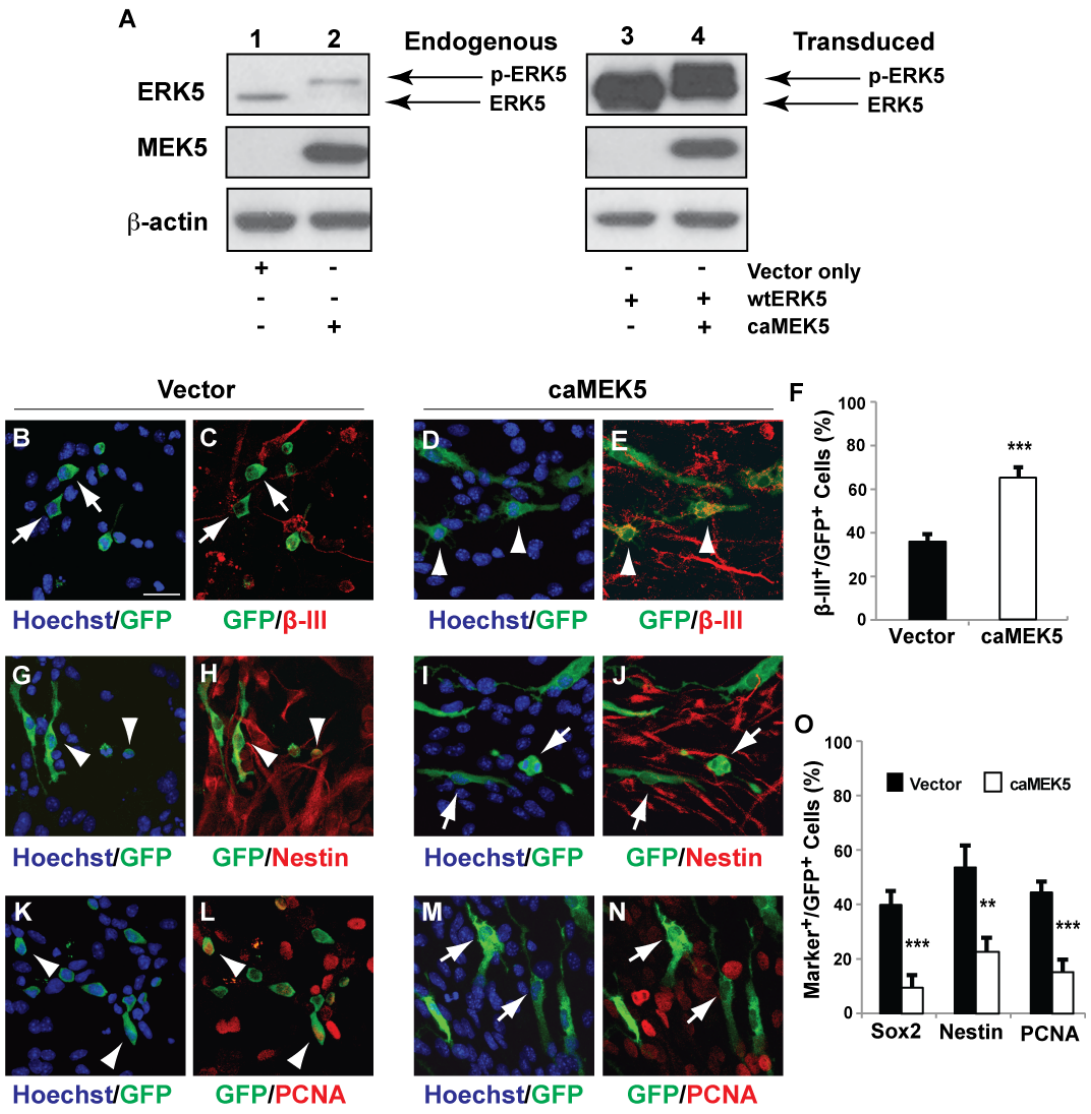


Figure 2.5. Activation of endogenous ERK5 signaling is sufficient to promote neurogenesis of dentate gyrus-derived aNPCs in culture. (A) Anti-ERK5 and MEK5 Western analysis of NIH-3T3 cells infected with retroviruses expressing caMEK5 and/or wild-type (wt-) ERK5. Cells infected with GFP retrovirus (vector only) were used as controls. β-Actin was used as a loading control. Expression of caMEK5 caused reduced elecrophoretic mobility of endogenous ERK5 (lanes 1 and 2) as well as the co-infected wtERK5 (lanes 3 and 4), indicative of ERK5 phosphorylation (p-ERK5) and activation. These data ascertain the constitutive active nature of the caMEK5 virus. (B–O) aNPCs were infected with control retroviral vector expressing eGFP only or expressing caMEK5-IRES-eGFP. One day after virus infection, cells were washed and then maintained in regular EGF- and bFGF-containing medium for 5 d before co-immunostaining for GFP (green) and various cellular markers (red) including β-III tubulin (B–E), Nestin (G–J), and PCNA (K–N). The percentage of GFP^+ cells that also express β-III tubulin (F), Sox2, Nestin, or PCNA (O) was quantified. Scale bar in B represents 25 μm and applies to all images. Arrowheads point to GFP^+ cells co-labeled with various cell markers, while arrows point to GFP^+ cells that are marker negative.

These data suggest a critical role for ERK5 in promoting neurogenesis in cultured aNPCs. Furthermore, aNPCs that did not differentiate into neurons upon ERK5 inhibition remained in the proliferating and stem/progenitor stage rather than undergoing precocious glial differentiation (data not shown).

Neurog2 may be a downstream target of ERK5 in adult neurogenesis

To elucidate downstream mechanisms mediating the neurogenic effect of ERK5 in SGZ cells, we investigated if ERK5 regulates the transcriptional activity of Neurog2, a basic helix-loop-helix (bHLH) transcription factor expressed in SGZ progenitors (Ozen et al., 2007; Hodge et al., 2008). A dual luciferase reporter assay was performed to measure Neurog2-stimulated transcription initiated from the NeuroD2-Luc reporter (Cundiff et al., 2009). Expression of Neurog2 alone stimulated NeuroD2-Luc activity 4-fold; this transcription was suppressed by co-transfection of shERK5 (Fig. 2.6). Neurog2 is essential for neurogenesis in the dentate gyrus during development (Galichet et al., 2008); however, its function in adult hippocampal neurogenesis has not been elucidated. Consequently, we examined if ectopic expression of Neurog2 is sufficient to promote neuronal differentiation of SGZ cells and if this is regulated by ERK5 signaling. SGZ-derived aNPCs were infected with lentiviruses expressing Neurog2-IRES-GFP; lentiviral-GFP was used as a control. Expression of Neurog2 was sufficient to increase the number of β-III tubulin$^+$ neurons even in the presence of bFGF and EGF (Fig. 2.7A–I). In contrast, Neurog2 decreased the number of Sox2$^+$ neural stem cells. Significantly, co-infection of shERK5 retroviruses blocked the effect of Neurog2 on neuronal differentiation (Fig. 2.7J–R). These data suggest that Neurog2 exhibits pro-neural activity in SGZ cells and may act as a downstream target of ERK5 during adult hippocampal neurogenesis.

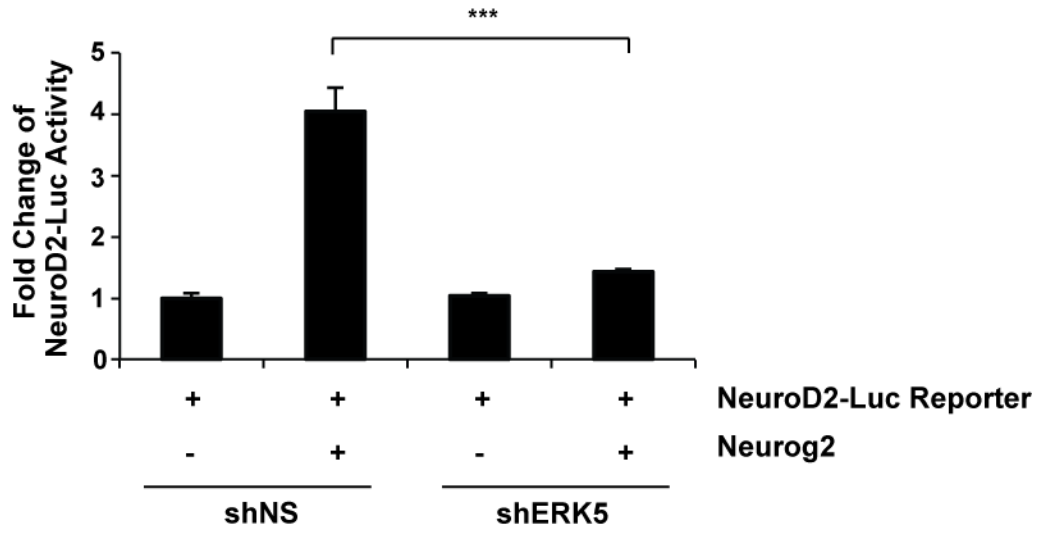


Figure 2.6. Neurog2 transcriptional activity requires ERK5 activity. Rat embryonic day 15 (E15) primary cortical cells were transfected with plasmids for NeuroD2-Luc reporter only, or together with a Neurog2 expression vector in pcDNA, shERK5 retroviral plasmid or shNS control plasmid.

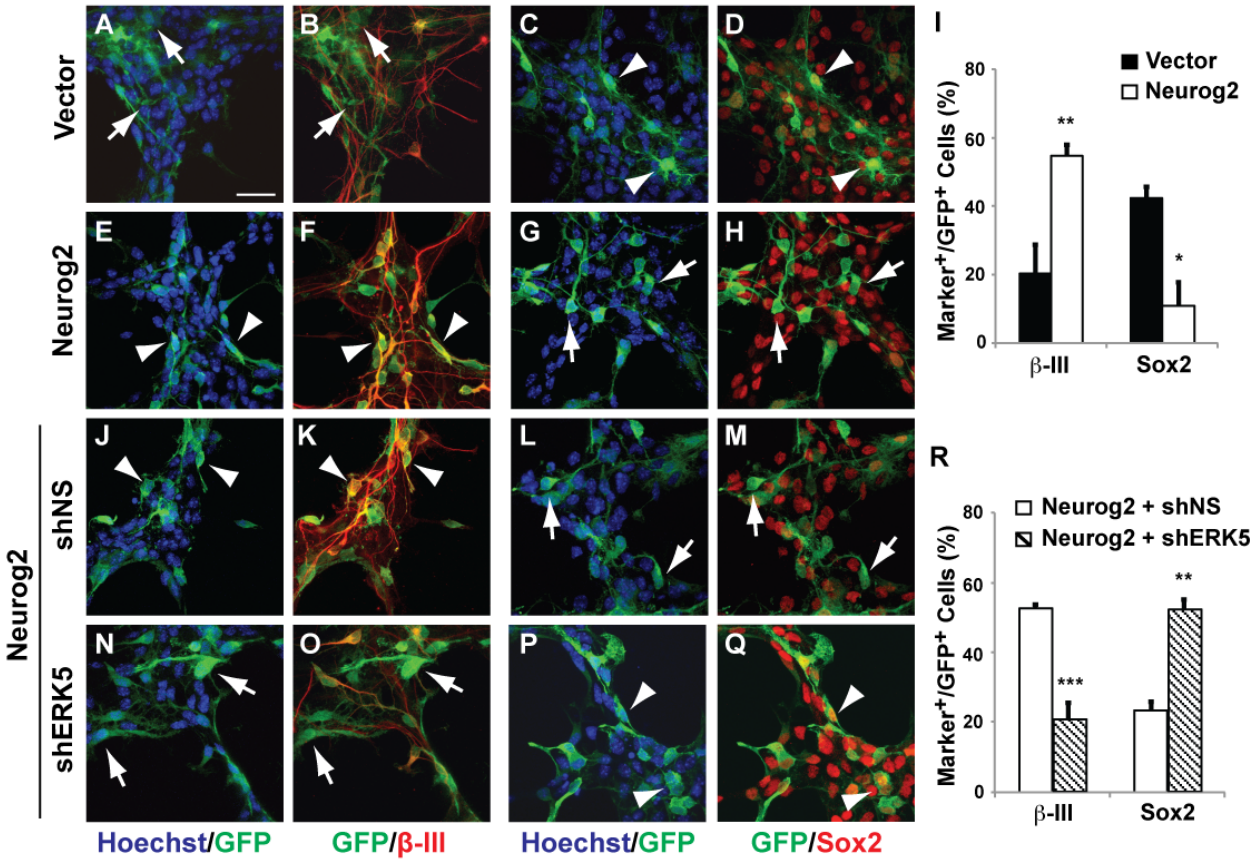


Figure 2.7. Neurog2 confers ERK5-dependent pro-neural activity in SGZ-derived aNPCs. SGZ-derived aNPCs were infected with control retroviral and lentiviral vectors expressing eGFP only (A–D), lentiviral Neurog2 only (E–H), lentiviral Neurog2 together with control retroviral shNS (J–M) or shERK5 (N–Q). One day after virus infection, cells were washed and then maintained in regular EGF- and bFGF-containing medium for 5 d before co-immunostaining for GFP (green) and various cellular markers (red) including β-III tubulin or Sox2 as indicated. The percentage of GFP^+ cells that also express β-III tubulin or Sox2 were quantified (I, R). Scale bar in A represents 25 μm and applies to all images. Arrowheads point to GFP^+ co-labeled cells, while arrows point to GFP^+ cells that are not co-labeled with cell-type specific markers.

NT3 promotes neuronal differentiation of SGZ-derived aNPCs in an ERK5-dependent manner

To begin to identify upstream activators of ERK5 signaling that regulate SGZ adult neurogenesis, we determined if ERK5 is activated by neurotrophins in aNPCs. Treatment with either BDNF or NT3 induced phosphorylation of ERK5 (p-ERK5) in SGZ-derived aNPCs (Fig. 2.8A), indicative of ERK5 activation. NT3 also increased the number of β-III tubulin$^+$ neurons (Fig. 2.8B), suggesting that NT3 stimulates neuronal differentiation of these cells. Significantly, the effect of NT3 on neuronal differentiation was completely blocked by shERK5. Additionally, shERK5 inhibited neuronal differentiation when aNPCs were pre-treated with BDNF or NT3 to prime neuronal differentiation (Fig. 2.9). These data suggest that NT3 stimulates hippocampal neurogenesis by activating the ERK5 signaling pathway.

Activation of endogenous ERK5 promotes SGZ neurogenesis in vivo

To investigate if activation of ERK5 promotes adult neurogenesis *in vivo*, we delivered retroviruses encoding caMEK5-IRES-eGFP and/or wtERK5-IRES-eGFP as well as the vector-control retrovirus to the dentate gyrus of adult mice using a stereotaxic surgery protocol (Athos and Storm, 2001).

Two weeks following stereotaxic surgery, mice were sacrificed and their brains processed for immunohistochemistry to identify GFP$^+$ infected cells and cells expressing NeuroD (Fig. 2.10A–I). Retroviral expression of caMEK5 alone or together with wtERK5 *in vivo* significantly increased the number of NeuroD$^+$ cells in the retrovirus-infected cell population (GFP$^+$) along the SGZ (Fig. 2.10J). The fact that co-expression of wtERK5 with caMEK5 did not further increase the number of NeuroD$^+$ cells relative to caMEK5 alone suggests that caMEK5 is sufficient to activate enough endogenous ERK5 to stimulate neurogenesis *in vivo*.

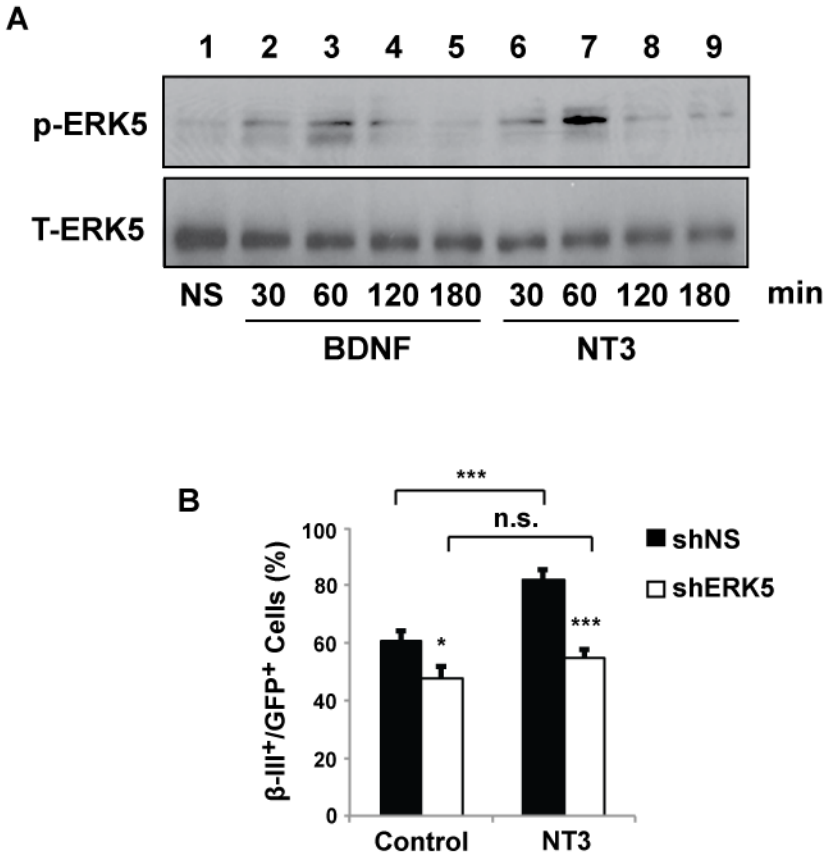


Figure 2.8. NT3 activates ERK5 and stimulates neuronal differentiation of SGZ-derived aNPCs through ERK5. (A) BDNF and NT3 induce ERK5 phosphorylation, indicative of ERK5 activation. SGZ-derived aNPCs were treated with vehicle control (NS, lane 1), 50 ng/mL BDNF (lanes 2–5), or 100 ng/mL NT3 (lanes 6–9) for indicated times. Total ERK5 (T-ERK5) was used as a loading control. (B) NT3 stimulates neuronal differentiation of SGZ-derived aNPCs through a process that requires ERK5. SGZ-derived aNPCs were infected with retroviruses encoding a non-specific control (shNS) or shERK5. Cells were then incubated in EGF/bFGF-free medium and treated with NT3 (100 ng/ml) for 5 d to induce neuronal differentiation. Cells treated with BSA (1 μg/ml) were used as a control.

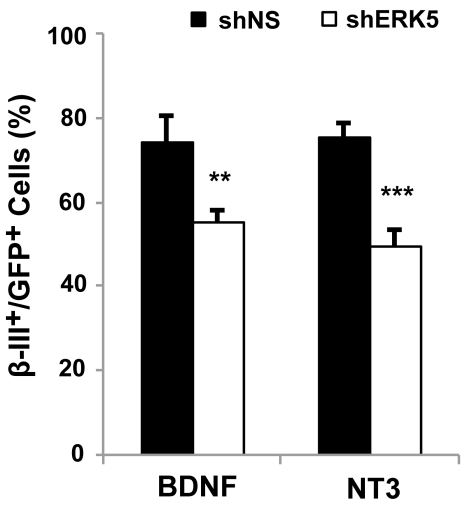


Figure 2.9. shRNA knock down of ERK5 inhibits neuronal differentiation in more differentiated aNPCs. SGZ-derived aNPCs were cultured for 3 d in culture medium containing BDNF (50 ng/mL) or NT3 (100 ng/mL) to prime cells for neuronal differentiation. EGF and bFGF were then withdrawn from the medium and cells were cultured in the continued presence of BDNF or NT3 for an additional 3 d to allow differentiation to begin. Cells were then infected with control (shNS) or shERK5 retroviruses, and maintained in BDNF- or NT3-containing media (free of EGF and bFGF) for an additional 5 d. Neuronal differentiation was examined by β-III tubulin expression in virus-infected cells (GFP^+)

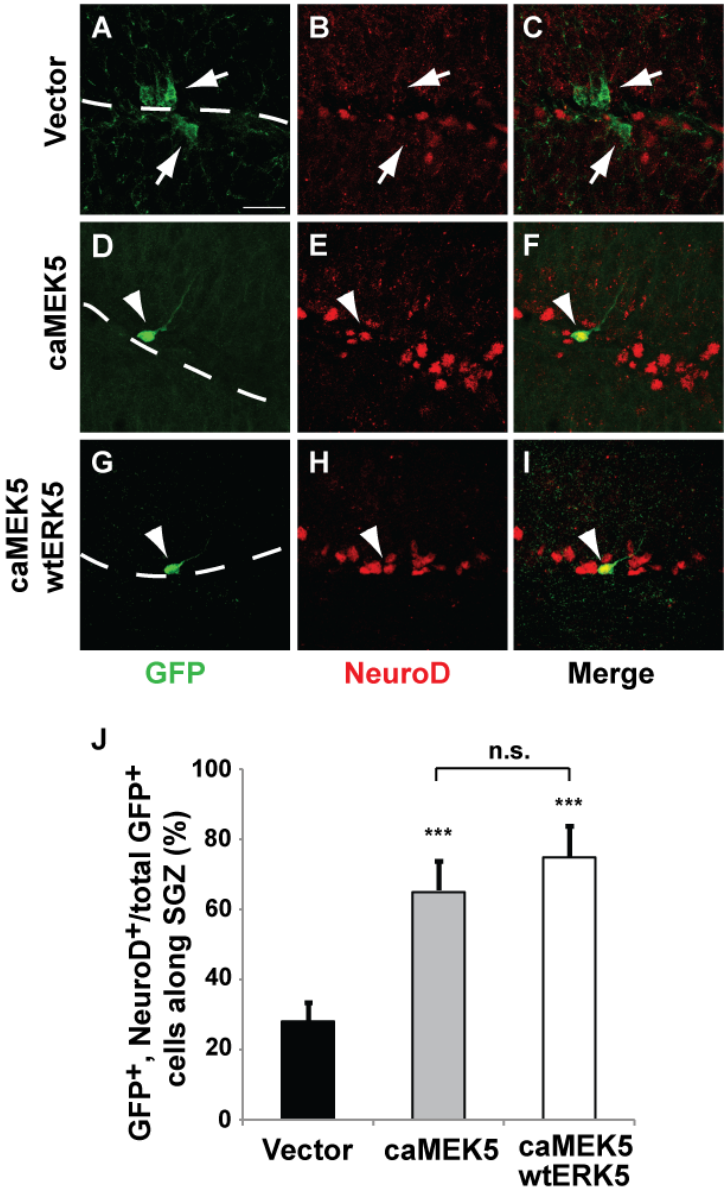


Figure 2.10. Ectopic activation of ERK5 promotes SGZ neurogenesis *in vivo*. Retroviral eGFP control (vector) (A–C), caMEK5-IRES-eGFP alone (D–F) or together with wtERK5-IRES-eGFP (G–I) was stereotaxically injected into the dentate gyrus of 8-10 week old mice. Mice were sacrificed 2 weeks later and brain sections immunostained for GFP (green) or NeuroD (red). Scale bar in A represents 25 μm and applies to all images. Dashed lines outline the SGZ layer of the dentate gyrus. Arrowheads point to $GFP^+/NeuroD^+$ co-labeled cells, while arrows point to GFP^+ cells that are negative for NeuroD. (J) The percentage of GFP^+ and $NeuroD^+$ co-labeled cells along the SGZ was quantified. Expression of caMEK5 alone or together with wtERK5 greatly increases the number of $NeuroD^+$ cells in total GFP^+ population.

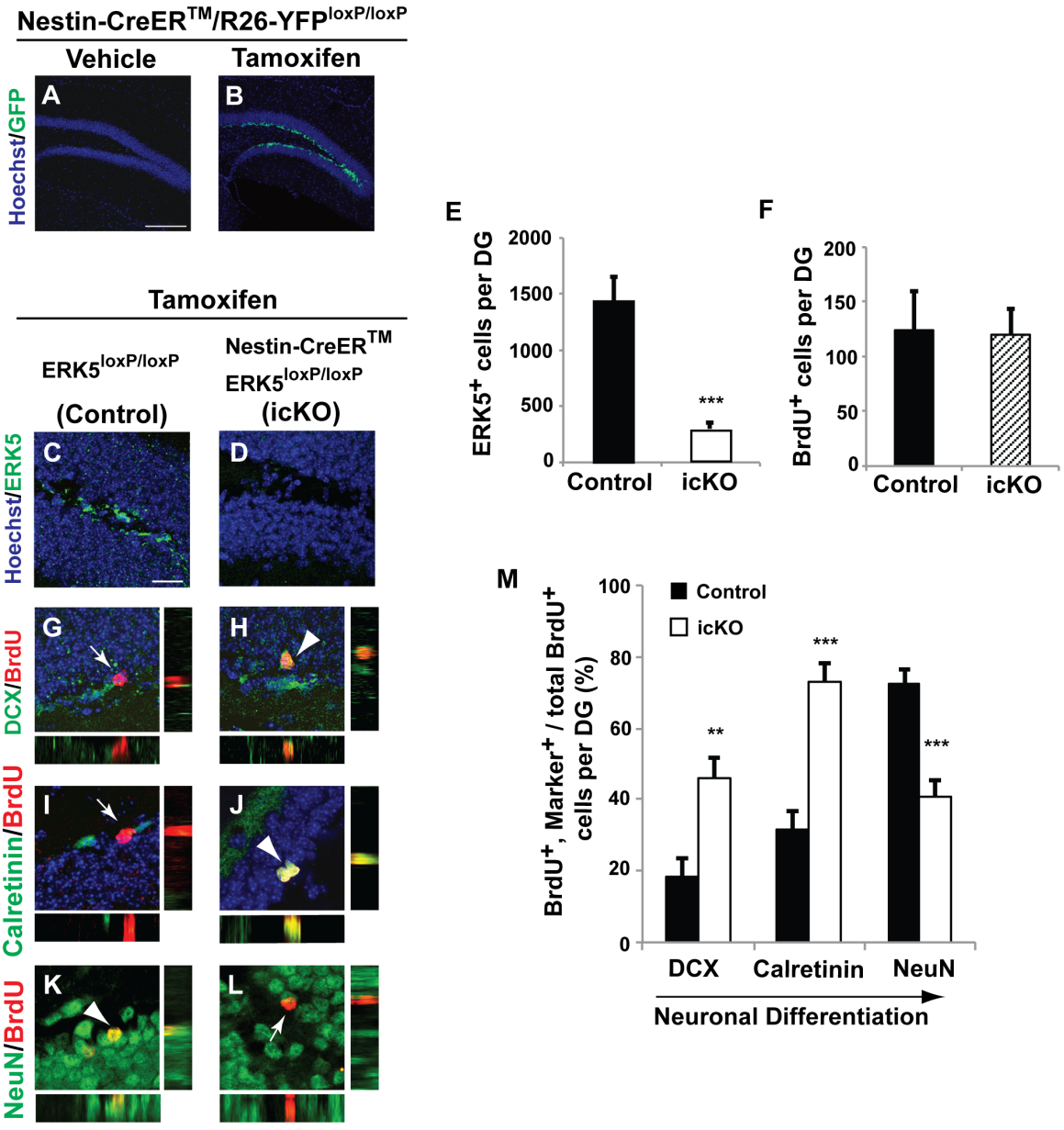
Inducible and conditional deletion of ERK5 in adult neurogenic regions reduces adult hippocampal neurogenesis by attenuating neuronal differentiation

To determine if ERK5 signaling is required for adult neurogenesis *in vivo*, we utilized the inducible and conditional ERK5 knock out (icKO) mice we recently generated (Pan et al., 2012a). Tamoxifen administration into adult Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice specifically deletes the *erk5* gene in Nestin- expressing neural stem cells, thereby avoiding potential deleterious effects on the entire brain during embryonic development. Male Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ and female ERK5$^{loxP/loxP}$ mice were mated to generate experimental animals (Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ and ERK5$^{loxP/loxP}$ mice). The general health and overall appearance were not different among littermates before or after tamoxifen treatment.

To confirm the specificity of tamoxifen-induced, Nestin-CreER^{TM}-mediated recombination, we crossed Nestin-CreER^{TM} with R26-YFP reporter mice where Cre-mediated recombination removes a transcriptional STOP to allow YFP expression (Srinivas et al., 2001). Tamoxifen or vehicle was administered to Nestin-CreER^{TM}/R26-YFP$^{loxP/loxP}$ mice, and animals were sacrificed 10 d after the last dose of tamoxifen. We observed no YFP expression in the mouse brain of vehicle control treated mice (Fig. 2.11A). In contrast, there were abundant YFP^{+} cells along the SGZ of tamoxifen treated mouse brains (Fig. 2.11B). Furthermore, YFP^{+} cells in tamoxifen treated mice were restricted to the adult neurogenic regions only (data not shown). These data suggest that Cre-ER^{TM}-mediated recombination is specific to adult neurogenic regions and there is no discernible leakiness of the Cre-recombinase from the Nestin-CreER^{TM} driver.

Tamoxifen was administered to male Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ (ERK5 icKO) and ERK5$^{loxP/loxP}$ (control) mice to induce Cre-mediated recombination of *erk5* in Nestin-expressing neural stem cells.

Figure 2.11. Conditional deletion of ERK5 in adult neurogenic regions attenuates neuronal differentiation and SGZ neurogenesis. (A, B) YFP immunostaining of Nestin-CreER^{TM}/R26-YFP$^{loxP/loxP}$ reporter animals treated with tamoxifen or vehicle control, demonstrating the specificity and effectiveness of Nestin-Cre-ER^{TM}-mediated recombination. (C, D) Representative confocal images of ERK5 immunostaining (green) in the SGZ for tamoxifen-treated control (ERK5$^{loxP/loxP}$) or ERK5 inducible and conditional knockout (icKO) mice (Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$). Hoechst staining (blue) was used to visualize all nuclei. (E) Quantification of total ERK5^{+} cells per dentate gyrus. The number of ERK5^{+} cells in the SGZ was reduced in ERK5 icKO mice. (F) Quantification of total BrdU^{+} cells per dentate gyrus 4 weeks following BrdU administration. (G–L) Representative confocal images of immunostaining in the SGZ of control and ERK5 icKO mice for BrdU (red) and DCX (green) (G, H), Calretinin (green) (I, J), or NeuN (green) (K, L). Cells co-labeled with BrdU and DCX, Calretinin, or NeuN are yellow (arrow heads). Side panels to G–L are orthographic views of the corresponding cells in the main panels. Arrows point to BrdU^{+} cells that are negative for DCX, Calretinin, or NeuN. (M) ERK5 gene deletion increases the total number of immature adult-born neurons (DCX and BrdU double-positive; Calretinin and BrdU double-positive cells among total BrdU^{+} cells) while concomitantly decreasing the total number of adult-born mature neurons (BrdU and NeuN double-positive cells among total BrdU^{+} population) along the SGZ. Scale bar in panel A represents 100 μm and applies to B while scale bar in panel C represents 25 μm and applies to D–L.


Nestin-CreER^{TM}/R26-YFP$^{loxP/loxP}$
Vehicle
Tamoxifen
Hoechst/GFP
A
B
Tamoxifen
ERK5$^{loxP/loxP}$
(Control)
Nestin-CreER^{TM}
ERK5$^{loxP/loxP}$
(icKO)
Hoechst/ERK5
C
D
DCX/BrdU
G
H
Calretinin/BrdU
I
J
NeuN/BrdU
K
L
E
ERK5^{+} cells per DG
2000
1500
1000
500
0
***
Control
icKO
F
BrdU^{+} cells per DG
200
150
100
50
0
Control
icKO
M
BrdU^{+}, Marker^{+} / total BrdU^{+} cells per DG (%)
100
80
60
40
20
0
Control
icKO
**
***
***
DCX
Calretinin
NeuN
Neuronal Differentiation

To label adult-born cells, BrdU was administered 7 d after the last dose of tamoxifen treatment and mice were sacrificed 4 weeks later. Treatment with tamoxifen effectively decreased the number of $ERK5^+$ cells in the SGZ of ERK5 icKO mice by 75% compared to control animals (Fig. 2.11C–E). There was no difference in the total number of $BrdU^+$ cells in the dentate gyrus of control vs. ERK5 icKO mice (Fig. 2.11F). However, deletion of the *erk5* gene reduced the number of adult-born, mature neurons (NeuN and BrdU double-positive cells among total $BrdU^+$ population) in the dentate gyrus (Fig. 2.11K–M). Concomitantly, there was an increase in the number of $BrdU^+$ cells co-labeled with DCX or Calretinin, a marker for immature granular neurons (Fig. 2.11G–J, M). This suggests that although ERK5 deletion does not change the total number of adult-born cells in the SGZ, it reduces the total number of adult-born mature neurons by delaying neuronal differentiation.

DISCUSSION

New neurons are continuously born in the adult dentate gyrus of the hippocampus. Although these adult-born neurons have been characterized at the cellular level, signaling mechanisms regulating adult hippocampal neurogenesis are not well defined. The goal of this study was to investigate the role of ERK5 MAP kinase in the regulation of adult hippocampal neurogenesis.

Despite its abundant presence in the developing brain, ERK5 expression declines as the brain matures (Liu et al., 2003a) and it is generally thought to be absent in the adult brain ((Di Benedetto et al., 2007) and Allen Brain Atlas). We report here, that although ERK5 expression is generally absent in most areas of adult brain, it is prominently expressed in the two neurogenic regions. The expression of ERK5 MAP kinase is quite unique and distinct from other signaling molecules implicated in adult neurogenesis, such as NeuroD, sonic hedgehog, Wnt, PI3K-Akt and BDNF, which are more widely expressed in the brain (Kenney et al., 2004; Lie et

al., 2005; Jiao and Chen, 2008; Bruel-Jungerman et al., 2009; Gao et al., 2009; Jessberger et al., 2009; Kuwabara et al., 2009; Ma et al., 2009; Suh et al., 2009). It also suggests that ERK5 may be critical in the regulation of adult neurogenesis. Indeed, shRNA knockdown of ERK5 in cultured aNPCs or conditional deletion of the *erk5* gene specifically in the neurogenic regions of the adult mouse brain reduces neurogenesis *in vitro* and *in vivo*, respectively. By contrast, ectopic activation of endogenous ERK5 signaling via expression of caMEK5 promotes neurogenesis in cultured aNPCs as well as in the dentate gyrus of mouse brains. These data suggest that ERK5 signaling is an important regulator of adult neurogenesis in SGZ cells both *in vitro* and *in vivo.*

Although ERK5 expression in the SGZ of the adult mouse brain is found in cells expressing markers for neural stem/progenitor cells, actively proliferating cells, transiently amplifying progenitors and/or newborn neurons, the majority of ERK5-positive cells express markers for transiently amplifying progenitors and/or newborn neurons. These data indicate that ERK5 may primarily regulate adult hippocampal neurogenesis through its action on these cell populations, including affecting neuronal differentiation and maturation. Indeed, shRNA knockdown of ERK5 in cultured aNPCs reduces the number of newborn neurons while simultaneously increasing the number of proliferating cells and progenitor cells. These data, coupled with the fact that shERK5 did not increase the number of $GFAP^+$ astrocytes suggest that inhibition of ERK5 attenuates neuronal differentiation without causing precocious glial differentiation. Using transgenic mouse technology, we conditionally deleted the *erk5* gene specifically in Nestin-expressing neural stem cells in the adult brain. Although this inducible and conditional gene targeting of *erk5* did not affect the total number of adult-born cells ($BrdU^+$) in the dentate gyrus *in vivo*, it reduced the number of adult-born mature neurons ($BrdU^+$ and $NeuN^+$) while concomitantly increasing the number of cells expressing immature neuron

markers DCX and Calretinin. These data suggest that loss of ERK5 causes a delay in the normal progression of neuronal differentiation and maturation during adult neurogenesis.

Neural progenitors in the SGZ express a cascade of transcription factors including Neurog2 (Ozen et al., 2007; Hodge et al., 2008). Neurog2 belongs to a family of bHLH transcription factors that also includes Neurog1 and are critical for neuronal fate specification during development (Ross et al., 2003). Neurog2 is essential for neurogenesis in the dentate gyrus during development (Galichet et al., 2008). However, its role in adult SGZ neurogenesis has not been demonstrated. Here we report that ectopic expression of Neurog2 is sufficient to promote neuronal differentiation of SGZ-derived aNPCs in culture, providing evidence that Neurog2 may confer pro-neural activity during adult hippocampal neurogenesis. We previously reported that ERK5 regulates both the transcriptional and pro-neural activities of Neurog1 during cortical development (Cundiff et al., 2009). This prompted us to investigate if ERK5 regulates adult hippocampal neurogenesis through Neurog2. Indeed, shRNA suppression of ERK5 signaling inhibited both the transcriptional and pro-neural activities of Neurog2 in cultured SGZ-derived aNPCs. Although it is possible that ERK5 could act downstream, in parallel, or upstream of Neurog2, the fact that ERK5 activity is required for Neurog2-stimulated transcription favors the interpretation that ERK5 is an upstream regulator of Neurog2.

What are the upstream extracellular signals that ERK5 responds to in regulating adult hippocampal neurogenesis? We have published evidence that ERK5 is activated by neurotrophins including brain-derived neurotrophic factor (BDNF) and NT3 in neurons (Cavanaugh et al., 2001). Interestingly, BDNF/TrkB signaling has been implicated in regulating adult hippocampal neurogenesis both under normal physiological conditions and after experimental manipulations such as voluntary exercise and chronic treatment with antidepressants (Takahashi et al., 1999; Lee et al., 2002; Shirayama et al., 2002; Russo-

Neustadt et al., 2004; Sairanen et al., 2005; Scharfman et al., 2005; Duman and Monteggia, 2006; Rossi et al., 2006; Warner-Schmidt and Duman, 2006; Bergami et al., 2008; Chan et al., 2008; Li et al., 2008). However, its downstream mechanisms have not been elucidated. We hypothesized that neurotrophins may activate ERK5 in SGZ-derived aNPCs and promote neuronal differentiation through ERK5. Indeed, ERK5 is activated by both BDNF and NT3 in these cells, and shRNA inhibition of ERK5 signaling suppresses the neuronal differentiation effect of NT3.

In summary, findings in this study identify ERK5 MAP kinase as a novel signaling pathway regulating adult hippocampal neurogenesis, especially in neuronal differentiation. Furthermore, Neurog2 may be a downstream target while NT3 may be an upstream activator of ERK5 in this process. Because ERK5 gene deletion is temporally and spatially regulated and specific to adult neural stem cells in the brain of the Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mouse, this ERK5 icKO mouse strain provides a unique and powerful tool to investigate the relationship between adult neurogenesis and hippocampus-dependent learning and memory.

Chapter 3

Inhibition of Adult Neurogenesis by Inducible and Targeted Deletion of ERK5 MAP Kinase Specifically in Adult Neurogenic Regions Impairs Contextual Fear Memory Extinction and Remote Fear Memory

INTRODUCTION

The mammalian brain has the remarkable capacity to process and store information. Since deficits in memory are prominent features of aging and many mental disorders, there is intense interest in the molecular and cellular basis of learning and memory. Several signal transduction pathways in neurons, including calcium, cAMP, and ERK1/2 MAP kinase have been implicated in the regulation of memory formation (Silva et al., 1992; Yin et al., 1994; Wu et al., 1995; Atkins et al., 1998; Taubenfeld et al., 1999; Wong et al., 1999; Athos et al., 2002). Interestingly, recent studies support the idea that hippocampus-dependent memory may also depend on newly generated neurons in the adult dentate gyrus.

Adult neurogenesis occurs in the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) along the lateral ventricles in mammalian brains (Altman and Das, 1965; Alvarez-Buylla et al., 1988; Ming and Song, 2005). Adult-born neurons in the dentate gyrus functionally integrate into the hippocampal circuitry (Ming and Song, 2005; Deng et al., 2010), suggesting a role in neuroplasticity. Hippocampus-dependent, but not hippocampus-independent learning increases adult neurogenesis in the dentate gyrus (Leuner et al., 2004). Furthermore, training for hippocampus-dependent memory selectively adds or removes adult-born neurons depending on the birthdate of the neurons relative to training (Dobrossy et al., 2003; Dupret et al., 2007). These data support the hypothesis that newly generated adult-born neurons of the dentate gyrus contribute to hippocampus-dependent memory (Deng et al., 2010; Leuner and Gould, 2010). Despite these exciting discoveries, signaling mechanisms regulating

adult neurogenesis are not fully understood. Additionally, although a number of studies have attempted to assess the role of adult neurogenesis in hippocampus-dependent memory, data from different studies are contradictory, and the function of adult-born neurons in the dentate gyrus remains controversial (Deng et al., 2010; Leuner and Gould, 2010).

ERK5 is a member of the MAP kinase family that includes ERK1/2 (Lee et al., 1995; Zhou et al., 1995). Neurotrophin activation of ERK5 promotes the survival of newborn neurons during embryonic development (Cavanaugh et al., 2001; Watson et al., 2001; Liu et al., 2003a; Shalizi et al., 2003; Wang et al., 2006b; Finegan et al., 2009). ERK5 also specifies cortical stem/progenitor cells toward a neuronal lineage during development by phosphorylating and modulating the activity of neurogenin (Neurog 1) (Liu et al., 2006; Cundiff et al., 2009). ERK5 expression in the brain is high during early embryonic development but declines as the brain matures (Liu et al., 2003a). It is generally thought that ERK5 is not expressed in the adult brain (Di Benedetto et al., 2007). However, upon closer examination we discovered selective ERK5 expression in the adult neurogenic regions, the SGZ and SVZ. This pattern of expression is unique and suggests an important role for ERK5 in the regulation of adult neurogenesis.

Here, we generated ERK5 icKO mice to delete *erk5* specifically in the neurogenic regions of the adult brain. This mouse strain provides a unique and powerful tool to investigate the role of ERK5 in adult neurogenesis and to delineate the relationship between adult neurogenesis and hippocampus-dependent learning and memory.

MATERIALS AND METHODS

Animals

Nestin-CreER^{TM} (Kuo et al., 2006) mice and ERK5$^{loxP/loxP}$ (Wang et al., 2005) mice were crossed to yield Nestin-CreER^{TM}/ERK5$^{loxP/+}$ animals. Nestin-CreER^{TM}/ERK5$^{loxP/+}$ mice were

further crossed with ERK5$^{loxP/loxP}$ mice to yield homozygous Nestin-CreER^{TM}/ERK5$^{lox/P/loxP}$ animals, which were used for experimental breeding. All animal experiments were performed with identically treated and handled littermate controls. Animals were housed under standard conditions (12 h light/dark cycle) with food and water provided *ad libitum* except where indicated. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Reagents

The following primary antibodies and dilutions were used for immunohistochemistry: rat monoclonal anti-BrdU (1:500, AbD Serotec); mouse monoclonal anti-NeuN (1:500, Millipore); and goat polyclonal anti-NeuroD (1:200, Santa Cruz Biotech Inc.). Rabbit polyclonal ERK5 antibody (1:500) was generated previously (Cavanaugh et al., 2001) and affinity purified using recombinant MBP-ERK5 protein.

BrdU and tamoxifen administration

Mice received 100 mg/kg BrdU (Sigma) by intraperitoneal (IP) injection 5 times (every 2 h for 10 h) in one day followed by sacrifice 3 weeks later to identify BrdU-retaining, adult-born cells. Tamoxifen (Sigma) was made fresh daily and dissolved in 2% glacial acetic acid in corn oil solution (Sigma). To activate Cre-mediated recombination, 5 mg of pre-warmed tamoxifen was administered orally to 10–12 week-old male mice daily for 7 days (for studies presented in Fig. 3.2 and Fig. 3.3A–F, M) or once per day for 4 d in each cycle, for 3 cycles with 2-week inter-cycle intervals (for studies presented in Fig. 3.3G–M, and Fig. 3.4–3.11).

Immunohistochemistry (IHC)

Mice were perfused intracardially with ice cold solutions of 20 ml PBS followed by 20 ml 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were harvested and post-fixed in 4% PFA/PBS overnight at 4°C, followed by 30% (w/v) sucrose in PBS solution at 4°C until brains sunk. Immediately after sucrose embedding, brains were frozen at -80°C until IHC processing. IHC was performed on 30 µm-thick coronal brain sections using a free-floating antibody staining method. Briefly, brain sections were washed 4 x 10 min with 0.1 M, pH 7.4 PBS, followed by 3 x 10 min with PBST (PBS + 0.25% Triton X-100). Brain slices were then incubated in blocking buffer (PBST + 0.1% bovine serum album (BSA) + 10% normal serum) for >2 h at room temperature. Where IHC for BrdU visualization was required, brain sections were washed 4 x 10 min with 0.1 M, pH 7.4 PBS, then subjected to HCl treatment by sequential incubation in water for 5 min, ice-cold 1 N HCl for 10 min, 2 N HCl for 30 min at 37°C, and finally neutralized by rinsing 2 x 5 min in 0.5 M borate buffer (pH 8.5). This was followed by PBST washes and blocking as stated above. Brain sections were incubated with primary antibodies for 48–60 h at 4°C on a platform shaker. Brain sections were washed in PBST 4 x 10 min after primary antibody incubation and incubated with secondary antibodies conjugated with Alexa Fluor dyes (1:500 dilution, Invitrogen) in blocking buffer overnight at 4°C on a platform shaker. Brain sections were then washed 4 x 10 min in PBST and incubated with 2.5 µg/mL Hoechst 33342 (Invitrogen) for 30 min and washed 3 x 5 min with PBST. Unless otherwise stated, all IHC procedures were carried out at room temperature. Brain sections were then mounted on gelatin-coated Superfrost plus slides (VWR) with anti-fade Aqua Poly/Mount (Polysciences).

Confocal imaging and analysis

All images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with numerical aperture (NA) 0.75, 20X lens, NA 1.3, 40X oil lens, or NA 1.35, 60X oil immersion lens. Optical Z-sections (0.5–1 µm) were collected and processed using ImageJ

software (NIH). Images were uniformly adjusted for color, brightness, and contrast with Adobe Photoshop CS4 (Adobe Systems, Inc).

Quantification of immunostained cells

One in every 8 serial brain slices through the entire rostral-caudal extent of the granule cell layer was immunostained for each marker. Immunopositive cells were analyzed and quantified using a modified unbiased stereology technique (West et al., 1991; Kempermann et al., 1997b; Malberg et al., 2000) by confocal analysis of the granule cell layer of one half of the brain with the experimenter blinded to treatment conditions. Resulting numbers were multiplied by 8 to obtain an estimated total number of cells per dentate gyrus. For colocalization analysis, cells were analyzed per marker combination using confocal microscopy. At least 50 immunopositive cells were selected randomly and the presence of overlapping fluorescent signals in a Z-series stack of a single cell was considered to be a double-positive cell.

Statistical analysis

All data are expressed as mean ± standard error of the means (s.e.m.) from at least 2 independent experiments ($n \geq 10$ for each treatment group/genotype) for *in vivo* behavior experiments, or $n \geq 3$ for *in vivo* cellular quantification data. Student's *t*-test was used to analyze *in vivo* cellular data. Two-way ANOVA with repeated measures was used to analyze data for all the water maze tests (except the probe test) and fear extinction assays. One-Way ANOVA with *Fisher's LSD* post-hoc analysis (Alpha=0.05) was used to analyze all other behavior data. n.s. not significant ($p>0.05$); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Behavior Assays

Open field test and habituation

Mice were placed in a 10”(W) x 10”(D) x 16”(H) TruScan Photo Beam Tracking arena with clear sidewalls and infrared beams spaced 0.6” apart providing a spatial resolution of 0.3” (Coulbourn). Mice were not pre-habituated to the arena to permit baseline locomotor activity and anxiety level analysis. Twenty minutes of free exploratory time were allotted per mouse on day one for the open field test. Data was collected and analyzed using TruScan 2.02 software (Coulbourn). To analyze habituation in the open field, mice were placed in the same arena 24 h and 48 h later for 20 min each.

Novel object recognition

Mice were placed into a 10”(W) x 10”(D) x 16”(H) contextual arena with clear sidewalls (Coulbourn) containing 2 objects (A and B) made of plastic material for a 5 min training session and then returned to their home cages. For short-term memory retention, mice were placed back into the same arena one hour later with one of the previous objects (i.e. A) and a novel object (C) for a 5-min testing session. For longer term memory retention at 24 h or 48 h, a different cohort of mice were trained as above except that training lasted 10 min and testing was performed either 24 h or 48 h later, respectively, during a 10-min testing session. A different set of objects was used for the 24 h and 48 h memory tests. Exploratory activity of each object was recorded for both training and testing sessions and analyzed by experimenters blinded to genotype and treatment group.

Standard cued and contextual fear conditioning and contextual fear extinction

Mice were placed in a 10”(W) x 10”(D) x 16”(H) square-shaped arena fitted with a metal grid shock floor (Coulbourn). On the day of training, each mouse was placed in the training context (with striped wallpaper) and allowed to freely explore for 2 min. A 90 dB tone, the

conditioned stimulus (CS), was then presented for 30 s. During the final 2 s of tone presentation, a 0.7 mA foot shock, the unconditioned stimulus (US), was delivered. CS and US were delivered automatically using tone generator and shocker controlled by TruScan software (Coulbourn). Mice were then returned to their home cages. Twenty-four hours later, contextual fear conditioning test was performed in the training room, where mice were placed in the same context arena without any foot shock for 2 min. Two hours after the contextual test, mice were subjected to a cued test. Mice were placed in a novel context (triangular shaped arena with solid grey plastic walls) in a different room and allowed to freely explore for 2 min. The CS (tone) was then presented for 2 min. Two hours after the cued test, mice were subjected to a control test in which they were placed in another novel context (hexagonal shaped arena with clear plastic walls) in a third room for 2 min with no presentation of either tone or foot shock. Freezing behavior was recorded manually every 5 s for each of the 2 min assessment periods for the three tests. Freezing behavior was defined as lack of bodily movement with all 4 paws remaining stationary on the floor except normal respiration. Data was collected and analyzed with experimenters blinded to genotype and treatment group.

One day following cued and contextual fear conditioning tests, mice were placed in the training context without the foot shock for two extinction trials each day, with a 4 h inter-trial interval. Each extinction trial lasted a total of 10 min and freezing behavior was recorded during the final 2 min of each trial. One day after the completion of the 8 d fear extinction trials, mice were placed in a novel context (trapezoid shaped arena with solid grey plastic walls) and freezing behavior was recorded for 2 min.

Modified cued and contextual fear conditioning using weaker electric shock

The same training and testing setup for cued and contextual fear conditioning was used as described above except that animals were subjected to 1-3 successive foot shocks, 0.3 mA, 2 s each and separated by 2 min inter-shock intervals.

Morris water maze assay

This was done as described with slight modifications (Wu et al., 1995; Zhang et al., 2008b). Mice were placed in a circular pool of water at room temperature (25°C) (1.2 meter diameter, 25 cm deep). The water was made opaque using non-toxic white paint. Three extra-maze cues, different in shape and size were uniformly spaced and placed on the wall surrounding the water tank. A small escape platform (13 cm x 8 cm) made of clear plexiglass was submerged just below the surface of the water and maintained in a fixed location for the entire training session. To begin a trial or test, mice were randomly started in drop zones, facing the wall, in any of the 3 quadrants without the platform. A total of 32 trials (4 trials per day for 8 consecutive days) were performed during the training session. Mice were allowed to swim for 40 s to find the platform, or guided to the platform after 40 s of the allotted maximum swim time was reached. Each trial ended after mice were allowed to stay on the escape platform for 15 s. A probe test was performed 24 h after the last training trial in which the escape platform was removed and mice were allowed to swim for 60 s in search of the escape platform. Reversal training ensued 24 h after the probe test for a total of 28 trials (4 trials per day for 7 consecutive days) where the escape platform was placed in the opposite quadrant from the initial training session. A reversal probe test was performed 24 h after the last trial of reversal training. Five hours after the reversal probe test, a visible platform test consisting of 4 trials was performed where a visible platform was placed above the water surface in a new quadrant other than the initial or reversal quadrants and mice were allowed to swim to locate the visible platform. In all sessions and tests, mice were allowed an inter-trial interval of 30 min. All data were collected

using ANYmaze software (San Diego Instruments) and analyzed offline with the experimenter blinded to treatment and genotypes.

Pattern separation assay

This was measured by the delayed-non-matching-to-place test using the 8-arm radial maze (Clelland et al., 2009; Guo et al., 2011). Mice were food restricted 4 d prior to beginning the assay until 85-90% of free-feeding body weight was reached. Each mouse was subjected to 4 trials per day for 5 consecutive days, with an inter-trial interval of 30 min. Each trial was divided into two phases, a training phase and a choice phase. During the training phase, 6 of 8 arms of the radial arm maze were blocked, leaving the sample arm and start arm open, separated by 90-degrees from each other. Food reward was placed at the end of the sample arm. Mice were placed in the maze facing the end of the start arm and allowed to freely explore for 3 min and then guided to the sample arm unless they retrieved the food reward from the sample arm before the 3 min cutoff time. Mice were allowed to eat for 60 s in the sample arm before being placed back in their home cage for approximately 30 s, during which time the maze was rotated 45 degrees and the arms cleaned with 5% acetic acid to minimize odor reference. The choice phase test was conducted immediately after cleaning, during which new sample and start arms were opened and the original arms were blocked, but the relative location of the arms during the training phase remained fixed. Additionally, a test arm was opened and separated by either 1-arm (separation 2) or 3-arms (separation 4) from the new sample arm. Each mouse was subjected to the same total number of separation 2 or 4 tests, although the two separations were presented randomly in sequence each day. Food reward was placed at the end of the test arm during the choice phase. Mice were placed in the maze facing the end of the start arm and allowed to make a choice in the maze. When a mouse entered the test arm, it was scored as a correct choice and the mouse was allowed to eat for 60 s before being removed from the maze. When a mouse entered the sample arm, re-entered the start arm, or did not make a choice

within 3 min, it was scored as an incorrect choice. On day 1 and day 2, mice that made an incorrect choice were allowed to self-correct and explore the maze for a maximum of 3 min before being guided to the test arm and allowed to eat for 60 s. On days 3-5, mice that made an incorrect choice were removed from the maze immediately and not allowed access to food reward. Correct or incorrect choices during the choice phase were scored manually with the experimenter blinded to experimental conditions.

Passive avoidance and remote memory assay

A light/dark chamber (Coulbourn) with a guillotine trap door separating the two chambers was used. A 60 W light bulb was placed over the opening of the light chamber, while the dark chamber was completely covered with black film. Mice were placed in the light chamber with the trap door closed and allowed to freely explore for 1 min. After which, the trap door was opened and the latency to cross over was recorded. Once mice crossed over into the dark chamber, the trap door was closed immediately followed by delivery of a mild foot shock (0.7 mA, 2 s); mice then remained in the dark chamber for an additional 1 min before being removed and placed in their home cage. Twenty-four hours later, mice were placed in the light chamber with the trap door closed and allowed to freely explore for 1 min. After 1 min, the trap door was opened and latency to cross over to the dark chamber was recorded with a maximum crossover latency cutoff of 5 min. For remote memory test, mice were tested 21 d post-training using the same procedure as the 24 h test.

RESULTS

ERK5 expression in the adult mouse brain

ERK5 expression was examined by immunohistochemistry using an affinity-purified ERK5-specific antibody. ERK5 protein was found along the SVZ and the SGZ of the dentate gyrus in the adult mouse brain (Fig. 3.1A–D). However, ERK5 was not expressed outside of the

adult neurogenic regions, including the cortex and striatum (Fig. 3.1E–H). This unique pattern of expression suggested an important function for ERK5 in adult neurogenesis.

Conditional targeting of ERK5 in adult neurogenic regions attenuates adult neurogenesis in the SGZ

To investigate a role for ERK5 signaling in adult neurogenesis *in vivo*, we crossed Nestin-CreER^{TM} mice (Kuo et al., 2006) with e*rk5* floxed allele mice (Wang et al., 2006a) to yield Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice. Upon tamoxifen treatment, the *erk5* gene is conditionally knocked out (ERK5 icKO) specifically in Nestin-expressing neural stem cells in the adult brain of this mouse strain. Thus, *erk5* deletion is temporally and spatially regulated and restricted to the adult neurogenic regions, limiting potential side effects on other areas of the brain during embryonic development.

Male Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice were treated with vehicle (control) or tamoxifen to induce Cre-mediated recombination of *erk5* in Nestin-expressing neural stem cells. In a separate set of experiments, tamoxifen administration to Nestin-CreER^{TM}/R26-YFP$^{loxP/loxP}$ reporter mice confirmed the specificity of tamoxifen-induced, Nestin-CreER^{TM}-mediated recombination (data not shown). Tamoxifen treatment did not affect the general health and overall appearance among littermates. A 7-day tamoxifen treatment decreased the number of ERK5^{+} cells in the SGZ by 65% compared to vehicle controls (Fig. 3.2A–D; *t*-test, $p<0.001$). This was accompanied by a significant reduction in the total number of NeuroD^{+} cells (Fig. 3.2E–G; *t*-test, $p=0.028$) and adult-born, mature neurons (NeuN and BrdU double-positive cells among total BrdU^{+} population) along the SGZ (Fig. 3.2H–J; *t*-test, $p=0.023$). NeuroD is a marker for transiently amplifying progenitors and/or newborn neurons while NeuN is a marker for mature neurons. These data suggest a critical role for ERK5 in promoting adult neurogenesis in the SGZ *in vivo*.

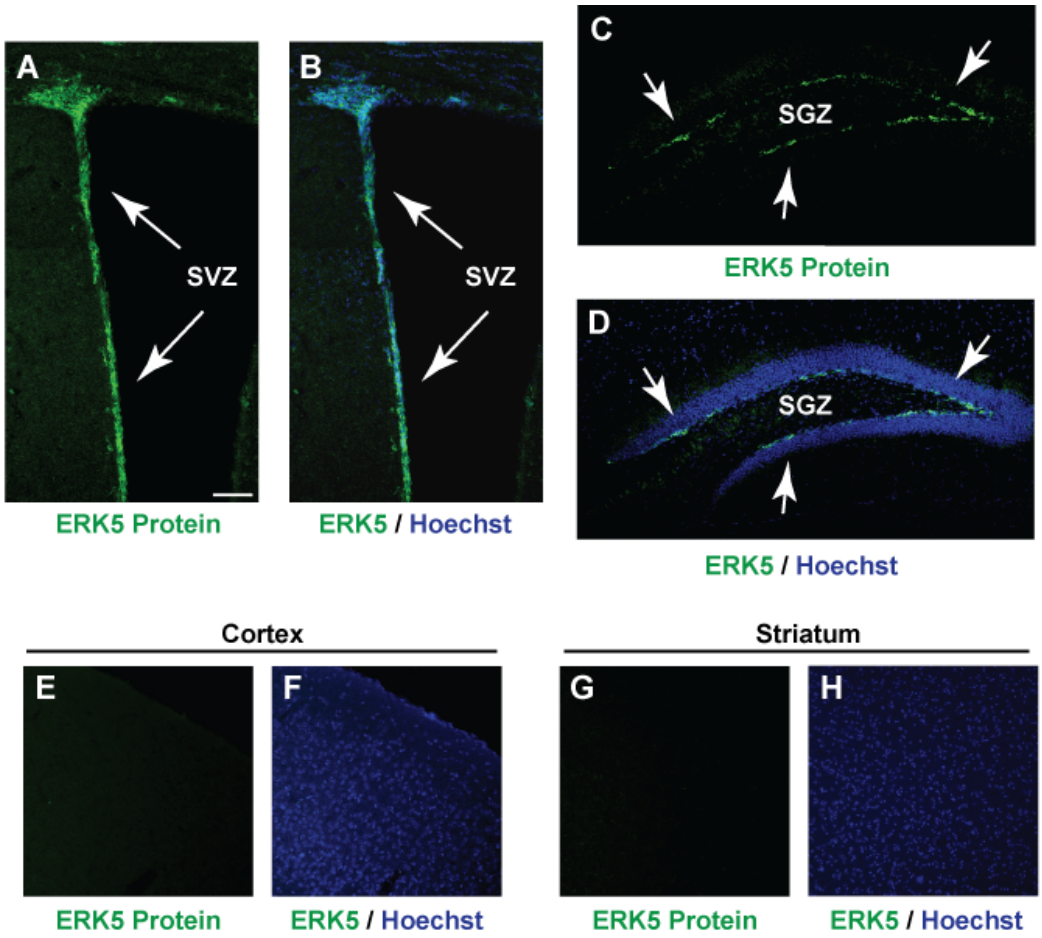
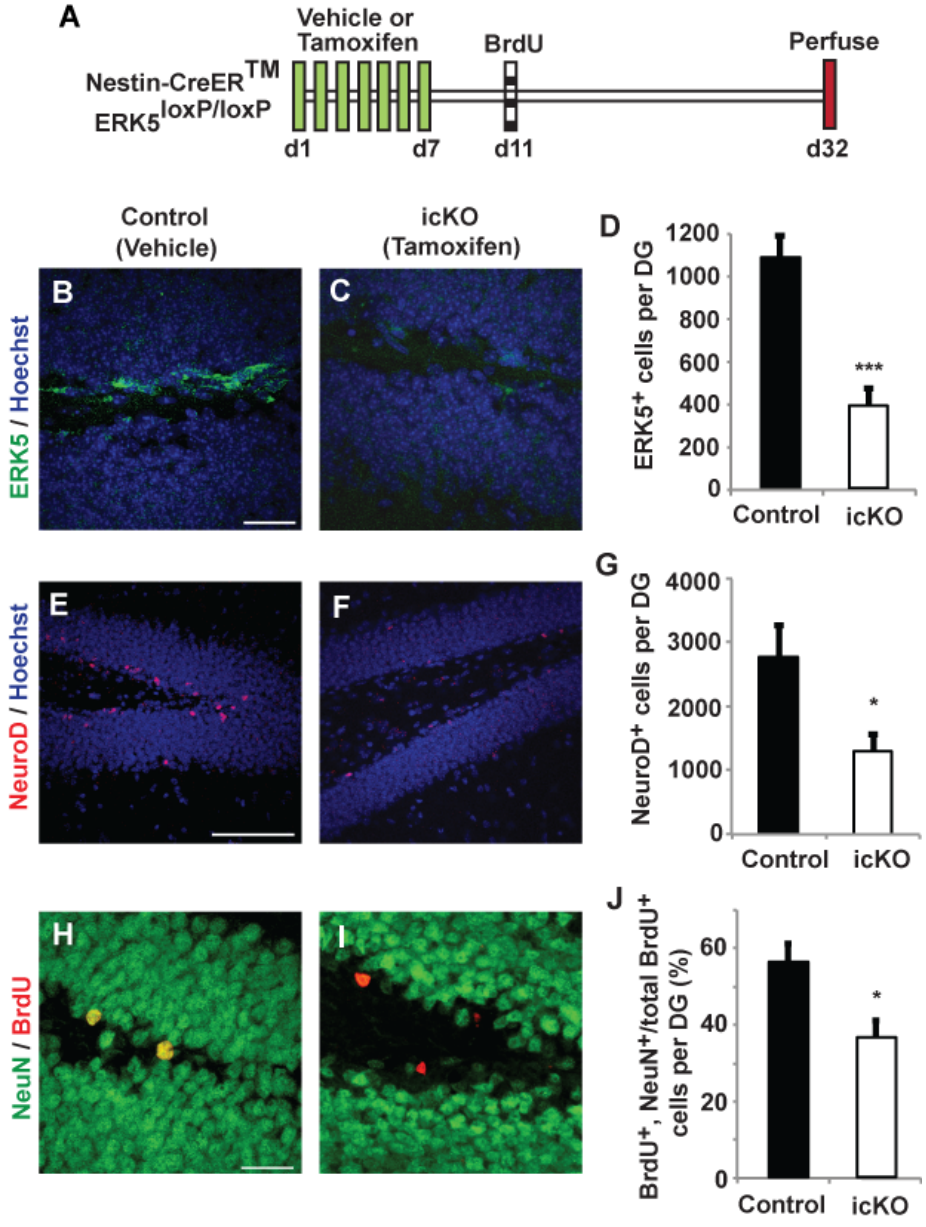


Figure 3.1. ERK5 is specifically expressed in the adult neurogenic regions in the mouse brain. Immunostaining of coronal sections of adult mouse brain tissue shows ERK5 protein expression (green) primarily in the SVZ (A, B) and SGZ of the hippocampus (C, D) but is absent in other brain regions such as the cortex (E, F) and striatum (G, H). Hoechst staining (blue) was used to identify all cell nuclei (B, D, F, H). Scale bar in A represents 100 μm and applies to all panels.

Figure 3.2. Targeted deletion of *erk5* specifically in adult neurogenic regions reduces SGZ neurogenesis. (A) Schematic illustration of experimental design. Tamoxifen or vehicle control was administered to 12-week old Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice orally, once per day for 7 d. BrdU was administered 4 d after the last dose of tamoxifen to label newborn neurons. Animals were sacrificed 3 weeks after BrdU injection for immunostaining. (B, C) ERK5 immunostaining (green) in the SGZ for vehicle-treated (control) or tamoxifen-treated (ERK5 icKO) Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice. (D) Quantification of total ERK5^{+} cells per dentate gyrus. The number of ERK5^{+} cells in the SGZ is reduced in ERK5 icKO mice. (E, F) Representative NeuroD immunostaining (red) in the SGZ of control and ERK5 icKO mice. Hoechst staining (blue) was used to visualize all nuclei. (G) Conditional ERK5 deletion in adult neurogenic regions greatly reduces the number of total NeuroD^{+} cells in the SGZ. (H, I) Representative BrdU (red) and NeuN (green) immunostaining in the SGZ of control and ERK5 icKO mice. BrdU and NeuN co-labeled cells are yellow. (J) *erk5* gene deletion decreases the total number of adult-born neurons (BrdU and NeuN double-positive cells among total BrdU^{+} population) along the SGZ. Graphs represent mean count of quantified cells ± (s.e.m.) from two independent experiments (n = 3 per treatment group per experiment). *, $p<0.05$; ***, $p<0.001$. Scale bars in panels B and H represent 25 μm and apply to panels C and I. Scale bar in E represents 100 μm and applies to F.


A
Vehicle or Tamoxifen
BrdU
Perfuse
Nestin-CreER^TM
ERK5^loxP/loxP
d1
d7
d11
d32
Control (Vehicle)
icKO (Tamoxifen)
B
C
ERK5 / Hoechst
D
ERK5+ cells per DG
1200
1000
800
600
400
200
0
***
Control
icKO
E
F
NeuroD / Hoechst
G
NeuroD+ cells per DG
4000
3000
2000
1000
0
*
Control
icKO
H
I
NeuN / BrdU
J
BrdU+, NeuN+/total BrdU+ cells per DG (%)
60
40
20
0
*
Control
icKO

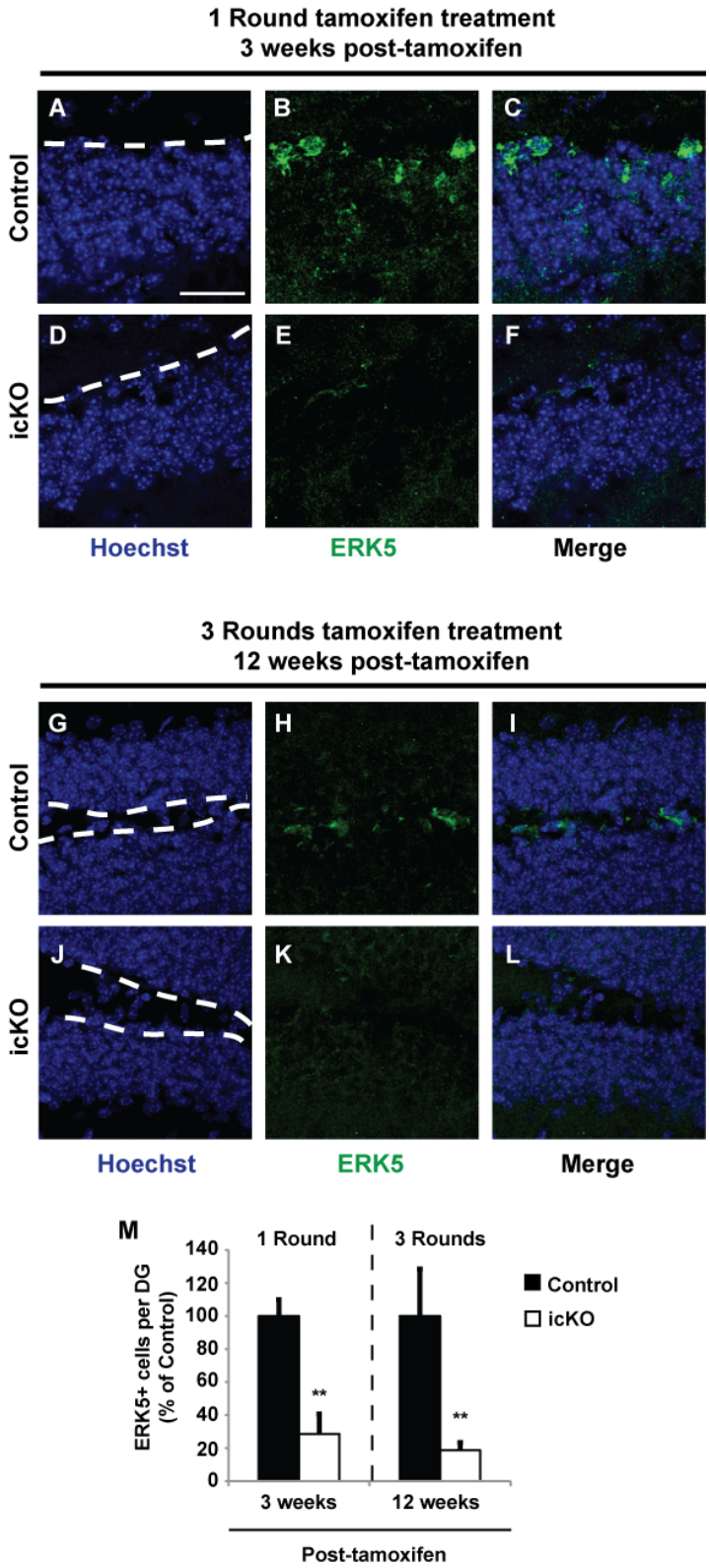
ERK5 icKO mice had no memory for novel objects measured 48 h after training

To examine the effect of conditional *erk5* deletion on hippocampus-dependent animal behavior, we used a modified tamoxifen dosing regimen (Imayoshi et al., 2008) to ensure prolonged *erk5* gene deletion and a cumulative loss of $ERK5^+$ cells over a period of time in the neurogenic regions. Tamoxifen was administered once per day for 4 d in each cycle, for a total of 3 cycles with 2 weeks inter-cycle interval (Fig. 3.3). The number of $ERK5^+$ cells along the SGZ was reduced 80% even 3 months after the last dose of tamoxifen treatment (Fig. 3.3M; *t*-test, p=0.005 for 3-week and p=0.007 for 3-month analysis), which provided a relatively large time window (at least 3 months) for behavior tests.

We first assessed motor function of ERK5 icKO and control mice and found no statistically significant differences in their open field activities or their ability to habituate to the open field in three consecutive days (Fig. 3.4; One-Way ANOVA, $F_{(1,2)}$=22.16, p=0.13 for panel B; $F_{(1,2)}$=1.32, p=0.46 for panel C; $F_{(1,2)}$=29.51, p=0.12 for panel D; $F_{(1,2)}$=29.51, p=0.12 for panel E). Furthermore, ERK5 icKO mice exhibited no defects in memory for novel objects measured 1 h after training (Fig. 3.5A, B; One-Way ANOVA, $F_{(1,27)}$=58.6, p<0.001 for control mice and $F_{(1,19)}$=42.11, p<0.001 for ERK5 icKO mice in panel B) or 24 h after training (Fig. 3.5C, D; One-Way ANOVA, $F_{(1,13)}$=11.5, p=0.005 for control and $F_{(1,19)}$=9.08, p=0.007 for ERK5 icKO in panel D). The lack of any deficits in memory for novel objects measured 1 h and 24 h after training, in the open field test and open field habituation suggests that ERK5 icKO mice have no defects in vision, general mobility and are not ataxic.

Interestingly, ERK5 icKO mice had no memory for novel objects measured 48 h after training (Fig. 3.5E, F; One-Way ANOVA, $F_{(1,13)}$=30.36, p<0.001 for control and $F_{(1,19)}$=0.0062, p=0.94 for ERK5 icKO in panel F), suggesting that ERK5 icKO mice may be deficient in longer-term memory.

Figure 3.3. ERK5-positive cells along the SGZ are significantly reduced 3 weeks and 12 weeks following tamoxifen treatment. (A–F) ERK5 immunostaining 3 weeks after a 7 d treatment paradigm with vehicle control (A–C, Control) or tamoxifen (D–F, icKO). (G–L) ERK5 immunostaining 12 weeks after a 3 x 4 d treatment paradigm with vehicle control (G–I, Control) or tamoxifen (J–L, icKO). Scale bar in A represents 25 μm and applies to all images. Dashed lines outline the SGZ layer of the dentate gyrus. (M) Quantification of total ERK5-positive cells per dentate gyrus. Data represent mean percentage from two independent experiments, error bars represent (s.e.m.) with n ≥ 2 animals per treatment group per time point per experiment. **, $p<0.01$.


1 Round tamoxifen treatment
3 weeks post-tamoxifen
A
B
C
Control
D
E
F
icKO
Hoechst
ERK5
Merge
3 Rounds tamoxifen treatment
12 weeks post-tamoxifen
G
H
I
Control
J
K
L
icKO
Hoechst
ERK5
Merge
M
1 Round
3 Rounds
ERK5+ cells per DG
(% of Control)
140
120
100
80
60
40
20
0
**
**
Control
icKO
3 weeks
12 weeks
Post-tamoxifen

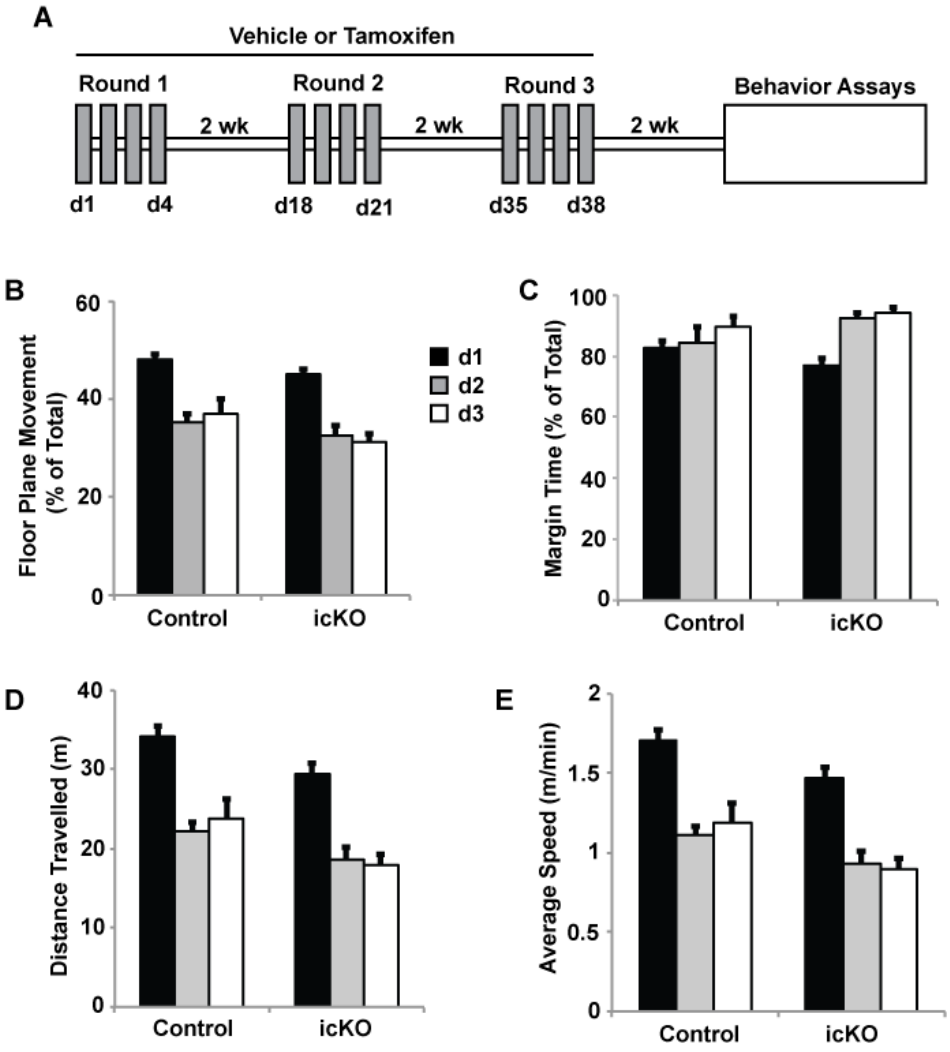
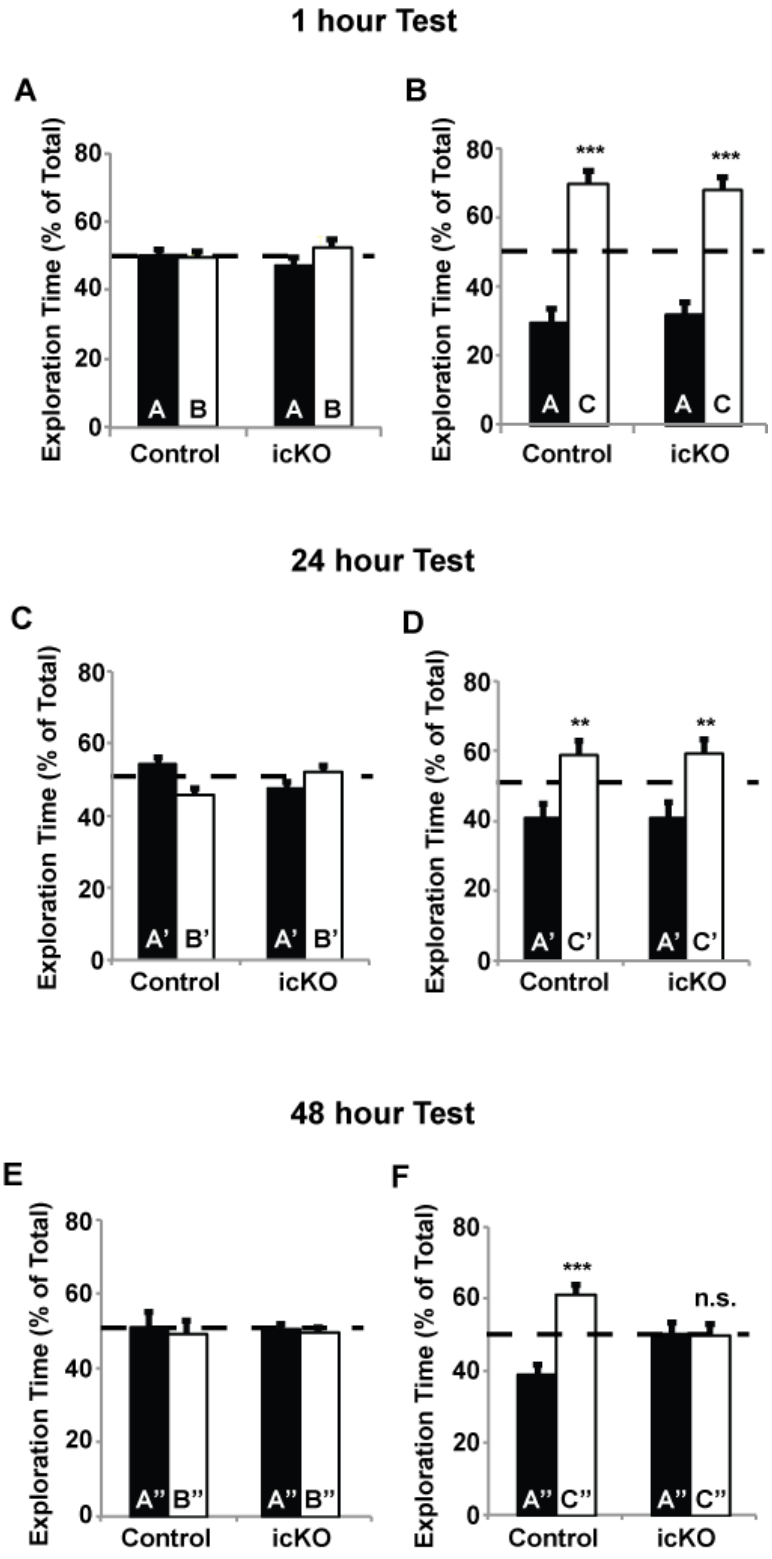


Figure 3.4. Baseline motor activity and habituation, monitored via the open field activity test, is not affected in ERK5 icKO mice. (A) Schematic illustration of the experimental design. Tamoxifen or vehicle was administered to 12-week old Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice orally, 4 d in each cycle, for a total of 3 cycles with 2-week inter-cycle intervals. Animals were subjected to behavior assays 2 weeks after the last dose of tamoxifen. (B) Total floor plane movement on day 1 (d1), d2, and d3. (C) Total time spent along the margins of the open field. (D) Total distance traveled. (E) Average traveling speed. Data represent mean of two independent experiments with n ≥ 10 animals per treatment group per experiment.

Figure 3.5. ERK5 icKO mice are deficient in the memory for novel object tested at 48 h after training. (A, B) Memory for novel object 1 h after training is not affected in ERK5 icKO mice. During the training session, control and ERK5 icKO mice were presented with 2 objects, A and B, for 5 min and the percent of time spent exploring each object was quantified (A). During the test session, conducted 1 h later, control and ERK5 icKO mice were presented again with 2 objects, A (familiar) and C (novel), for 5 min and the percent of time spent exploring each object was quantified (B). (C, D) Memory for novel object 24 h after training is not affected in ERK5 icKO mice. A new cohort of mice was used. Training session was conducted for 10 min with objects A' and B' and exploration time was quantified (C). Test session was conducted 24 h later with objects A' and C' for 10 min (D). (E, F) ERK5 icKO mice have no memory for novel object 48 h after training. After the 24 h test, animals were trained with objects A'' and B'' for 10 min (E). Test session with presentations of objects A'' and C'' was performed 48 h later (F"). Data represent mean exploration time from three independent experiments with n ≥ 8 animals per treatment group per experiment. n.s.,not significant; **, $p<0.01$; ***, $p<0.001$.


1 hour Test
A
B
Exploration Time (% of Total)
80
60
40
20
0
A
B
C
***
Control
icKO
24 hour Test
C
D
A'
B'
C'
**
48 hour Test
E
F
A"
B"
C"
n.s.

Inducible and conditional deletion of the erk5 gene interferes with a challenging form of contextual fear memory and the extinction of fear memory

Animals were subjected to contextual fear conditioning (Fig. 3.6A), a form of hippocampus-dependent learning and memory. In initial experiments, mice were trained with a single 0.7 mA, 2 s foot shock, which is a standard method used in contextual fear conditioning (Shan et al., 2008). Using this training protocol, ERK5 icKO mice did not exhibit defects in memory for contextual fear measured 24 h after training (Fig. 3.6B; One-Way ANOVA, $F_{(1,17)}$=1.30, p=0.27). The contextual memory for both ERK5 icKO and control mice was context specific since the mice did not show training-induced decreases in mobility when tested in a novel context 24 h after training (Fig. 3.6C; One-Way ANOVA, $F_{(1,17)}$=1.31, p=0.27). These data are consistent with other data using the 0.7 mA foot shock paradigm reporting that adult neurogenesis is not required for contextual fear conditioning (Dupret et al., 2008; Zhang et al., 2008a; Deng et al., 2009). Furthermore, memory for auditory-cued fear conditioning, a form of amygdala-dependent but hippocampus-independent learning and memory, was also unaffected in ERK5 icKO mice (Fig. 6D; One-Way ANOVA, $F_{(1,17)}$=0.15, p=0.71).

Although targeted deletion of ERK5 did not affect the formation of contextual fear memory, ERK5 icKO mice were impaired in contextual fear extinction (Fig. 3.6E; Two-Way ANOVA, $F_{(1,8)}$=17.68, p=0.003). This is consistent with another report implicating adult neurogenesis in the extinction of contextual fear memory (Deng et al., 2009). The contextual memory that persisted after fear extinction trials was context dependent since neither ERK5 icKO nor control mice showed increased freezing when exposed to a novel context after the 8-day fear extinction training (Fig. 3.6F; One-Way ANOVA, $F_{(1,17)}$=3.27, p=0.09). Contextual fear memory generated with a single 0.7 mA foot shock is a very strong (thus relatively easy) form of episodic memory that persists up to 11 weeks (Shan et al., 2008).

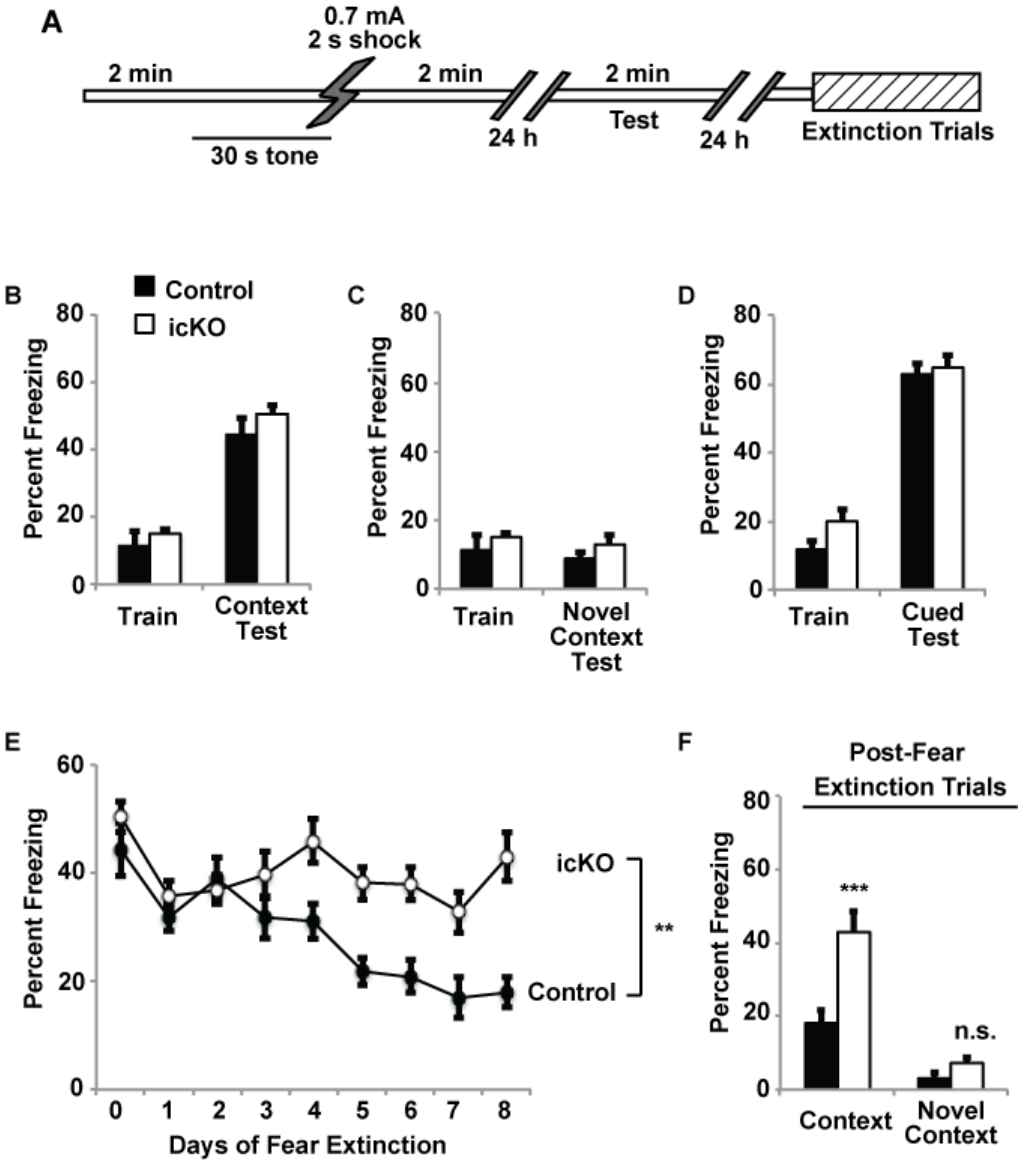


Figure 3.6. ERK5 icKO mice are compromised in contextual fear memory extinction. (A) Schematic illustration of the standard fear conditioning paradigm where animals were subjected to one 0.7 mA, 2 s foot shock. (B) Control and ERK5 icKO mice have similar levels of contextual fear memory 24 h post-training. (C) Neither control nor ERK5 icKO mice froze when placed in a novel context 24 h after training. (D) Control and ERK5 icKO mice showed similar cued-fear response 24 h post-training. (E) However, fear extinction was impaired in ERK5 icKO mice. Day 0: the day of contextual fear testing. Day 1: the first day animals were subjected to fear extinction training. (F) The freezing response that persisted after fear extinction trials was context specific because animals did not freeze in a novel context on Day 9, one day after the completion of the fear extinction trials. Data are mean ± (s.e.m.) from two independent experiments with n ≥ 8 animals per treatment group per experiment. n.s., not significant; **, $p<0.01$; ***, $p<0.001$.

Contextual fear extinction is a distinct form of hippocampus-dependent learning and memory (Fischer et al., 2007). It may be more challenging than contextual fear conditioning since it is an active form of forgetting in which the animals learn to dissociate the context from the foot shock. For example, while it took only a single 0.7 mA foot shock to establish the initial contextual fear memory, it took repeated trials of context exposure over the course of 8 days without the foot shock for this memory to undergo extinction in control mice. This prompted us to investigate if targeted deletion of ERK5 interferes with contextual fear memory when mice are subjected to a more challenging form of contextual memory training. We examined the sensitivity of wild type C57/BL6 mice to contextual fear conditioning using a weaker aversive stimulus, 0.3 mA foot shock. When C57/BL6 mice were trained for contextual fear memory using a 2 s, 0.3 mA foot shock, they had to be shocked three times to generate contextual memory as strong as that generated with a single 2 s, 0.7 mA foot shock (Fig. 3.7A, B). Thus, contextual fear training using the 0.3 mA foot shock is a weaker training paradigm and more difficult compared to the single 0.7 mA foot shock.

Interestingly, when ERK5 icKO mice were trained for contextual fear memory using this more challenging protocol (3X 0.3 mA foot shocks, 2 s each), they responded to the shocks as well as control mice during the three-shock training session (data not shown). However, memory for contextual fear, measured 24 h after training, was significantly reduced in ERK5 icKO mice (Fig. 3.7C; One-Way ANOVA, $F_{(1,18)}$=7.13, p=0.01). The remaining fear memory was still context specific since animals did not freeze in a novel context (Fig. 3.7D; One-Way ANOVA, $F_{(1,12)}$=0.72, p=0.41) and *erk5* deletion did not affect cued-fear conditioning (Fig. 3.7E; One-Way ANOVA, $F_{(1,12)}$=2.00, p=0.18).

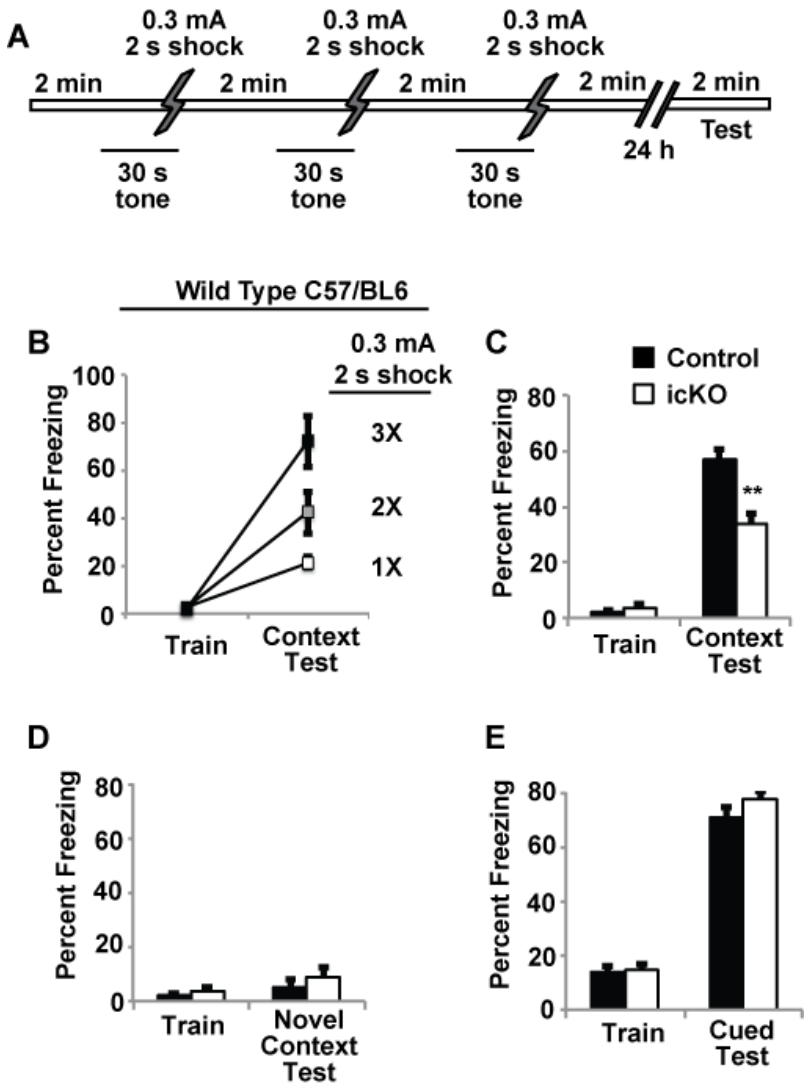


Figure 3.7. ERK5 icKO mice exhibit reduced contextual fear memory when animals were trained with weaker electric foot shocks. (A) Schematic illustration of the modified cued-contextual fear conditioning paradigm using 0.3 mA shock intensity delivered up to 3 times. (B) C57Bl/6 mice were subjected to one, two, or three foot shocks (0.3 mA, 2 s each), with 2 min inter-shock intervals to generate a dose-dependent fear conditioning response. Freezing behavior in the same context but without foot shock was recorded 24 h later. For subsequent experiments in panels C–E, animals were subjected to 3 foot-shocks (0.3 mA, 2 s each), with 2 min inter-shock intervals. (C) When placed into the same shocking context 24 h later, contextual fear memory was reduced in ERK5 icKO mice. (D) The fear memory was context specific since animals did not freeze when placed into a novel context 24 h after training. (E) Memory for the hippocampus-independent, auditory-cued fear conditioning measured 24 h after training was unaffected in ERK5 icKO mice. Data are mean ± (s.e.m.) from two independent experiments with $n \geq 6$ per treatment group per experiment. **, $p<0.01$.

The fact that the freezing response immediately after each shock and cued memory were normal in ERK5 icKO mice suggests that the mutant mice exhibit shock sensitivity comparable to control mice. Collectively these data indicate that targeted deletion of *erk5* attenuates extinction of contextual fear memory and the formation of contextual memory generated by a more demanding training protocol using a weaker shock paradigm than normally used in contextual fear conditioning.

Effects of conditional *erk5* deletion on spatial learning and memory

Animals were subjected to the Morris water maze test (Fig. 3.8), a spatial learning and memory task that is also hippocampus-dependent. Although ERK5 icKO mice initially took a little longer to locate the hidden platform on day 1, they learned to locate the hidden platform almost as well as control mice overall over the course of an 8-day training period (Fig. 3.8B; Two-Way ANOVA, $F_{(1,7)}$=3.10, p=0.12). There was also no significant difference when ERK5 icKO and control mice were subjected to a probe trial 24 h after training (Fig. 3.8C; One-Way ANOVA, $F_{(7,146)}$=58.71, p=0.12).

We then subjected animals to reversal training in which the hidden platform was moved to the opposite location. Reversal training may be more demanding than the initial hidden platform training because the mice need to actively forget the old location of the hidden platform and learn to find it in a new location. Interestingly, ERK5 icKO mice swam longer distances than control mice to find the new platform during a 7-day period of reversal training (Fig. 3.8D; Two-Way ANOVA, $F_{(1,6)}$=5.56, p=0.05). Furthermore, while control mice spent much more time in the new target quadrant than in the old one during the subsequent probe test, ERK5 icKO mice spent the same amount of time searching in both quadrants (Fig. 3.8E, G; One-Way ANOVA, $F_{(7,145)}$=14.31, p=0.002 for panel E between control and ERK5 icKO mice; $F_{(7,145)}$=14.31, p<0.001 for control mice between Old and New quadrants; $F_{(7,145)}$=14.31, p=0.143 for ERK5 icKO mice

between Old and New quadrants). Consistently, ERK5 icKO mice spent less time in the target platform zone than control mice during the reversal probe test (Fig. 3.8F; One-Way ANOVA, $F_{(1,31)}$=0.85, p=0.36 for probe test and $F_{(1,31)}$=8.26, p=0.007 for reversal probe test). These data suggest that ERK5 icKO mice exhibit impaired acquisition of newer spatial information and did not learn the new location of the hidden platform as well as control mice. However, ERK5 icKO mice swam similar total distances with similar speed as control mice measured during the first probe test and the reversal probe test (Fig. 3.9A, B, and data not shown). Furthermore, ERK5 icKO mice performed equally well as the control mice in the visible platform test, carried out one day after the reversal probe test (Fig. 3.9C). Thus, the deficiency of ERK5 icKO mice in acquisition of newer spatial information in the reversal hidden platform test was not due to a procedural learning deficit, their lack of ability to swim, or motivation to escape from the water.

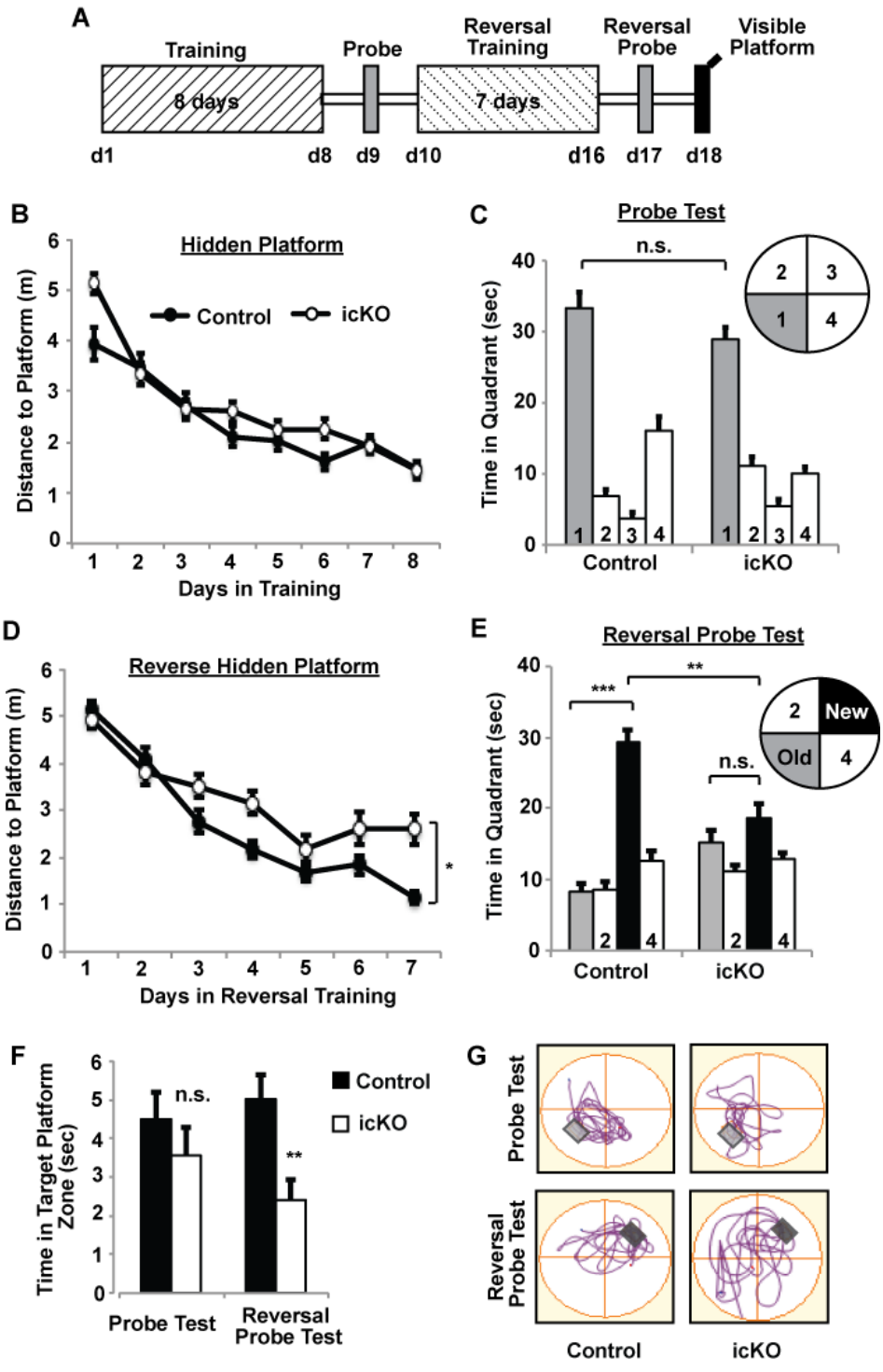
ERK5 icKO mice are deficient in pattern separation

ERK5 icKO mice were subjected to the radial arm maze pattern separation assay, a form of spatial learning and memory that is dentate gyrus-dependent (Farioli-Vecchioli et al., 2008; Clelland et al., 2009; Guo et al., 2011) (Fig. 3.10A). Pattern separation is the process of transforming similar representations or memories into highly dissimilar, non-overlapping representations. Thus, it is also considered a difficult form of hippocampal memory formation (Clelland et al., 2009; Guo et al., 2011). ERK5 icKO mice displayed a deficit in this form of pattern separation at both separations 2 and 4 (Fig. 3.10B; One-Way ANOVA, $F_{(3,63)}$=3.94, p=0.012).

Remote fear memory is impaired in ERK5 icKO mice

Passive avoidance is another form of hippocampus-dependent fear conditioning. We analyzed ERK5 icKO mice for their ability to learn this task and to retain remote fear memories (Fig. 3.11).

Figure 3.8. ERK5 icKO mice show impaired learning of newer spatial information. (A) Schematic depiction of the experimental design. Mice were subjected to 8 d of training (4 sessions/day, 30 min inter-session interval, from d1 to d8) in the hidden platform water maze. A probe test was conducted 24 h later. Mice were then subjected to 7 d of reversal training (4 sessions/day, 30 min intersession interval) where the escape hidden platform was relocated to the opposite quadrant of the water maze. This was followed by reversal probe test and visible platform test. (B) Target (in quadrant 1) acquisition in the hidden platform, quantified as swim distance to platform, was similar in control and ERK5 icKO mice, demonstrating similar spatial learning. (C) ERK5 icKO mice spent similar amount of time in the virtual target quadrant as control mice in the probe test, suggesting that ERK5 icKO mice retained the spatial memory. (D) Swim distance to escape in the reversal hidden platform training where the hidden platform was moved to quadrant 3. ERK5 icKO mice swam longer distances, suggesting impaired learning of newer spatial information. (E) Control but not ERK5 icKO mice spent significantly more time in the new virtual target quadrant 3 than in the previous virtual target quadrant 1 during the reversal probe test. (F) ERK5 icKO mice spent less time in the target platform zone than control mice during the reversal probe test. (G) Representative computer-generated tracing of the swim and search pattern of control versus ERK5 icKO mice in the initial probe test and the reversal probe test. Data are mean ± (s.e.m.) from two independent experiments with n ≥ 10 per treatment group per experiment. n.s., not significant, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.


A
Training
Probe
Reversal Training
Reversal Probe
Visible Platform
8 days
7 days
d1
d8
d9
d10
d16
d17
d18
B
Hidden Platform
Control
icKO
Distance to Platform (m)
Days in Training
C
Probe Test
n.s.
Time in Quadrant (sec)
Control
icKO
D
Reverse Hidden Platform
Distance to Platform (m)
Days in Reversal Training
*
E
Reversal Probe Test
***
**
n.s.
New
Old
Time in Quadrant (sec)
Control
icKO
F
Time in Target Platform Zone (sec)
n.s.
**
Control
icKO
Probe Test
Reversal Probe Test
G
Probe Test
Reversal Probe Test
Control
icKO

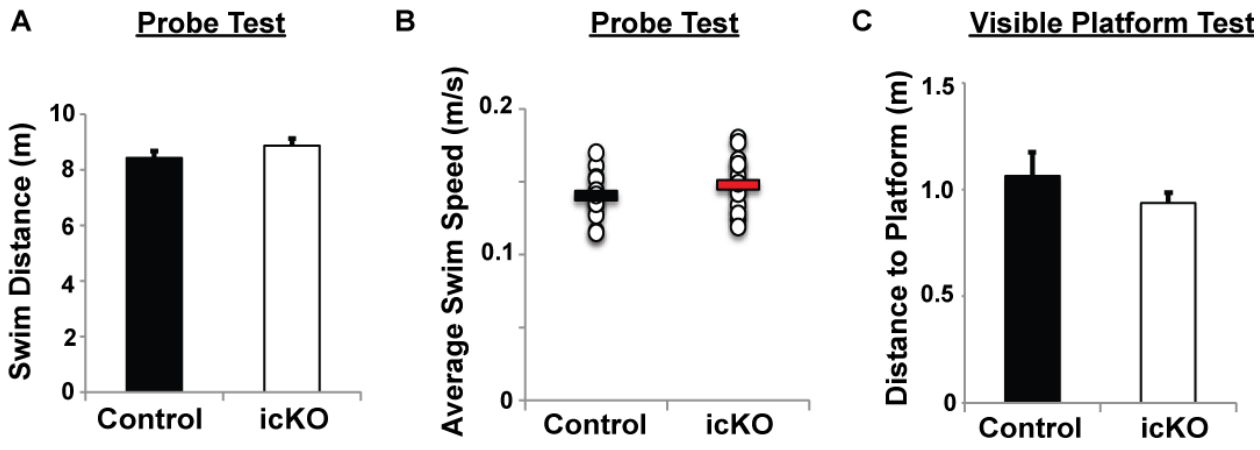


Figure 3.9. ERK5 icKO mice are not deficient in their ability to swim or in their motivation to escape water in the Morris water maze assay. (A) Total swim distance was measured during the 60 s period of the probe test. (B) Average swim speed during the 60 s probe test. (C) Swim distance to platform during the visible platform test. Data represent mean from two independent experiments with n ≥ 10 animals per treatment group per experiment.

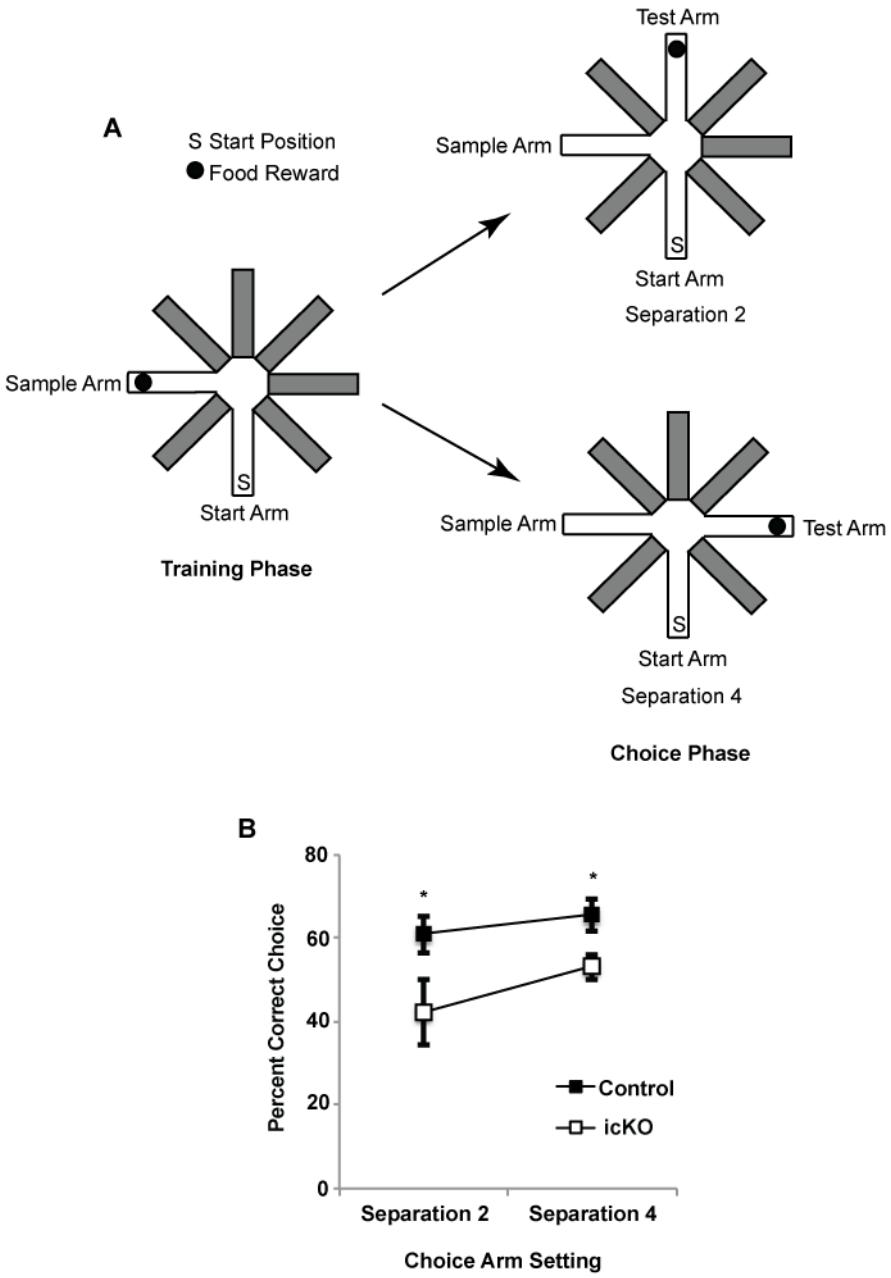


Figure 3.10. ERK5 icKO mice are deficient in pattern separation. (A) Schematic illustration of the experimental design. (B) ERK5 icKO mice made fewer correct choices in both the easier (separation 4) and more difficult (separation 2) pattern separations. Data are from the last day of a 5 d-experimental paradigm and represent mean ± (s.e.m.) with n ≥ 10 per treatment group. *, $p<0.05$.

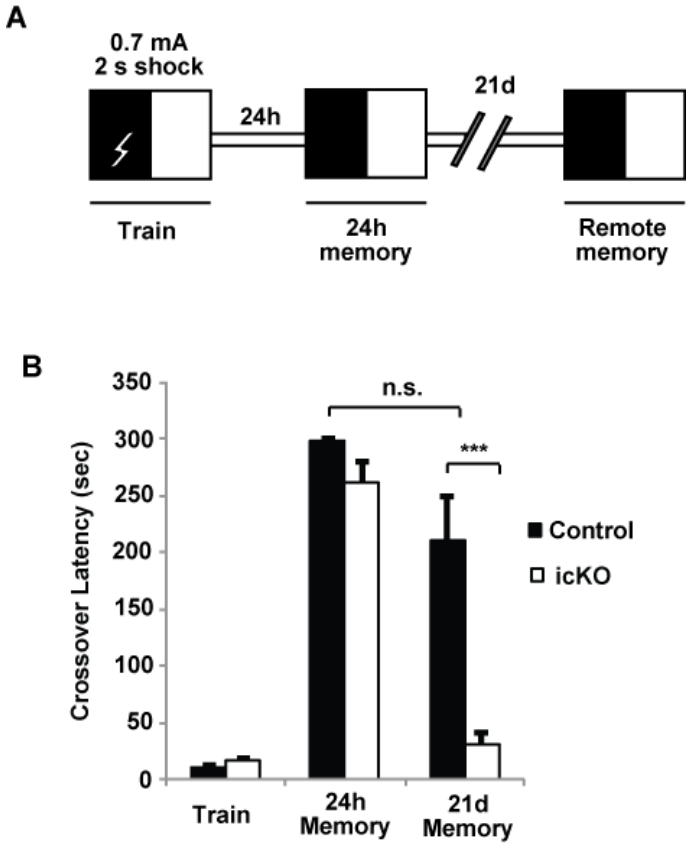


Figure 3.11. Remote memory in the passive avoidance assay is impaired in ERK5 icKO mice. Mice were trained with one 0.7 mA, 2 s foot shock in the passive avoidance assay. (A) Schematic illustration of the experimental design. (B) Crossover latency was measured 24 h or 21 d post-training. Data represent mean ± (s.e.m.) with n ≥ 10 per treatment group. n.s., not significant; ***, $p<0.001$.

A mild foot shock (0.7 mA, 2 s) was delivered once mice crossed over into the dark chamber during training. ERK5 icKO mice showed similar crossover latency as control mice when tested 24 h post-training, suggesting that they learned the task and can retrieve the memory. Interestingly, ERK5 icKO mice showed no remote memory measured 21 d after training (One-Way ANOVA, $F_{(5,40)}$=40.34, p<0.001). These data define a new role for adult neurogenesis in the expression of remote memory.

DISCUSSION

New neurons are constantly born in the dentate gyrus of the hippocampus in the adult mammalian brain. However, the functional impact of these adult-born neurons on hippocampus-dependent functions is still an open question. Furthermore, signaling mechanisms regulating adult neurogenesis are incompletely defined. Our goal was to investigate the role of ERK5 MAP kinase in the regulation of adult hippocampal neurogenesis and associated physiological functions.

Here we report that the expression of ERK5 MAP kinase is restricted to the two neurogenic regions in the adult mouse brain. This pattern of expression distinguishes ERK5 from other signaling molecules implicated in adult neurogenesis, including NeuroD, sonic hedgehog, and Wnt which are more widely expressed in the brain (Kenney et al., 2004; Lie et al., 2005; Jiao and Chen, 2008; Bruel-Jungerman et al., 2009; Gao et al., 2009; Jessberger et al., 2009; Kuwabara et al., 2009; Ma et al., 2009; Suh et al., 2009). It also suggests that ERK5 may regulate adult neurogenesis. Indeed, conditional deletion of the *erk5* gene specifically in the neurogenic regions attenuated adult hippocampal neurogenesis *in vivo*.

Although several studies have attempted to assess the role of adult neurogenesis in hippocampus-dependent memory formation, the results have been inconsistent (Deng et al., 2010; Leuner and Gould, 2010). For example, although some studies showed that ablation of adult neurogenesis interferes with contextual fear response (Imayoshi et al., 2008), contextual fear memory (Saxe et al., 2006), contextual trace memory (Guo et al., 2011), contextual fear memory extinction (Deng et al., 2009), and contextual fear memory transfer out of the hippocampus (Kitamura et al., 2009), other studies suggest that inhibition of adult neurogenesis does not affect contextual fear conditioning (Shors et al., 2002; Dupret et al., 2008; Zhang et al., 2008a; Deng et al., 2009; Jaholkowski et al., 2009). Several factors may contribute to these seemingly contradictory results, including design of the behavior studies. Here, we sought to examine the effects of targeted *erk5* deletion on contextual fear memory by directly comparing two paradigms that have been used in previous reports, 1X 0.7 mA, 2 s, vs. 3X 0.3 mA, 2 s each (Imayoshi et al., 2008; Deng et al., 2009). Interestingly, ERK5 icKO mice show reduced contextual fear memory using the 3X 0.3 mA, 2 s foot shock but not the 1X 0.7 mA, 2 s foot shock.

Adult neurogenesis has also been implicated in spatial learning and memory in some studies (Dupret et al., 2008; Farioli-Vecchioli et al., 2008; Imayoshi et al., 2008; Zhang et al., 2008a; Clelland et al., 2009; Garthe et al., 2009; Jessberger et al., 2009; Creer et al., 2010; Guo et al., 2011), but not in others (Shors et al., 2002; Meshi et al., 2006; Saxe et al., 2006; Denis-Donini et al., 2008; Jaholkowski et al., 2009). In addition to the differences in experimental design of behavior assays, several other factors may contribute to these contradictory results, including the specificity of the methods used to ablate adult neurogenesis. Many early studies used X-irradiation or anti-mitotic drugs to ablate adult neurogenesis. Although these methods are effective at reducing adult neurogenesis, they are not specific to aNPCs and can affect other neurons. These treatments may also lead to significant side effects, including neural

inflammation. More recent studies employed transgenic expression of a lethal gene, such as diphtheria toxin using the Nestin-CreER^{T2};NSE-DTA mice (Imayoshi et al., 2008), thymidine kinase (Saxe et al., 2006; Deng et al., 2009), or the pro-apoptotic protein Bax (Dupret et al., 2008) to kill adult-born cells. Although these approaches are more specific to aNPCs than irradiation or anti-mitotic drugs, greatly increased cell death in the dentate gyrus may interfere with normal hippocampal function. In other studies, a specific gene such as cyclin D2, Wnt, TLX, or PI3K was deleted or inhibited (Denis-Donini et al., 2008; Zhang et al., 2008a; Jaholkowski et al., 2009; Jessberger et al., 2009). However, these genes are also expressed in neurons of the brain and the deletion or inhibition of these genes in mice may not be restricted to aNPCs. The off-target effects associated with these approaches may contribute to ambiguity in interpretation of the results.

The contradictory results in the literature regarding the functional impact of adult-born neurons on hippocampus-dependent function necessitated studies using more specific genetic approaches with less off-target side effects to manipulate adult neurogenesis. One strategy uses transgenic mouse technology to conditionally disrupt a specific gene both temporally and spatially in adult neurogenic regions. Indeed, a recent study used this approach to delete fragile X mental retardation protein (FMRP), a protein that is enriched in neurons but also expressed in aNSCs. This study reported evidence that adult neurogenesis contributes to learning and memory (Guo et al., 2011). A superior strategy to disrupt adult neurogenesis would be to identify a signaling molecule that is only expressed in neurogenic regions of the brain that is required for adult neurogenesis, and to conditionally disrupt that gene in adult neurogenic regions. The present study identifies ERK5 MAP kinase as such a molecule. We have demonstrated spatially and temporally controlled deletion of *erk5* in the neurogenic regions of the adult brain, which led to significant inhibition of adult neurogenesis in the dentate gyrus.

Our data showed that ERK5 icKO mice do not exhibit general behavioral defects in the open field activity assay or open field habituation, memory for novel object at 1 h and 24 h, cued-fear conditioning, contextual fear conditioning using a 0.7 mA foot shock, or the acquisition of the initial spatial learning and memory in Morris water maze training. Thus, these mice have no defects in vision, general mobility, general curiosity, ability to swim, motivation to escape from the water, or procedural learning, and they are not ataxic.

However, it is interesting that targeted deletion of *erk5* interfered with novel object memory measured 48 h after training, contextual fear extinction, contextual fear memory from weaker foot shocks (0.3 mA), reversal water maze test, and pattern separation. These data suggest that ERK5 knockout mice are deficient in several forms of hippocampus-dependent memory, which share the property of being more challenging. Several studies have implicated the dentate gyrus in pattern separation (Clelland et al., 2009; Creer et al., 2010; Guo et al., 2011). Data presented in this study provide additional genetic evidence supporting a role for adult neurogenesis in this dentate gyrus-dependent function. The fact that *erk5* deletion interferes with fear memory extinction and reversal of hidden platform training suggests that adult neurogenesis may be important for learning that requires active forgetting of a prior memory.

How enduring long-term, or remote memory is formed is an important question. Although recent studies have shed light on mechanisms responsible for the formation of recent hippocampus-dependent memories, much less is known regarding how recent memories are transformed into remote memories (Frankland and Bontempi, 2005). We discovered that although ERK5 icKO mice have normal fear memory one day after training in the passive avoidance assay, their remote memory at 21 days was completely disrupted. This data is the first demonstration that adult neurogenesis contributes to the expression of remote memory.

Together, our data identify ERK5 MAP kinase as a novel signaling pathway regulating adult neurogenesis in the dentate gyrus, and demonstrates a causal relationship between ERK5-regulated adult neurogenesis and the expression of remote memory as well as several challenging forms of hippocampus-dependent memory formation including fear memory extinction. This study also provides the first evidence for a physiological function of ERK5 *in vivo* in the adult mammalian brain.

Chapter 4

The Maintenance of Established Remote Contextual Fear Memory Requires ERK5 MAP Kinase and Ongoing Adult Neurogenesis in the Hippocampus

INTRODUCTION

Adult neurogenesis occurs in distinct regions in the mammalian brain under normal physiological conditions including the subgranular zone (SGZ) in the dentate gyrus of the hippocampal formation (Ming and Song, 2011). Adult neurogenesis in the SGZ has been implicated in several forms of hippocampus-dependent learning and memory (Deng et al., 2010). We previously reported that ERK5 expression in the adult brain is limited to the neurogenic regions and that adult neurogenesis in the SGZ is regulated by the ERK5 MAP kinase both *in vitro* and *in vivo* (Pan et al., 2012b). Interestingly, deletion of *erk5* specifically in the adult neurogenic regions impairs adult neurogenesis in the hippocampus (Pan et al., 2012b), and disrupts the more demanding forms of hippocampus-dependent memory including contextual fear memory generated by a weak foot shock as well as contextual fear extinction (Pan et al., 2012a).

Whether the maintenance of remote contextual memory depends on the hippocampal formation is controversial with some evidence supporting its involvement (Debiec et al., 2002; Goshen et al., 2011; Sutherland and Lehmann, 2011). However, other data have suggested that the hippocampus only plays a temporary role in the formation of new contextual fear memories and that remote memories are independent of the hippocampus and are permanently stored in extra-hippocampal regions such as the neocortex (Squire and Zola-Morgan, 1991; Kim and Fanselow, 1992; Squire and Alvarez, 1995; Anagnostaras et al., 1999; Frankland and Bontempi, 2005). Furthermore, although adult neurogenesis occurs throughout adult life in the dentate gyrus and may modulate the duration of hippocampus-dependent memory (Kitamura et al.,

2009), its role in the maintenance of remote memory has not been reported. Here, we present evidence that remote contextual memory depends upon ongoing adult neurogenesis even after remote memory is established.

MATERIALS AND METHODS

Ethics statement

All animals used in this study were approved by the University of Washington Institutional Animal Care and Use Committee. Experimental conditions and procedures were performed with direct approval under protocol 3041-04.

Animals

The generation of Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice has been previously described (Pan et al., 2012a). All experiments were performed using identically handled littermates. Standard housing conditions were applied (12 h light/dark cycle) with food and water provided *ad libitum.* All experimental procedures were approved and performed in accordance with the guidelines under the University of Washington Institutional Animal Care and Use Committee.

Administration of tamoxifen

Male and Female 10–12 week old adult mice were used. Tamoxifen was freshly dissolved in 2% glacial acetic acid and corn oil (Sigma) at 37°C and vortexed periodically. To initiate Cre-mediated *erk5* gene deletion, 5 mg of pre-warmed tamoxifen was delivered via oral gavage by either of two paradigms: A) once per day for 4 d in each cycle for 3 total cycles (3 x 4 d) with a 2 week inter-cycle interval for Figures 1 and 2, or B) once per day for 7 d for Figures 3 and 4. Vehicle control animals were dosed in an identical manner with corn oil solution.

Fear memory conditioning using a weak foot shock

This was performed as described (Pan et al., 2012a). Briefly, The foot shock context is a 10”(W) x 10”(D) x 16”(H) square-shaped box fitted with metal grid shock floor (Coulbourn). On training day, mice were placed in this context box with striped wall paper and allowed to habituate to the environment for 2 min. Immediately following habituation, animals were subjected to 3 successive foot shocks, 0.3 mA, 2 s each and separated by 2 min inter-shock intervals. Freezing behavior was scored immediately after each shock for 2 min. Mice were returned to their home cages for a 2 min period while the context box was cleaned, and then back to the cleaned context box for 2 min, during which time their freezing behavior was assessed as a measure of contextual fear memory acquisition. Six days post-training, mice were brought back to the context box for 2 min and freezing behavior scored. Two hours later, mice were placed in a novel context (hexagonal shaped arena with clear side walls) in a different room and assessed for freezing behavior for 2 min. Freezing behavior is defined by lack of bodily movement with all 4 paws on the grid floor except for normal respiration.

Remote memory assessment

Mice were trained for contextual fear and cued-fear conditioning using the standard 1 x 0.7 mA foot shock paradigm as described (Pan et al., 2012a). The establishment of contextual fear memory was confirmed by scoring freezing behavior in the context box for 2 min at 24 h after training. Freezing behavior in response to the cue or in a novel environment was also scored. Remote memory was assessed under three different conditions: A) *erk5* was deleted prior to training, B) *erk5* was deleted 6 d after training, and C) *erk5* was deleted 5 weeks following training. In all cases, remote contextual and cued-fear memories, as well as freezing behavior in a novel context were analyzed as described (Pan et al., 2012a).

Statistical analysis

Two-way ANOVA with repeated measures was used to analyze data in Figure 3E. One-way ANOVA with Fisher's LSD *post-hoc* analysis was performed for all remaining data. Data represent mean ± standard error of means (s.e.m.). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., not statistically significant ($p \geq 0.05$). $n \geq 6$ for each genotype/control in each experiment.

RESULTS

Conditional deletion of *erk5* causes deficits in contextual memory consolidation but does not affect memory acquisition or retrieval

Contextual fear memory is significantly reduced in the inducible and conditional ERK5 knockout (icKO) mice when they are trained using a challenging weak shock protocol (3 x 0.3 mA foot shocks, 2 s each) but not when trained with a stronger foot shock (1 x 0.7 mA. 2 s) (Pan et al., 2012a). The deficit in contextual memory when mice are trained with 3 x 0.3 mA foot shocks could result from impairments in memory acquisition, retrieval, or consolidation. To address this issue, we employed the ERK5 icKO mice in which tamoxifen treatment (3 x 4 d) of adult Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice selectively deletes the *erk5* gene in Nestin-expressing adult neural stem cells (Pan et al., 2012b). This leads to significant impairment of adult neurogenesis quantified by the reduction of the total number of BrdU, NeuN double-positive adult-born neurons in the SGZ (Pan et al., 2012b). Three groups of control mice were used: ERK5$^{loxP/loxP}$ mice treated with either vehicle control (control A) or tamoxifen (control B), and Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice treated with vehicle control (control C) (Table 4.1). When ERK5 icKO and control mice were trained for contextual fear conditioning using the weak shock paradigm (Fig. 4.1A), they all displayed similar degrees of freezing immediately after each foot shock during training, indicating comparable levels of shock sensitivity (Fig. 4.1B). Mice were then tested for contextual fear memory at 2 min and 6 d after training. ERK5 icKO mice behaved similarly as control mice when tested 2 min after training (Fig. 4.1C), indicating that they can

acquire and retrieve contextual fear memory. However, ERK5 icKO mice exhibited significantly less freezing at 6 d post-training (Fig. 4.1D). The contextual fear memory at 6 d was context-specific since all mice froze minimally when exposed to a novel context. Consolidation of contextual fear memory in mice occurs within 24 h and continues thereafter. Our data suggest that the impaired contextual fear memory of ERK5 icKO mice 6 d after training using the weak foot shock paradigm is not due to a deficit in acquisition or memory retrieval, but rather consolidation and persistence of contextual fear memory.

Targeted deletion of *erk5* prior to training for contextual fear conditioning interferes with the establishment of remote memory

Since ERK5 icKO mice exhibited impaired contextual fear memory 6 d after training using the 0.3 mA foot shock training paradigm (Fig. 4.1), we employed a commonly used, stronger shock training protocol (0.7 mA, 2 s) to assess the importance of adult neurogenesis for remote memory (Fig. 4.2A). ERK5 icKO and control mice exhibited equivalent levels of contextual fear memory 1 d after training (Fig. 4.2B). When assessed for remote contextual fear memory 5 weeks post-training, however, ERK5 icKO mice exhibited significantly reduced memory (Fig. 4.2C). This result was context-specific since all mice froze minimally when exposed to a novel context (Fig. 4.2D). Additionally, all mice froze at similar levels when assessed for cued-fear conditioning at 5 weeks, suggesting that amygdala-dependent fear memory was intact in ERK5 icKO mice (Fig. 4.2E). These data suggest that adult neurogenesis is required for the establishment of remote contextual fear memory.

Table 4.1. Description of animal genotypes and treatment groups

Name	Genotype	Treatment
Control A	$ERK5^{loxP/loxP}$	Vehicle
Control B	$ERK5^{loxP/loxP}$	Tamoxifen
Control C	Nestin-$CreER^{TM}$/$ERK5^{loxP/loxP}$	Vehicle
ERK5 icKO	Nestin-$CreER^{TM}$/$ERK5^{loxP/loxP}$	Tamoxifen

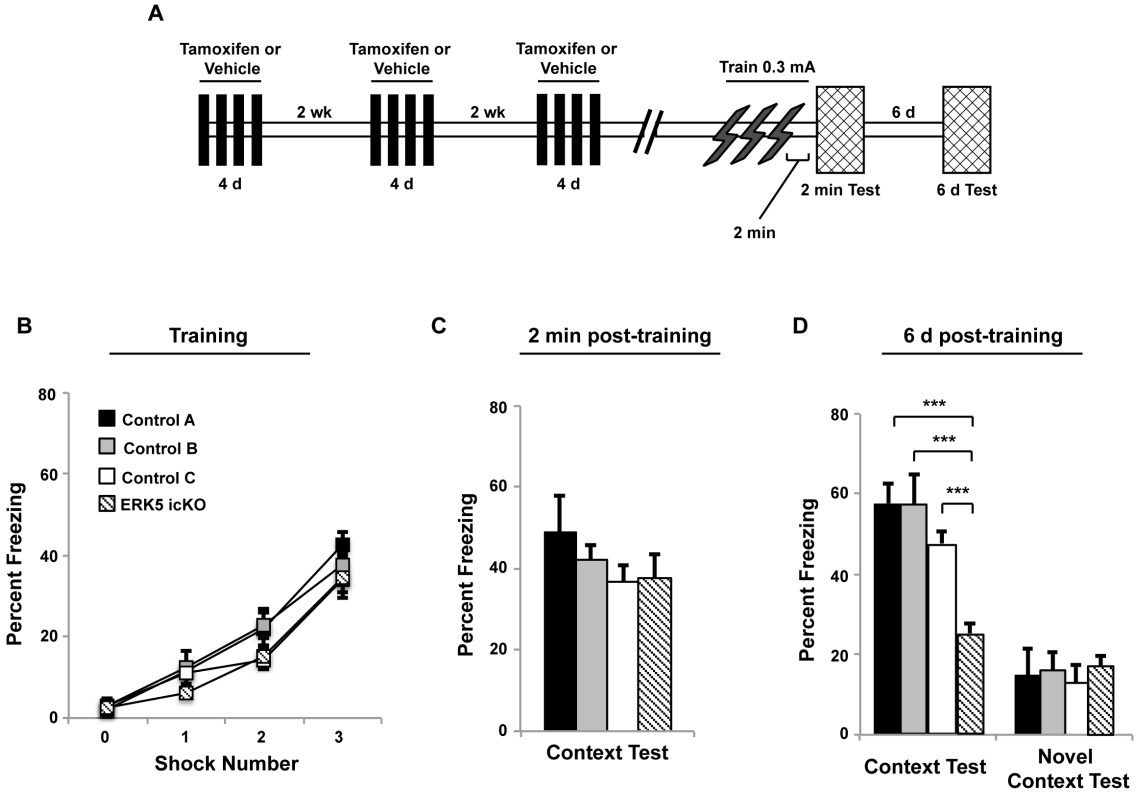


Figure 4.1. ERK5 icKO mice display normal shock sensitivity, acquisition and retrieval of contextual fear memory, yet show reduced contextual fear memory 6 d after training with the 3 x 0.3 mA foot shock paradigm. A) Schematic depiction of the experimental design. B) Freezing behavior displayed by ERK5 icKO and control mice immediately following each of the 3 successive 0.3 mA foot shocks during training. C) ERK5 icKO mice have acquired and can retrieve contextual fear memory 2 min after training. D) ERK5 icKO mice show reduced contextual fear memory 6 d after training while freezing behavior in the novel context was unaffected.

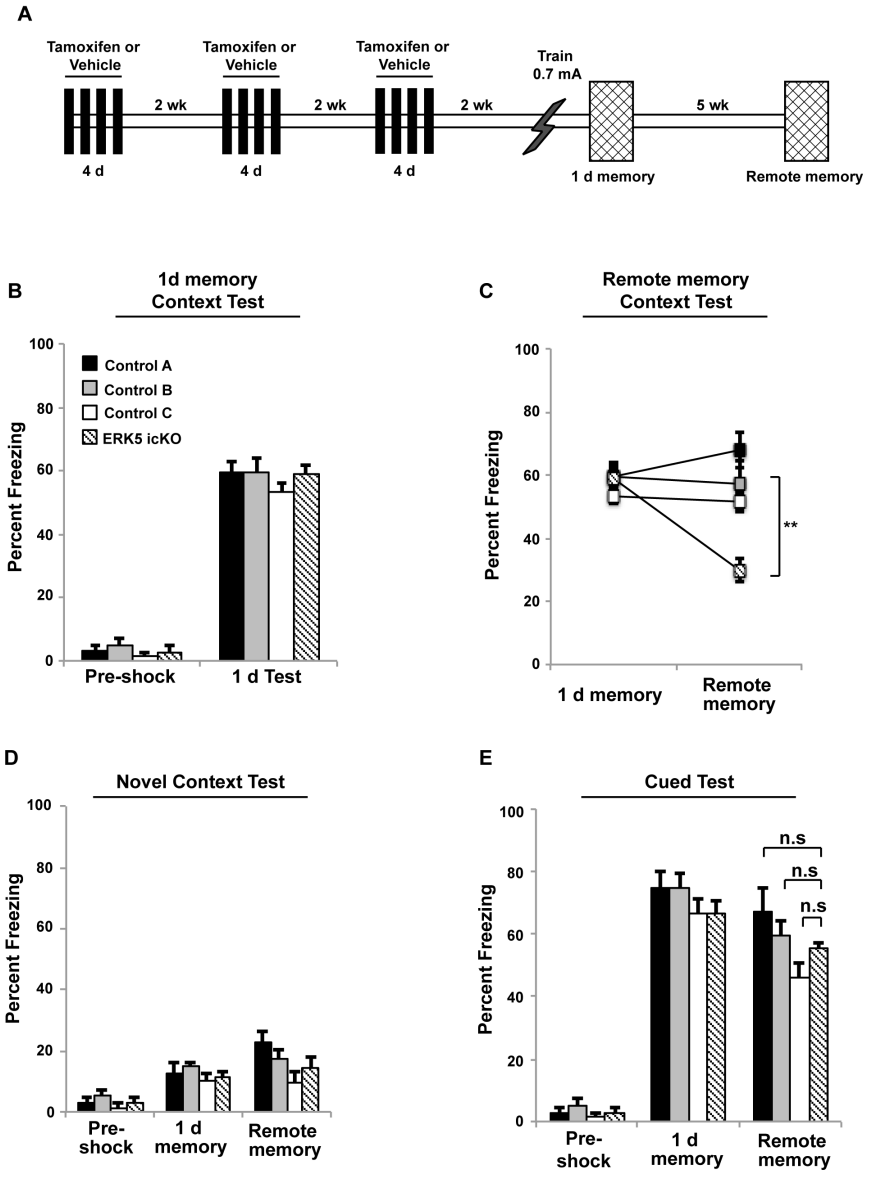


Figure 4.2. ERK5 icKO mice are impaired in remote memory when the *erk5* gene was inducibly deleted prior to training. A) Schematic depiction of the experimental design. Mice were treated with tamoxifen to delete *erk5* before contextual fear conditioning with the 1 x 0.7 mA foot shock paradigm. B) Freezing behavior in the shocking context 1 d after training does not differ between ERK5 icKO and control mice. C) Remote contextual memory is impaired in ERK5 icKO mice when tested 5 weeks post-training. D) Minimal freezing behavior is observed in a novel context at both 1 d and 5 weeks post-training for all treatment groups. E) Amygdala-dependent, cued-fear conditioning is normal in ERK5 icKO mice at both 1 d and 5 weeks post training

Conditional deletion of *erk5* six days after training for contextual fear conditioning causes remote memory decay

We next examined if new neurons born after initial acquisition and consolidation of contextual fear memory are necessary for the expression of remote memory. Mice were first trained and assessed 1 d after training for context-specific and cued-fear conditioning (Fig. 4.3A–D). Both ERK5$^{loxP/loxP}$ and Nestin-CreER^{TM}-ERK5$^{loxP/loxP}$ mice performed similarly suggesting that prior to tamoxifen treatment, no phenotypic differences exist. After mice had 6 d to consolidate contextual fear memory, they were treated with vehicle control or tamoxifen for 7 d to delete *erk5* thereby inhibiting adult neurogenesis. This treatment has been demonstrated previously to significantly reduce the total number of cells expressing ERK5, NeuroD, as well as BrdU and NeuN double-positive adult-born neurons in the SGZ (Pan et al., 2012a). Remote contextual memory was assessed at 5, 9, 12, and 16 weeks after initial training. ERK5 icKO mice froze significantly less than control mice, especially at 12 and 16 weeks post-training (Fig. 4.3E). This effect was context-specific and did not affect cued-fear conditioning (Fig. 4.3F, G).

Ongoing ERK5-regulated adult neurogenesis is required for the maintenance of remote memory, even after remote memory has been established

Another important question is whether ongoing adult neurogenesis is necessary for the maintenance and expression of remote contextual fear memory after the memory has transferred into extra-hippocampal regions, which usually takes approximately 4 wks in mice (Kitamura et al., 2009; Wiltgen et al., 2010; Goshen et al., 2011). To address this question, ERK5$^{loxP/loxP}$ and Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice were trained as in Figure 3 with 0.7 mA foot shock and their contextual fear memory tested and confirmed 1 d after training (Fig. 4.4A–D). Both groups of mice performed equally well after training. Five weeks after training, we treated mice with vehicle control or tamoxifen to delete *erk5* and disrupt adult neurogenesis. Mice were tested for context-specific freezing behavior 15 wks after training. Interestingly, ERK5 icKO mice

froze significantly less than control mice when assessed for persistence of remote contextual memory 15 wks post-training (Fig. 4.4E), but not in remote cued-fear memory or in the novel context (Fig. 4.4F, G). These data suggest a context-specific deficit in the maintenance of remote contextual fear memory even when *erk5* was deleted in adult neurogenic regions after remote memory had been established.

DISCUSSION

Deficits in learning and memory are prominent features of many mental disorders. Understanding molecular mechanisms underlying learning and memory are key to the development of therapies to correct cognitive disorders in the treatment of mental illness. The goal of this study was to investigate the role for ERK5 MAP kinase and ERK5-regulated adult neurogenesis in the memory formation of contextual fear and the maintenance of this memory.

We previously reported that contextual fear memory is significantly reduced in ERK5 icKO mice at 24 h after animals were trained using a challenging training protocol (3 x 0.3 mA foot shocks) (Pan et al., 2012a). Here, we demonstrate that under the same conditions, ERK5 icKO mice showed similar contextual fear memory as control mice when tested 2 min after training. Thus, the reduced contextual fear memory seen at 24 h using the 3 x 0.3 mA foot shocks training protocol is most likely due to a deficit in consolidation of the fear memory rather than deficits in memory acquisition and/or retrieval.

Since inhibition of adult neurogenesis may increase anxiety (Revest et al., 2009), it is possible that the deficits in remote contextual fear memory observed in ERK5 icKO mice may be due to increased anxiety. However, ERK5 icKO mice are not deficient in short-term (2 min, 1 day) contextual fear memory, nor in both short-term (1 day) and remote (5 wks, 15 wks, and 16 wks) cued fear memory.

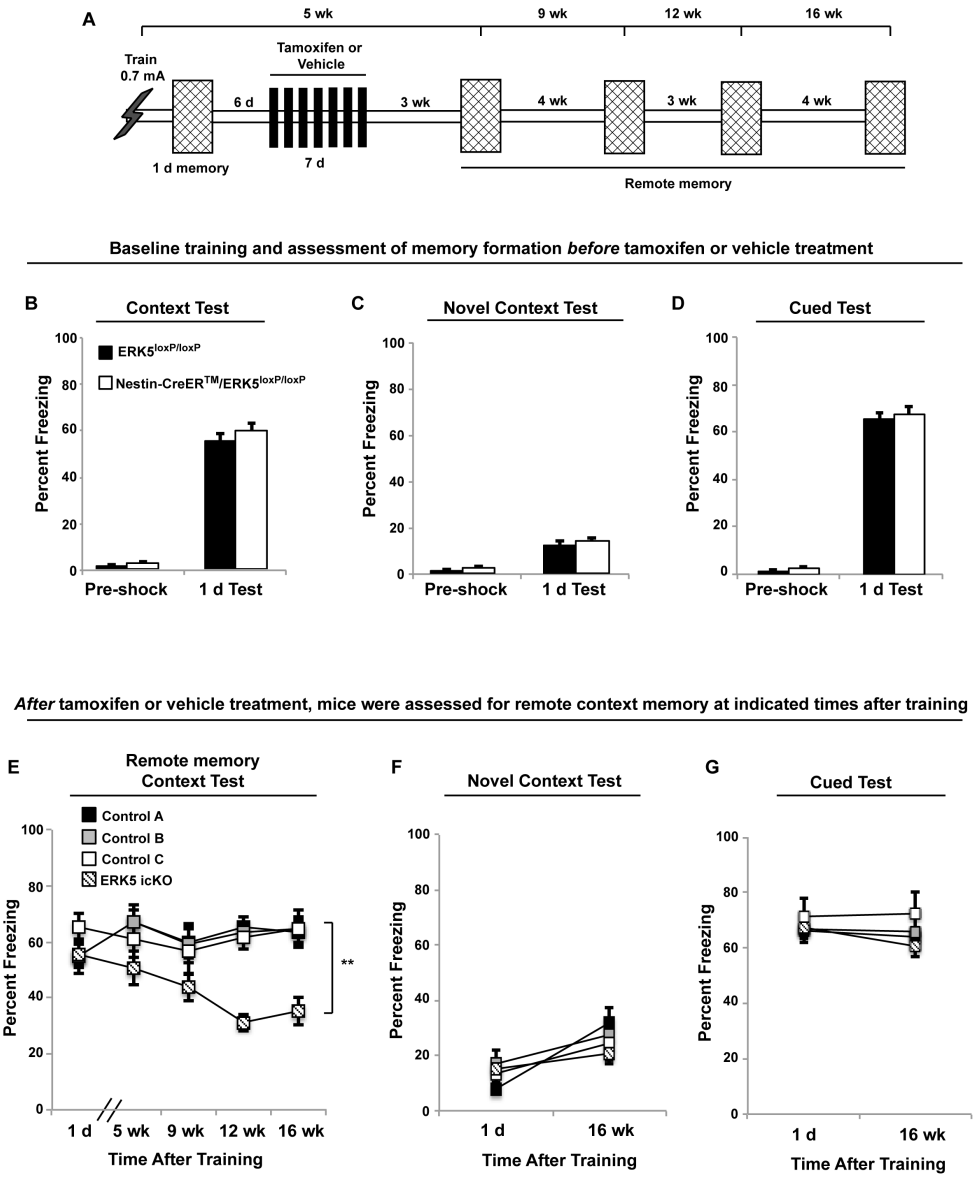


Figure 4.3. Deletion of the *erk5* gene 6 days after training causes decay of remote contextual fear memory. A) Schematic depiction of the experimental design. B–D) Assessment of memory formation for contextual fear conditioning (B), novel context (C), and cued fear (D) before *erk5* deletion. Mice were trained with the 1 x 0.7 mA foot shock paradigm and freezing behavior was assessed 1 d after training. E–G) Six days after training, mice were treated with tamoxifen for 7 d to delete *erk5* or with vehicle control. Freezing behavior was assessed in the training context (E), a novel context (F), or in a novel context paired with the cue (G) at 5, 9, 12, and 16 weeks after training.

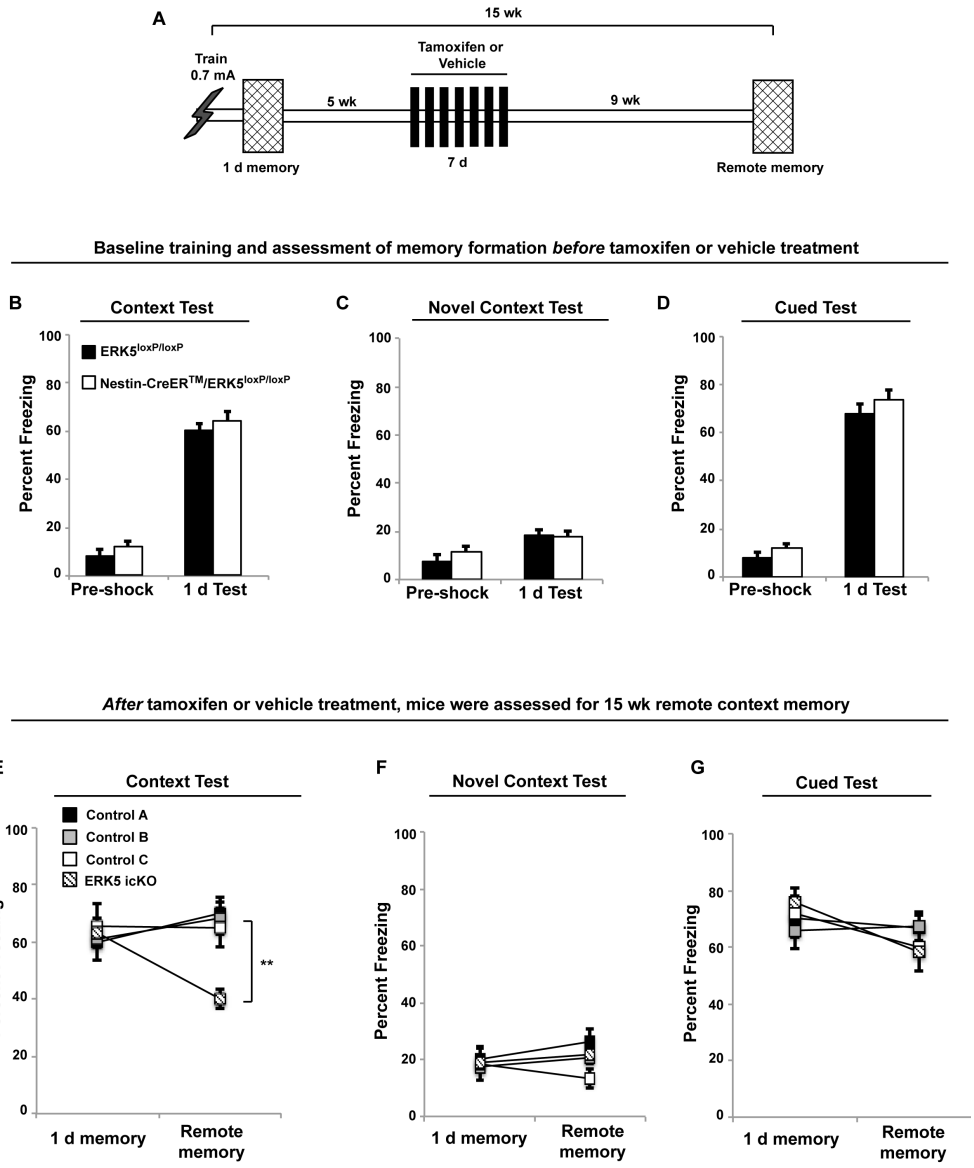


Figure 4.4. Deletion of the *erk5* gene 5 weeks post-training impairs remote memory. A) Schematic depiction of the experimental design. B–D) Assessment of memory formation for contextual fear conditioning (B), novel context (C), and cued fear (D) before *erk5* deletion as in Fig. 4.3. E–G) Five weeks after training, mice were treated with tamoxifen for 7 d to delete *erk5* or with vehicle control. Remote memory for the shocking context was significantly reduced 15 weeks after training in ERK5 icKO mice (E), while their response to a novel context (F) or the cue (G) was unaffected.

If increased anxiety significantly affects remote contextual fear memory, then it should have also affected short-term contextual and cued fear memory, as well as remote cued fear memory. Therefore, it seems unlikely that the observed defect of remote memory in ERK5 icKO mice is due to increased anxiety.

It is generally agreed that contextual fear memory is initially stored in the hippocampus for the short term. Furthermore, interaction between the hippocampus and neocortex after the formation of recent memory is crucial for the storage of remote memory regardless of the exact storage location for remote memory (Frankland and Bontempi, 2005; Nakashiba et al., 2008; Nakashiba et al., 2009). Indeed, post-training deletion of *erk5*, which reduces adult neurogenesis (Pan et al., 2012b), during the hippocampus-dependent period (6 d post-training) caused retrograde amnesia of contextual fear memory. Together with a recent report using diphtheria toxin-based strategy to ablate mature, adult-generated neurons within the week after training (Arruda-Carvalho et al., 2011), these data suggest that interference with adult neurogenesis can cause retrograde amnesia.

Whether the hippocampal formation is required for the maintenance of remote contextual memory once the memory is established is controversial (Squire and Zola-Morgan, 1991; Kim and Fanselow, 1992; Squire and Alvarez, 1995; Anagnostaras et al., 1999; Debiec et al., 2002; Frankland and Bontempi, 2005; Frankland et al., 2006; Lehmann et al., 2007; Wang et al., 2009; Wiltgen et al., 2010; Goshen et al., 2011; Sutherland and Lehmann, 2011). Furthermore, before this study, it was not known if continuing adult neurogenesis plays a role in the expression and maintenance of remote contextual memory. For example, one study suggests that adult neurogenesis modulates the duration of hippocampus-dependent period of associative fear memory but inhibition of adult neurogenesis does not cause loss or reduction of remote memory

per se (Kitamura et al., 2009). Using our transgenic mouse line, we were able to specifically and conditionally target *erk5* in neural stem/progenitor cells in adult neurogenic regions, thus interfering with adult neurogenesis (Pan et al., 2012a; Pan et al., 2012b) to assess the importance of adult-born neurons in the hippocampus for the expression and persistence of remote memory. Our data indicate a critical role for adult-born neurons in both the expression and maintenance of remote contextual memory using three different paradigms, exemplified with observed deficits when *erk5* was conditionally deleted prior to training, 6 d after training, or 5 wks after training.

The cortex and basolateral amygdala are required for remote contextual fear memory (Gale et al., 2004; Frankland and Bontempi, 2005; Poulos et al., 2009). It is possible that the functional connectivity between the hippocampal formation and amygdala or cortex during memory retrieval and reconsolidation is important for the maintenance of remote contextual fear memory. Although this study does not directly address this functional connectivity, it is important to note that ERK5 is selectively expressed in the neurogenic regions but not other regions of the adult brain (Pan et al., 2012a; Pan et al., 2012b). Furthermore, the *erk5* gene deletion is restricted to neurogenic regions of the adult brain (Pan et al., 2012a; Pan et al., 2012b). Data presented in this study clearly suggest that the continual formation of new neurons in the dentate gyrus is integral for the establishment and maintenance of remote contextual fear memory, even after the memory has transferred out of the hippocampus. Our data support the general hypothesis that the hippocampus has a long-term role in the continued expression of contextual fear memory (Sutherland et al., 2008; Sparks et al., 2011), and may have important implications for the treatment of memory disorders.

Chapter 5

Inducible and Targeted Deletion of ERK5 MAP Kinase in Adult Neurogenic Regions Impairs Adult Neurogenesis in the Olfactory Bulb and Several Forms of Olfactory Behavior

INTRODUCTION

Humans and other mammals detect and distinguish between thousands of different odorants. Defects in olfaction cause loss of appetite and poor nutrition, particularly with older individuals. For example, the average human loses a significant proportion of their olfaction as they age, and olfactory dysfunction is associated with several neurodegenerative diseases including Alzheimer's and Parkinson's disease (Hawkes, 2006). Recent studies led to the exciting discovery of ongoing adult neurogenesis in the subventricular zone (SVZ) along the lateral ventricles in the adult brains of mammals (Alvarez-Buylla and Garcia-Verdugo, 2002; Ming and Song, 2005; Zhao et al., 2008; Ming and Song, 2011). Newly generated neuronal precursors in the SVZ migrate along the rostral migratory stream (RMS) to the core of the olfactory bulb (OB) where they begin radial migration and differentiation into inhibitory interneurons (Belvindrah et al., 2009; Whitman and Greer, 2009). Although these neurons have been extensively characterized at the cellular level, their functional impact on olfactory behavior is still an open question (Whitman and Greer, 2009; Bardy and Pallotto, 2010; Lazarini and Lledo, 2011; Breton-Provencher and Saghatelyan, 2012; Kageyama et al., 2012). Furthermore, signaling mechanisms regulating adult SVZ/OB neurogenesis are not fully defined. Consequently, it is critical to elucidate signaling mechanisms regulating adult SVZ/OB neurogenesis and to generate definitive evidence concerning the role of adult neurogenesis in olfactory behaviors.

ERK5 is a member of the MAP kinase super family of proteins that include ERK1/2 (Lee et al., 1995; Zhou et al., 1995). We recently discovered that although its expression in the adult brain is extremely low, ERK5 is prominently expressed along the neurogenic SVZ-RMS-core of the OB pathway. This striking pattern of expression suggests a unique and important role for ERK5 signaling in adult SVZ/OB neurogenesis. We have generated an ERK5 inducible and conditional knock out (icKO) mouse that allows us to delete the *erk5* gene specifically in neurogenic regions of the adult brain (Pan et al., 2012a). Here, we report that ERK5 icKO mice are impaired in adult neurogenesis in the SVZ/OB and in several forms of olfactory behavior.

MATERIALS AND METHODS

Ethics statement

All animals used in this study were approved by the University of Washington Institutional Animal Care and Use Committee. Experimental conditions and procedures were performed with direct approval under protocol 3041-04.

Animals

Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ and ERK5$^{loxP/loxP}$ transgenic animals have been previously described (Pan et al., 2012a; Pan et al., 2012b). Littermates were handled identically and housed under standard conditions (12 h light/dark cycle) with food and water provided *ad libitum* except where indicated.

Reagents

The following primary antibodies were used for immunohistochemistry: rat anti-BrdU (1:500, AbD Serotec), mouse anti-NeuN (1:500, Millipore), and polyclonal goat anti-doublecortin (DCX, 1:200, Santa Cruz Biotech. Inc.). Affinity-purified polyclonal ERK5 antibody (1:500) was described previously (Cavanaugh et al., 2001; Pan et al., 2012a). The following odorants were

used: citralva (International Flavors & Fragrances, Inc.), isoamyl acetate (IAA, Sigma), ethyl vanillin (SAFC Global), S-terpinen-4-ol (Sigma), acetophenone (Sigma), 1-octanol (Sigma), 2-heptanone (Sigma), farnesene (Wako USA), 2,3,5-trimethyl-3-thiazoline (TMT, Phero Tech).

Tamoxifen and BrdU administration

Adult (8–10 wk old) male mice were dosed daily with 200 mg/kg of freshly dissolved tamoxifen (Sigma) in 2% glacial acetic acid in corn oil solution (Sigma) to activate Cre-mediated recombination. All mice were administered tamoxifen either once per day for 7 d for 1 cycle for data in Fig. 5.2 (cellular studies) or once per day for 4 d in each cycle for 3 cycles, with a 2-week inter-cycle interval for Fig. 5.3–5.8 (behavior studies). BrdU (Sigma) was administered at a dose of 100 mg/kg by intraperitoneal injection 5 times (every 2 h for 10 h) in one day followed by sacrifice 3 weeks later to identify BrdU-retaining, adult-born cells.

Immunohistochemistry (IHC)

Brains from mice were harvested following intracardial perfusion and stored in -80°C until IHC processing as described (Pan et al., 2012a; Pan et al., 2012b). IHC was performed on 20 μm thick coronal olfactory bulb sections and either 30 μm thick coronal or 20 μm thick sagittal brain sections using a free-floating antibody staining method as described (Pan et al., 2012b).

Confocal imaging and analysis

Images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with numerical aperture (NA) 1.3, 40X oil immersion lens. Optical Z-sections (1 μm) were collected and processed using ImageJ (NIH). Images were uniformly adjusted for color, contrast, and brightness using Adobe Photoshop CS4 (Adobe Systems, Inc.).

Quantification of immunostained cells

One in every 8 coronal serial brain sections containing the SVZ or 1 in every 4 coronal serial olfactory bulb sections was immunostained with the indicated markers. Immunopositive cells were quantified as described (Pan et al., 2012b), using a modified unbiased stereology technique by confocal microscopy with the experimenter blind to treatment conditions (West et al., 1991; Kempermann et al., 1997b; Malberg et al., 2000). Hoechst staining of the nuclei was used to confirm corresponding marker expression associated with a specific cell nucleus. Resulting cell numbers were scaled relative to the number of sections per SVZ or olfactory bulb and reported as an estimated total number of cells per region. Approximately 6–10 sections were used for quantification per region per brain. For co-localization analysis, greater than 50 immunopositive cells were randomly selected and analyzed using confocal microscopy for the presence of overlapping fluorescent signal across all planes in an optical Z-series of a single cell. Only when overlapping signal was found in all planes was a cell considered to be double positive.

Behavior assays

Male mice were used for behavior assays. Mice were individually housed and handled at least 4 d before olfactory behavior assays and remained singly caged throughout all olfaction assays. With the exception of the TMT-based innate fear assay, all other olfactory behavior assays were conducted in mouse home cages. For all cotton-tip based behavior assays, cotton swabs dipped in vehicle control (mineral oil or water) or odorant solution were suspended from the wire top of the animal's home cage with the cotton tips 8 cm above the cage floor. The duration of animals' sniffing of the cotton swab was recorded. The sniffing was defined as animals' noses approaching to and within 1 cm distance to the swabs. The animal does not have to physically touch it. Odorants and corresponding concentrations used in various olfaction tests are listed in Table 5.1.

Olfactory habituation/dishabituation test with chemical odorants

This was conducted as described (Zou et al., 2012). Briefly, naïve animals were pre-trained with mineral oil-laced cotton swabs for four presentations (60 s each, 2 min intervals) to ensure that subsequent exposure to an odorant-laced cotton swab did not elicit a response due to object novelty. The odor habituation/dishabituation test was then performed by presenting citralva, IAA, and ethyl vanillin sequentially, with four presentations for each odorant (60 s each presentation, 2 min intervals). A significant decrease in the number of investigations during subsequent presentations of the same odorant indicates odor recognition and habituation. An increase in investigation of a new odorant indicates dishabituation.

Olfactory habituation/dishabituation test with diluted mouse urine

This was conducted as described above using diluted (1:50) normal female mouse urine, ovariectomized female mouse urine, male mouse urine, pregnant female mouse urine, and lactating female mouse urine, sequentially, with four presentations of each urine sample (60 s each presentation, 2 min intervals). A significant decrease in total investigation time during subsequent presentations of the same urine sample indicates recognition and habituation. An increase in investigation of the next, new urine sample indicates dishabituation.

Olfactory short-term memory

This was performed as described with modifications (Breton-Provencher et al., 2009). Mice were presented with a cotton swab laced with the same odorant during two different 5 min sessions separated by 30, 240, 300, or 360 min intervals. Odorant detection and investigation of the cotton swab was recorded for each 5 min session. A different odor was used for each interval time point, but only one time interval was tested on each day for each mouse to avoid cross interference of olfactory detection and memory. A significant decrease in total

investigation time of the cotton swab during the 2nd presentation of the odorant suggests olfactory memory for the 1st presentation of the same odorant (Rochefort et al., 2002; Mechawar et al., 2004; Breton-Provencher et al., 2009).

Odor detection threshold

This was performed as described with modifications (Breton-Provencher et al., 2009). Mice were presented with 2 cotton swabs simultaneously; one laced with vehicle control and the other laced with a specific odorant or pheromone as indicated. The relative location of the two cotton swabs was randomly switched between presentations to avoid spatial learning. One session was performed per day per concentration, with increasing concentrations each day. Each session lasted 3 min and total investigation time of each cotton swab was recorded during the entire 3 min session. Data are presented as the mean ratio between the time spent investigating the odor and the total sniffing of both cotton swabs (percent sniffing duration). A 50% sniffing duration indicates no detection of the odorant because the animals spent an equal amount of time investigating both cotton swabs. Sniffing durations greater than 50% of the time indicates that animals detected the odorant.

To measure detection sensitivity for TMT, the above assay was performed with slight modifications. Since TMT is a known fear-inducing odor component of fox feces (Fendt et al., 2005), we mixed it with odorants that mice are not averse to (1 mM 2-heptanone, 1-octanol, or S-terpinen-4-ol). Mice were then presented with 2 cotton swabs as above, one laced with just the odorant and the other laced with the odorant plus increasing concentrations of TMT. When mice detect TMT, they avoided this cotton swab; thus a sniffing duration less than 50% indicates that the animal detected the fear odor.

TMT-based innate fear assay

This was conducted in a 3-chamber testing apparatus as described (Hacquemand et al.; Buron et al., 2007). Briefly, the apparatus (60 cm x 22 cm x 12 cm) consisted of three 20 cm x 22 cm x 12 cm partitions. Dividers between chambers had a small opening to allow mice to cross freely between the three chambers. Mice were placed in the middle chamber and a small plastic dish was placed at the far end of each of the other two chambers; mice were allowed to habituate in the apparatus for 15 min. Following habituation, the two plastic dishes were retrieved and a filter paper (2 cm x 2 cm) containing 5 µL of water or 5 mM TMT was placed into each of the two dishes in a randomized manner between mice. The dishes were then placed back into the apparatus at their original location; the duration of investigation to each chamber and freezing behavior were recorded during a 5 min test session with the experimenter blind to treatment conditions. Avoidance of the TMT chamber and increased freezing behavior are indicative of innate fear responses.

Olfactory preference

Mice were presented simultaneously with two cotton swabs laced with undiluted mouse urine (10 µL). Mouse urine was collected from group-housed mice (n = 4–5 per cage) and pooled over a period of 2 weeks to minimize day-to-day fluctuations in basal pheromone and urine amount. Total investigation time of each cotton swab was recorded during a 2 min session. Cotton swab investigation is defined as the nose of the animal approaching to and within 1 cm from the cotton swab. The animal does not have to physically touch it. Data presented are percent sniffing duration for each odorant or urine relative to the total sniffing of both cotton swabs. A 50% sniffing duration indicates no preference for either urine.

Sand-digging based odor-cued associative olfactory learning

Following olfactory preference assay, mice were food restricted to maintain 85-90% of free-feeding body weight for 4–5 d prior to the beginning and throughout the entirety of associative olfactory discrimination assay, which was performed as previously described using the same apparatus, pre-training, and training protocols (Zou et al., 2012). Briefly, after mice learned to associate sand digging with a food reward at the bottom of the dish, mice were presented with 2 sand dishes in each trial, for 4 trials per block, and 2 blocks per day. One dish contained 100 μL of a urine sample associated with a food reward, while the other contained 100 μL of another urine sample (both urine samples were diluted 1:50) without food reward. To avoid spatial learning, the two dishes were placed on either the left or right side randomly as long as each dish was placed on each side twice per block but no more than three consecutive times in each day. Scoring for correct or incorrect choice was based entirely on the animal's first dig, either in the urine dish with the food reward (correct) or in the urine dish without the food reward (incorrect). Mice that dug in the incorrect dish were not allowed to self-correct. Two pairs of urine samples were used in this assay: 1) ovariectomized (enforced with food reward) vs. normal female urine; 2) pregnant (enforced with food reward) vs. lactating female urine.

Statistical analysis

Repeated measures ANOVA was used to analyze data in Fig. 5.3, 5.7A, and 5.8. Student's *t*-test was used to analyze all remaining data. Data represent mean ± standard error of means (s.e.m.). *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p<0.001$; n.s., not statistically significant ($p \geq 0.05$).

Table 5.1. List of odorants used in behavior assays.

Assay	Odorant	Concentration
Olfactory Habituation	Citralva	1 mM
	Isoamyl Acetate	50 μM
	Ethyl Vanillin	50 μM
	Adult Female Mouse Urine	1:50 Dilution
	Ovariectomized Female Mouse Urine	1:50 Dilution
	Adult Male Mouse Urine	1:50 Dilution
	Pregnant Female Mouse Urine	1:50 Dilution
	Lactating Female Mouse Urine	1:50 Dilution
Olfactory Memory	S-Terpinen-4-ol	1 mM
	Acetophenone	1 mM
	1-Octanol	1 mM
	Ethyl Vanillin	1 mM
Olfactory Threshold	1-Octanol	1 μM – 500 μM
	2-Heptanone	1 μM – 500 μM
	Farnesene	1 μM – 500 μM
	2,3,5-Trimethyl-3-thiazoline (TMT)	1 μM – 50 μM
Innate Fear	2,3,5-Trimethyl-3-thiazoline (TMT)	5 mM
Olfactory Preference	Male Mouse Urine	10 μL Undiluted
	Female Mouse Urine	10 μL Undiluted
	Pregnant Female Mouse Urine	10 μL Undiluted
	Lactating Female Mouse Urine	10 μL Undiluted
	Adult Female Mouse Urine	10 μL Undiluted
	Adult Ovariectomized Female Mouse Urine	10 μL Undiluted
Odor-cued Associative Olfactory Learning	Pregnant Female Mouse Urine	1:50 Dilution
	Lactating Female Mouse Urine	1:50 Dilution
	Adult Female Mouse Urine	1:50 Dilution
	Adult Ovariectomized Female Mouse Urine	1:50 Dilution

RESULTS

ERK5 is expressed along the SVZ-RMS-OB axis in adult mice

Brain sections from adult mice were processed for ERK5 immunohistochemistry using an affinity-purified polyclonal ERK5 antibody. Although ERK5 protein is not expressed in most regions of the adult mouse brain (Pan et al., 2012b), ERK5 expression was prominent along the SVZ, RMS, and in the center of the granular cell layer of the OB where adult born neurons exit the RMS (Fig. 5.1). Additionally, we performed co-labeling of ERK5 and doublecortin (DCX), a marker for transiently amplifying progenitors/newborn neurons, and found a high degree of co-labeling in both the SVZ and OB (Fig. 5.1C–J). This unique expression pattern of ERK5 suggests that ERK5 plays a role in regulating adult SVZ/OB neurogenesis.

Targeted deletion of *erk5* reduces adult SVZ/OB neurogenesis

Using a Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ transgenic mouse strain (Pan et al., 2012a), we conditionally deleted *erk5* in Nestin-expressing neural stem cells along both the subgranular zone (SGZ) of the dentate gryus (Pan et al., 2012a) and SVZ (Fig. 5.2) in adult animals upon tamoxifen treatment (ERK5 icKO mice). Three weeks after a 7-day tamoxifen treatment paradigm, the total number of ERK5^{+} cells was reduced by 60% in the SVZ of ERK5 icKO mice compared to vehicle control-treated littermates (control) (Fig. 5.2A–H; *t*-test, $p<0.001$). This was accompanied by a 50% reduction in the total number of DCX^{+} cells in the SVZ (Fig. 5.2I; *t*-test, $p=0.03$). To determine if the reduction of ERK5^{+} and total DCX^{+} cells in the SVZ had any impact on the number of adult-born neurons in the OB, we dosed animals with BrdU to label adult born, BrdU-retaining cells. Animals were sacrificed 3 weeks later; OB sections were immunostained for BrdU and NeuN, a marker expressed in mature neurons of the granular cell layer of the OB. There was a statistically significant reduction in the percentage of BrdU and NeuN double-positive cells among all BrdU^{+} cells in the granule cell layer of the OB (Fig. 5.2J; *t*-test, $p=0.05$), indicating a reduction in the total number of adult-born, mature neurons during this time window

(3 weeks after BrdU labeling). These data suggest that ERK5 plays a role in regulating adult SVZ/OB neurogenesis *in vivo*.

ERK5 icKO mice can detect discrete odorants in a habituation/dishabituation assay but show reduced olfactory short-term memory

To investigate the functional impact of conditional *erk5* deletion in ERK5 icKO mice on olfactory behavior, we first tested animals for an odor habituation/dishabituation assay using three distinct odorants: citralva, isoamyl acetate (IAA), and ethyl vanillin. This assay is commonly used to detect overt odor detection deficits in mice (Trinh and Storm, 2003). ERK5 icKO mice showed normal habituation/dishabituation and were indistinguishable from control mice (Fig. 5.3; $ANOVA_{mineral\ oil}$, p=0.06; $ANOVA_{citralva}$, p=0.22; $ANOVA_{IAA}$, p=0.06; $ANOVA_{ethyl\ vanillin}$, p=0.25).

When tested for short-term olfactory memory, both control and ERK5 icKO mice spent significantly less time sniffing the odor-laced cotton swab during the second exposure than the first exposure when the same odorant was presented 30 min apart (Fig. 5.4; $t\text{-test}_{Control}$, p<0.001; $t\text{-test}_{ERK5\ icKO}$, p<0.001). There was no statistically significant difference between control and ERK5 icKO mice in their sniffing duration during the second exposure (p=0.11). This suggests that the 30-min olfactory memory for ERK5 icKO mice was comparable to control mice. Control mice maintained a similar degree of memory at 240 min and 300 min as it did at 30 min. However, ERK5 icKO mice did not exhibit statistically significant olfaction memory from 240 min onward. Thus, the short term olfactory memory of ERK5 icKO mice decayed much faster than that of the control mice, suggesting that they have impaired short-term olfactory memory.

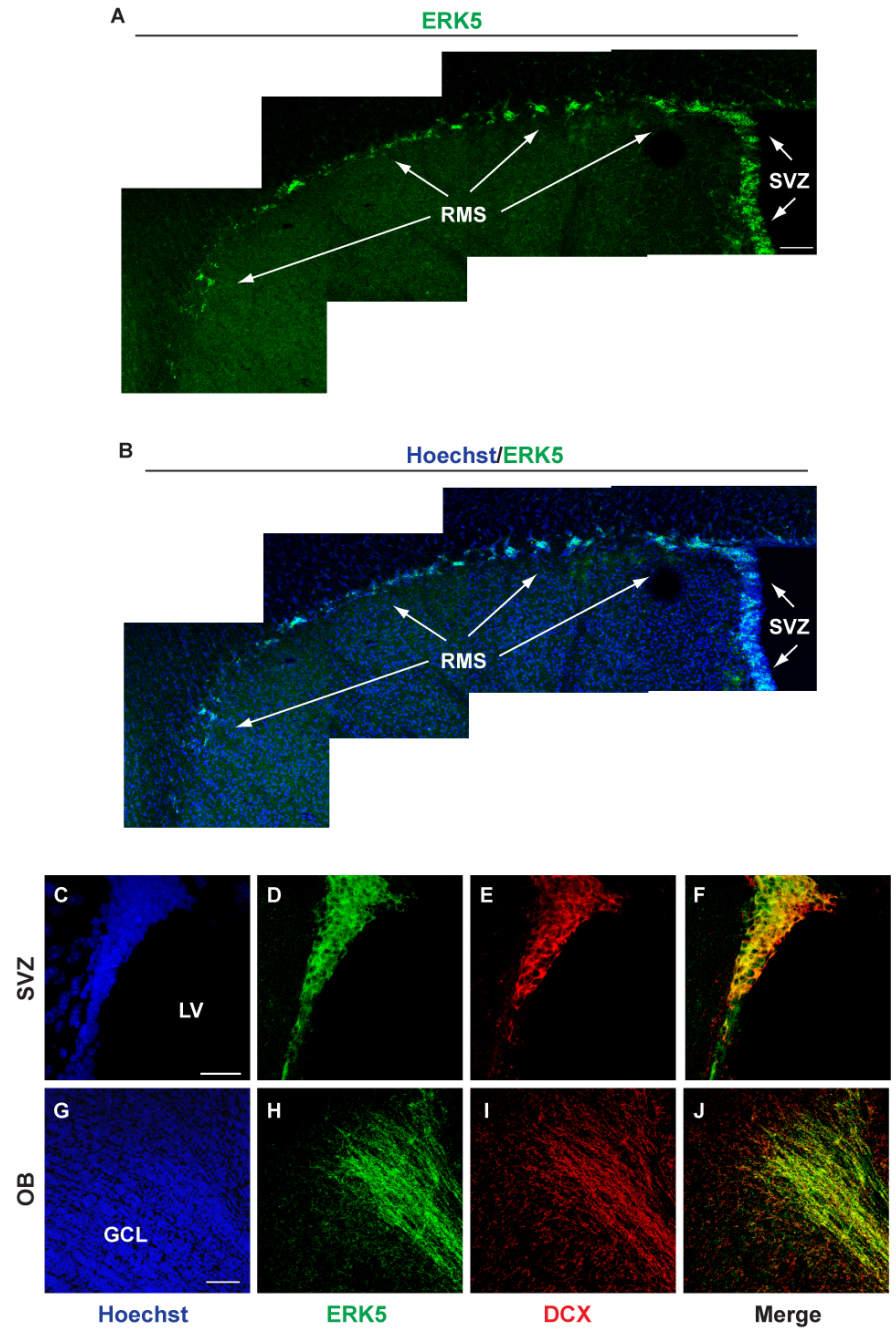


Figure 5.1. ERK5 is expressed along the entire SVZ/RMS/OB axis. A, B) ERK5 protein (green) was identified by IHC along the SVZ and RMS of adult mouse brain. Hoechst staining (blue) was used to identify all cell nuclei. Scale bar in A represents 100 μm and applies to B. C–J) ERK5 co-localizes with DCX^{+} cells (red) along the lateral ventricles (LV) and in the center of the granular cell layer (GCL) of the OB where adult born neurons exit the RMS. Scale bar in C represents 25 μm and applies to D–F, while scale bar in G represents 100 μm and applies to H–J. Three individual mouse brains were analyzed for ERK5 expression along the SVZ/RMS/OB axis.

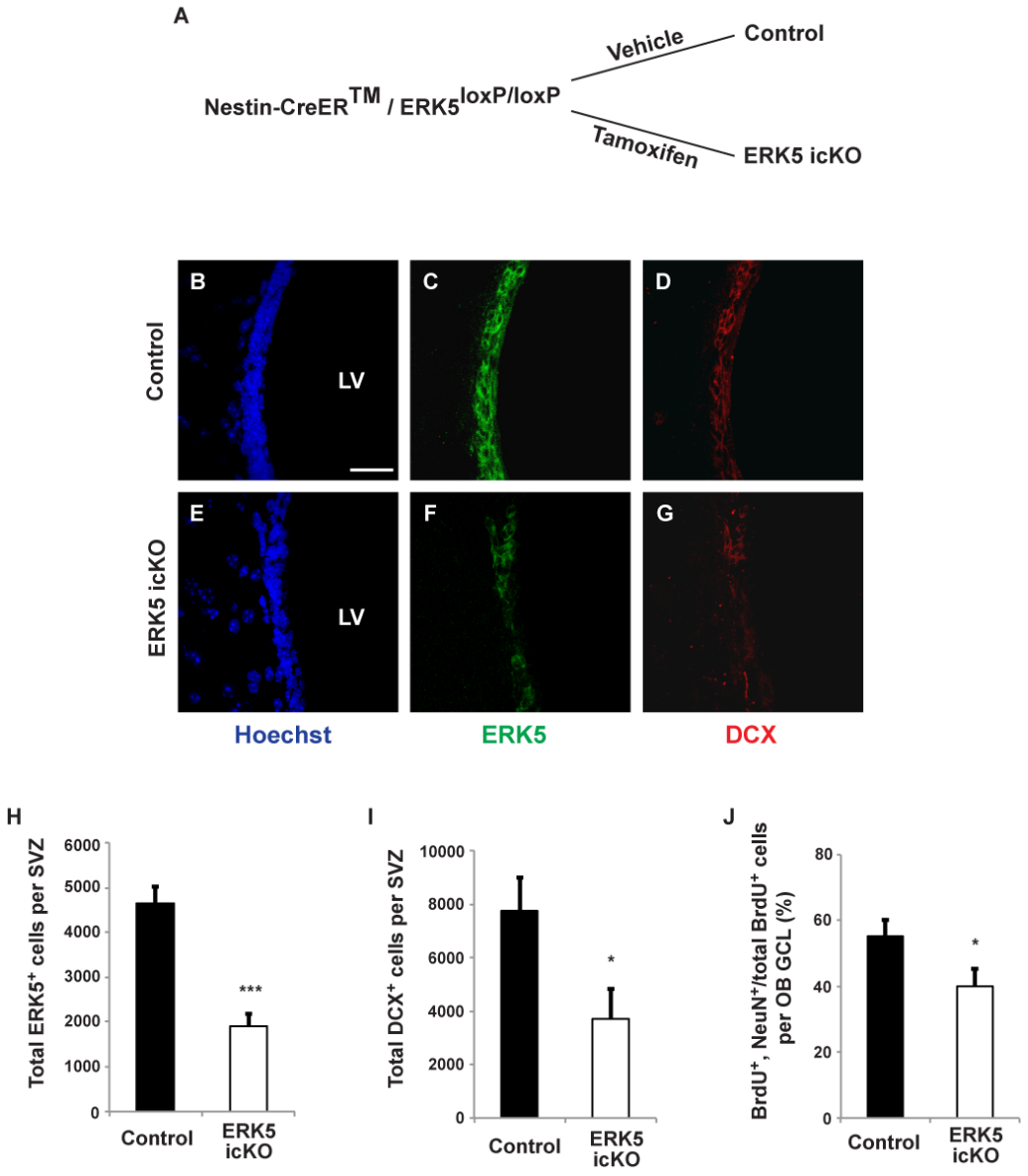


Figure 5.2. Inducible and conditional knock out of *erk5* significantly reduces ERK5 protein expression along the SVZ, the number of DCX$^+$ cells in the SVZ, and the number of adult-born neurons in the OB. A) Schematic illustration of the experimental design. B–G) Representative immunostaining photomicrographs of ERK5 (green) and DCX (red) in vehicle control-treated mice (B–D) as well as tamoxifen-treated ERK5 icKO mice (E–G). Scale bar in B represents 25 µm and applies to C–G. H) Quantification of total ERK5$^+$ cells along the SVZ. I) Quantification of total DCX$^+$ cells along the SVZ. J) The deletion of *erk5* decreases the total number of adult-born neurons (BrdU and NeuN double-positive cells among total BrdU$^+$ population) in the granule cell layer of the OB 3 weeks after BrdU injection. n = 5 individual mouse brains and olfactory bulbs per treatment condition.

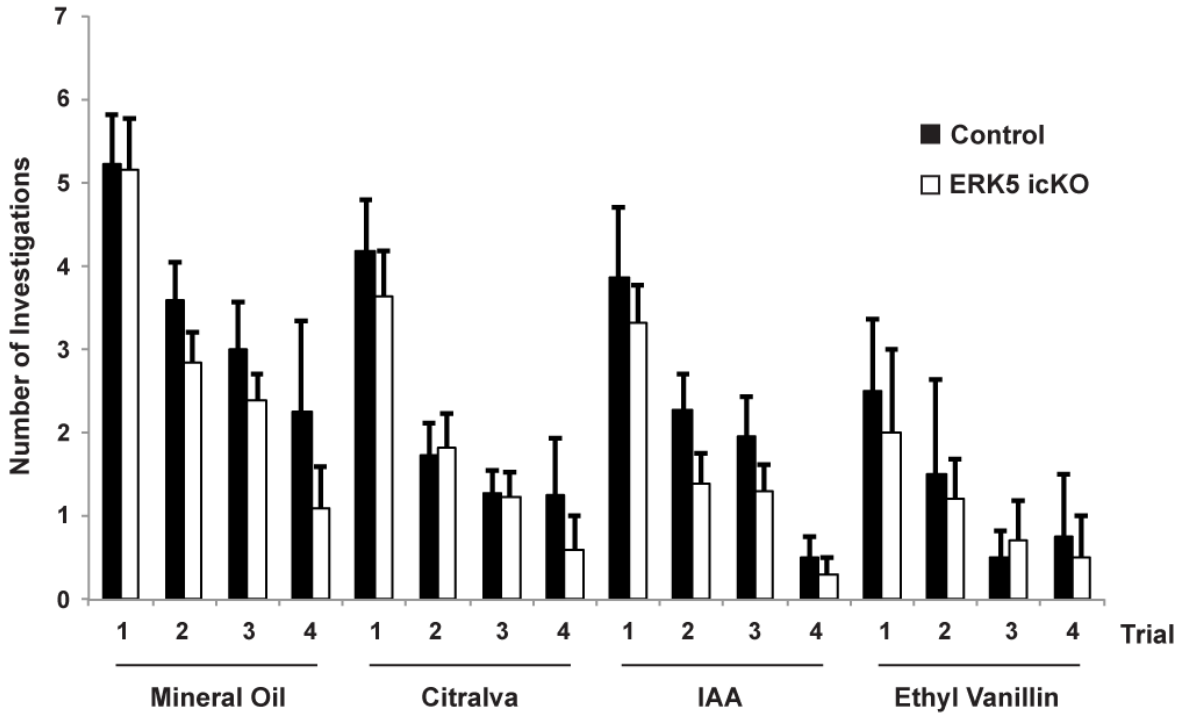


Figure 5.3. ERK5 icKO mice have normal olfactory detection to discrete odorants in a habituation/dishabituation assay. Naïve, adult mice were pre-trained with four presentations of mineral oil-soaked cotton swabs, then exposed to three structurally different odorants, citralva, isoamyl acetate (IAA), and ethyl vanillin with 4 trials per odorant. Step-wise decrease in the number of investigation during sequential presentations of the same odor followed by renewed interest in investigation of the first presentation of a new odorant suggests normal olfactory habituation/dishabituation behavior. n ≥ 16 mice per treatment group from 2 independent experiments.

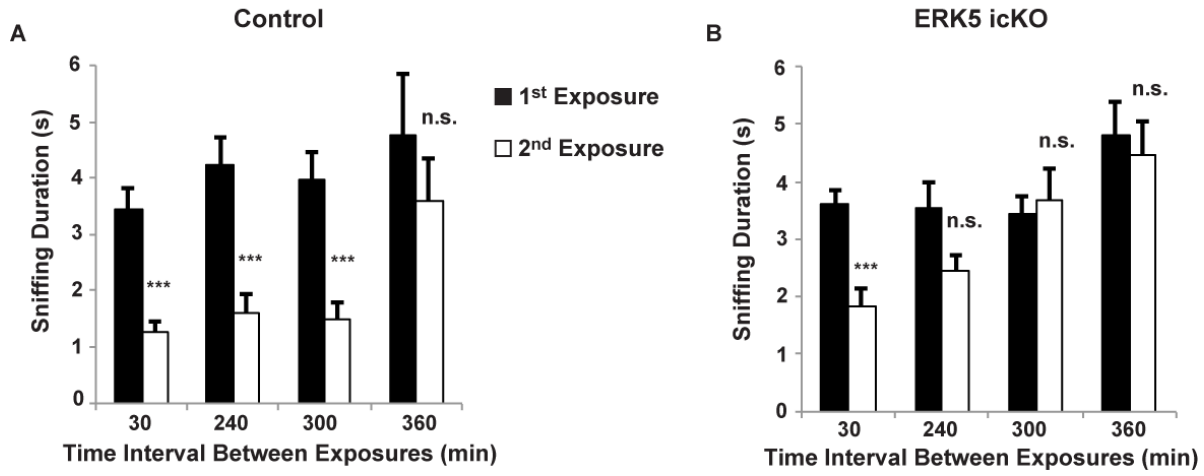


Figure 5.4. ERK5 icKO mice are deficient in olfactory short-term memory. Mice were presented with cotton swabs laced with the same odorant twice at the indicated time intervals. A different odor was used for each interval time point. The sniffing duration of the cotton swab was recorded for both exposure sessions. A decrease in investigation during the second exposure of the same odorant is suggestive of olfactory memory to the odorant. A) Short-term olfactory memory of control mice. B) Short-term olfactory memory of ERK5 icKO mice. n ≥ 7 mice per treatment group.

Reduced detection sensitivity to chemically defined odorants and pheromones in ERK5 icKO mice

We next examined the sensitivity of odor detection using an olfactory threshold assay. ERK5 icKO and control mice were presented with pairs of cotton swabs laced with mineral oil or increasing concentrations of 1-octanol (1-500 μM). Although both groups of mice detected 500 μM 1-octanol equally well, ERK5 icKO mice failed to detect 1-octanol at 100 μM while control mice clearly did (Fig. 5.5A; *t*-test, p=0.008). Similarly, control mice detected 2-heptanone, a mouse pheromone (Novotny et al., 1986; Leinders-Zufall et al., 2000; Trinh and Storm, 2003) at lower concentrations than ERK5 icKO mice did, and there was a statistically significant difference between the two animals at 100 μM 2-heptanone (Fig. 5.5B; *t*-test, p=0.004).

We also examined the sensitivity of ERK5 icKO mice to farnesene, a synthetic, aggression-evoking pheromone (Novotny et al., 1990; Wang et al., 2004). ERK5 icKO mice were only able to detect farnesene at the highest concentration tested (500 μM) while control mice detected it at 100 μM (Fig. 5.6A; *t*-test, p=0.009). Furthermore, mice were also examined for their detection of TMT, a component of fox scent that is known to elicit innate fear and alarm in rodents (Fendt et al., 2005; Kobayakawa et al., 2007; Sakamoto et al., 2011). When mice detect TMT, they will typically avoid it and may exhibit freezing behavior. Control mice avoided cotton swabs laced with TMT at 10 and 50 μM (Fig. 5.6B) while ERK5 icKO did not (t-test$_{10\ \mu M}$, p=0.04; t-test$_{50\ \mu M}$, p=0.05). It is possible that ERK5 icKO mice have reduced sensitivity to detect TMT at 10 and 50 μM. Alternatively, ERK5 icKO mice may have reduced intrinsic innate fear of TMT and therefore do not avoid the TMT-laced cotton swab as much as control mice. To distinguish between these possibilities, we used a separate cohort of mice and a 3-chamber apparatus to directly test their intrinsic innate fear of TMT at a higher concentration.

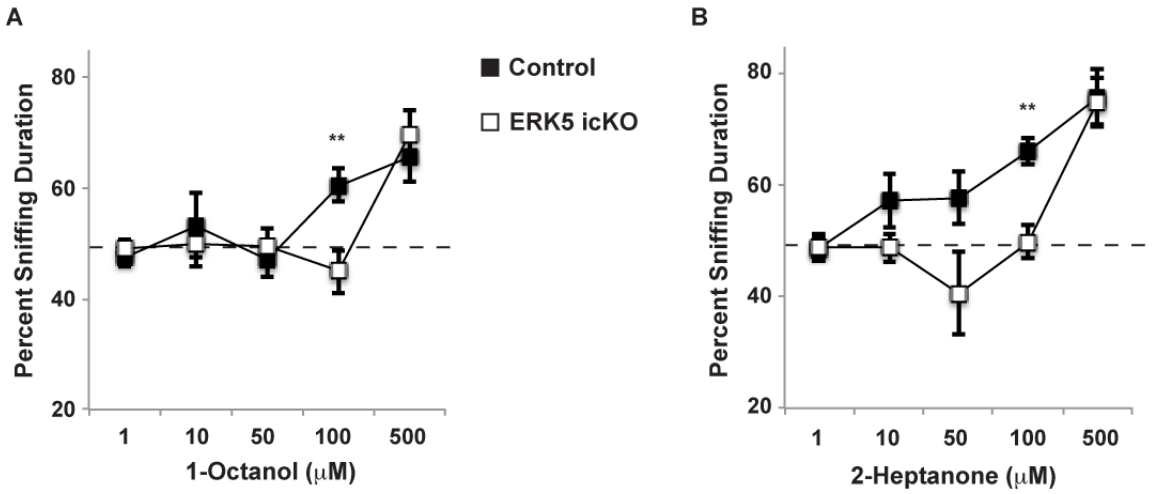


Figure 5.5. Reduced detection sensitivity to 1-octanol and 2-heptanone in ERK5 icKO mice. ERK5 icKO and control mice were presented with 2 cotton swabs, one laced with vehicle control and the other with increasing concentrations of 1-octanol or 2-heptanone. An above 50% sniffing duration (above chance) indicates detection of 1-octanol or 2-heptanone at that specific concentration. A) Dose-response of detection threshold to 1-octanol, a chemically defined odorant. B) Dose-response of detection threshold to 2-heptanone, a chemically defined mouse pheromone. n ≥ 14 mice per treatment group.

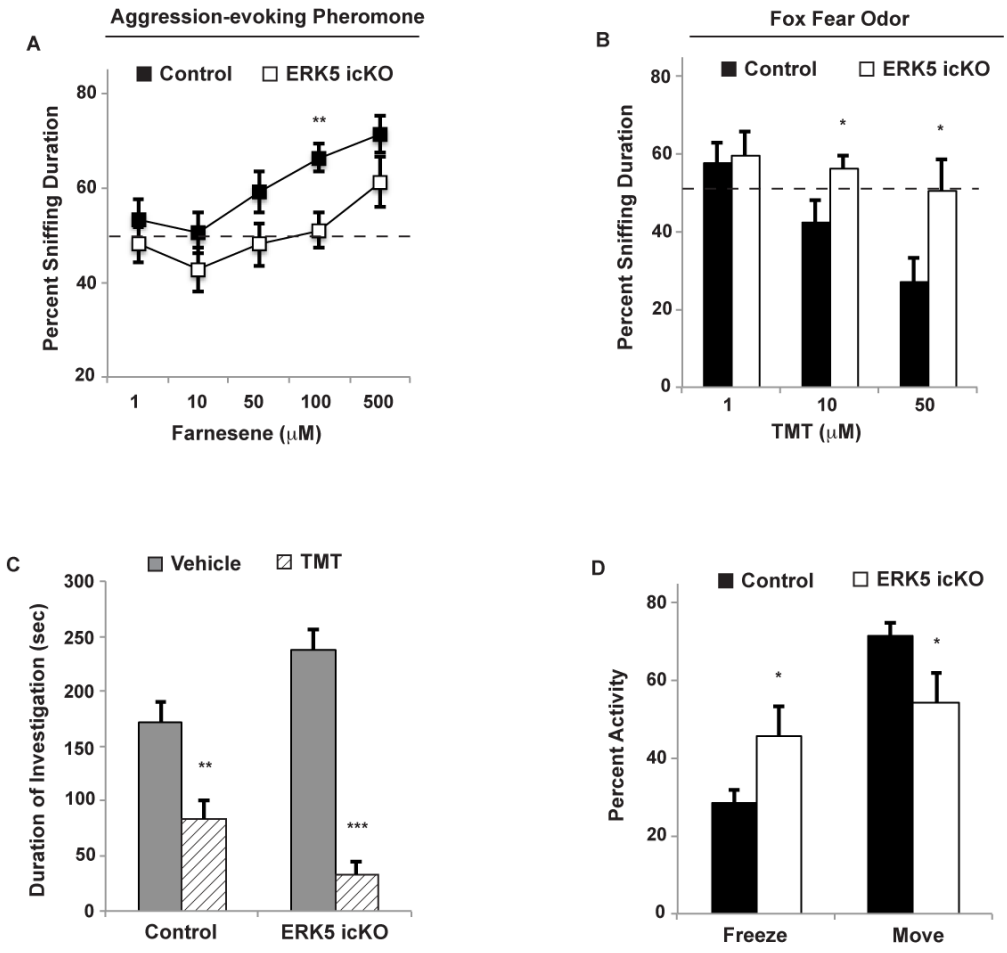


Figure 5.6. ERK5 icKO mice have reduced detection sensitivity to aggression-evoking and fear-inducing pheromones, but are not impaired in innate fear response *per se*. A) Dose-response of detection threshold to farnesene, a chemically defined, aggression-evoking mouse pheromone. ERK5 icKO and control mice were presented with 2 cotton swabs, one laced with vehicle control and the other with increasing concentrations of farnesene. An above 50% sniffing duration (above chance) indicates detection of farnesene. B) Dose-response detection to TMT, a fear-evoking odor found in fox feces. ERK5 icKO and control mice were presented with 2 cotton swabs, one laced with vehicle control and the other with increasing concentrations of TMT. Because TMT should elicit avoidance behavior when mice detect it under normal conditions, a below 50% sniffing duration (less than chance) indicates detection of TMT. C) ERK5 icKO and control mice were subjected to the innate fear assay toward TMT using a 3-chamber apparatus. The duration of investigation toward the chamber containing filter paper laced with TMT (5 mM) or vehicle control was quantified. Avoidance behavior toward the TMT chamber relative to the vehicle control chamber is a measure of TMT detection and innate fear. D) Quantification of percent time mice spent freezing, another measurement of innate fear, during the innate fear assay toward TMT using the 3-chamber apparatus. n ≥ 7 mice per treatment group.

Mice were placed in the middle chamber, TMT (5 mM) and vehicle control were placed in the other two chambers, separately. The duration of investigation toward each chamber and the percent time mice froze were quantified. Although both control and ERK5 icKO mice froze some of the time and spent less time investigating the chamber associated with TMT, ERK5 icKO mice clearly avoided TMT even more so than control mice (Fig. 5.6C; $t\text{-test}_{\text{Control}}$, p=0.002; $t\text{-test}_{\text{ERK5 icKO}}$, p<0.001). They also froze more and moved less than control mice (Fig. 5.6D; $t\text{-test}_{\text{Freeze}}$, p=0.05; $t\text{-test}_{\text{Move}}$, p=0.05). Thus, ERK5 icKO mice do not exhibit reduced intrinsic innate fear when a sufficient concentration of TMT was present; if anything, their innate fear is enhanced. Together, our data suggest that ERK5 icKO mice have reduced sensitivity to detect odorants and pheromones at lower concentrations. However, *erk5* deletion and subsequent reduction of adult neurogenesis do not impair innate fear.

ERK5 icKO mice do not prefer mouse urine collected from normal females to that from ovariectomized females

Since ERK5 icKO mice displayed deficits in detecting lower concentrations of chemically defined odorants and pheromones, we next evaluated their responses to various mouse urine samples, which contain complex mixtures of odorants and pheromones. We first performed the olfactory habituation/dishabituation assay with mouse urine (1:50 dilution) and found no distinguishable differences between control and ERK5 icKO mice in their ability to discriminate between various urine samples (Fig. 5.7A; $\text{ANOVA}_{\text{vehicle}}$, p=0.08; $\text{ANOVA}_{\text{normal female urine}}$, p=0.99; $\text{ANOVA}_{\text{ovariectomized female urine}}$, p=0.16; $\text{ANOVA}_{\text{male urine}}$, p=0.14; $\text{ANOVA}_{\text{pregnant female urine}}$, p=0.39; $\text{ANOVA}_{\text{lactating female urine}}$, p=0.08). Next we assessed if any overall deficits existed between groups of mice in the urine preference assay using different cohorts of mice. ERK5 icKO and control mice were presented with two cotton swabs laced with a pair of undiluted urine samples collected from groups of adult mice; their sniffing duration toward each cotton swab was recorded and quantified. Both male control and male ERK5 icKO mice showed a preference for

female mouse urine relative to male mouse urine; there was no difference between the two genotypes (Fig. 5.7B; *t*-test, p=0.68). Neither group of mice showed any preference between lactating and pregnant female mouse urine (Fig. 5.7C; $t\text{-test}_{\text{Control}}$, p=0.13; $t\text{-test}_{\text{ERK5 icKO}}$, p=0.76). Interestingly, however, although control mice preferred urine collected from normal female mice over that from ovariectomized females, ERK5 icKO mice showed no such preference (Fig. 5.7D; $t\text{-test}_{\text{Control}}$, p=0.003; $t\text{-test}_{\text{ERK5 icKO}}$, p=0.50).

ERK5 icKO mice are impaired in odor-cued associative olfactory learning

To examine if conditional deletion of *erk5* and subsequent impairment of adult neurogenesis adversely affects acquisition of odor-cued associative olfactory tasks, mice were trained in a sand-digging based associative olfactory discrimination assay as described previously (Zou et al., 2012). Mice were presented with two sand dishes, each laced with mouse urine (1:50 dilution) from pregnant females or lactating females, respectively. A food reward was buried in the sand at the bottom of the dish laced with pregnant female urine. Thus, animals had to use olfactory cues to discriminate between the two urine samples to retrieve the food reward. This type of odor-cued associative task is hippocampus-independent (Kaut and Bunsey, 2001; Kaut et al., 2003; Jonasson et al., 2004; Sultan et al., 2010). Over the course of 5 training days, control mice learned that the food reward was associated with pregnant female mouse urine but not with lactating female mouse urine; learning was evidenced by the increase in correct choices (Fig. 5.8A). However, ERK5 icKO mice showed no such learning and there was a statistically significant difference between the two groups of mice (ANOVA, p=0.03).

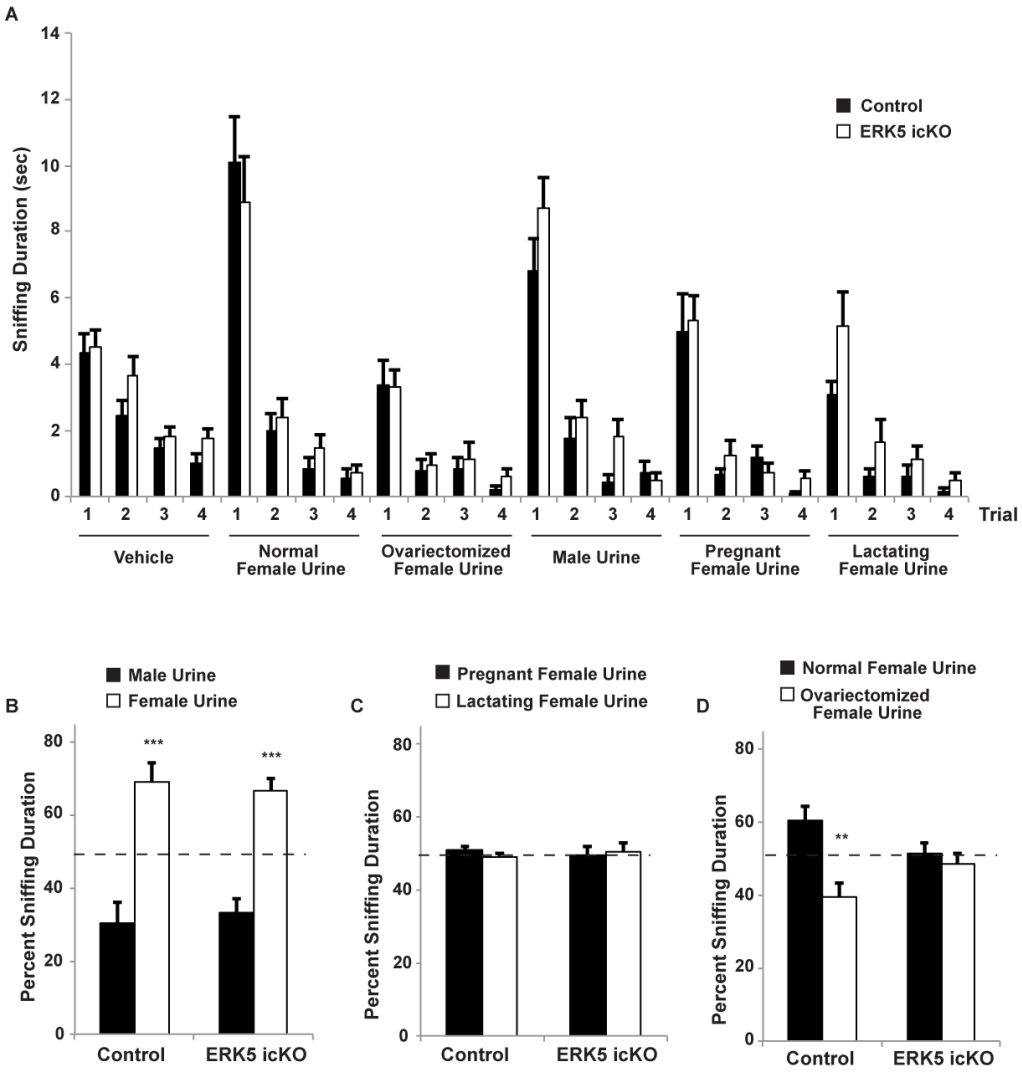


Figure 5.7. Unlike control mice, ERK5 icKO mice do not prefer mouse urine from normal females to that from ovariectomized females. A) Male ERK5 icKO and control mice were tested for their ability to discriminate between various mouse urine samples using the habituation/dishabituation assay. The duration of sniffing of the cotton swab was quantified. Both groups of mice were able to discriminate between urine samples over the course of 4 sequential presentations. B) Urine preference test between female mouse urine vs. male mouse urine, presented simultaneously with cotton swabs laced with female or male mouse urine. The duration of sniffing toward each cotton swab was quantified. Control and ERK5 icKO mice (both male) showed a significant preference sniffing towards female mouse urine vs. male mouse urine. C) Urine preference test between pregnant and lactating female mouse urine. D) Urine preference test between ovariectomized female mouse urine and age-matched, normal adult female mouse urine. Control mice preferred sniffing normal adult female mouse urine while ERK5 icKO mice showed no preference toward either. n ≥ 7 mice per treatment group.

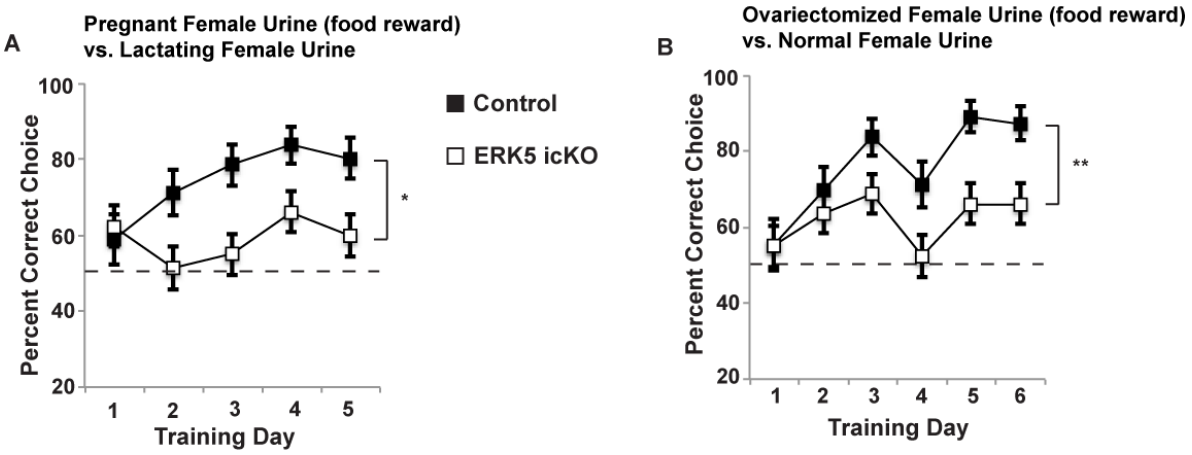


Figure 5.8. ERK5 icKO mice are impaired in the acquisition of a sand digging-based, odor-cued associative learning. A) Control, but not ERK5 icKO mice learned to associate a food reward with pregnant female mouse over a 5-day training course. B) ERK5 icKO mice did not learn as well as control mice to associate a food reward with ovariectomized female mouse urine over a 6-day training course. n ≥ 7 mice per treatment group.

This defect in odor-cued associative olfactory learning was confirmed with another pair of urine samples: normal adult female mouse urine vs. ovariectomized female mouse urine (Fig. 5.8B). Since control mice had a preference for normal adult female mouse urine over ovariectomized female mouse urine, we paired the food reward with ovariectomized female mouse urine. Over the course of a 6-day training paradigm, control mice learned significantly better than ERK5 icKO mice to discriminate between the urine pair and to associate ovariectomized female mouse urine with the food reward (ANOVA, p=0.01). These data suggest that ERK5 icKO mice are impaired in the acquisition of odor-cued associative olfactory learning.

DISCUSSION

The adult brain has the remarkable capacity to continuously generate new neurons in the SVZ and OB. However, the functional impact of these adult-born neurons on olfactory behavior is still elusive. Furthermore, signaling mechanisms regulating adult neurogenesis in the SVZ/OB are not well defined. The objective of this study was to investigate the role of ERK5 MAP kinase in the regulation of adult SVZ/OB neurogenesis and associated olfactory behavior.

Although several studies have attempted to address the role of adult neurogenesis in olfactory behavior, findings from different studies are contradictory and the physiological function of adult-born neurons in the OB is still unclear (Lledo et al., 2006; Whitman and Greer, 2009; Bardy and Pallotto, 2010; Lazarini and Lledo, 2011). For example, genetic expression of a lethal diphtheria toxin fragment (DTA) in adult-born neurons, which kills these neurons, led to a substantial loss of adult-born granule cells in the OB and a much smaller OB (Imayoshi et al., 2008). Surprisingly, despite these structural changes in the OB, no deficits in olfactory behavior were observed, casting doubt as to whether adult-born neurons in the OB influence olfactory behavior. However, a subsequent study using lateral ventricle infusion of AraC to ablate adult neurogenesis in the SVZ/OB in mice showed that a reduction in OB adult neurogenesis reduces

odor detection sensitivity and impairs short-term (hours) olfactory memory but does not affect odor discrimination or reward-associated long-term memory (days) (Breton-Provencher et al., 2009). Although results from this study generated much excitement in the field (Arenkiel, 2010; Bardy and Pallotto, 2010; Lazarini and Lledo, 2011), they contradicted results from other reports (Gheusi et al., 2000; Lazarini et al., 2009; Sultan et al., 2010). For example, left ventricle infusion of AraC in another study implicated OB adult neurogenesis in long-term olfactory memory (Sultan et al., 2010). Furthermore, cranial irradiation of the SVZ, which robustly impaired adult neurogenesis in the OB, showed that only long-term olfactory memory, but not the threshold for odor detection or short-term olfactory memory, is affected (Lazarini et al., 2009). In addition, NCAM knockout mice have diminished OB neurogenesis, and are deficient in the odor discrimination task while both the detection threshold for odors and short-term olfactory memory are unaltered (Gheusi et al., 2000). Thus, the exact function of adult-born OB neurons is still highly debated.

Several factors may contribute to this controversy including the specificity of methods used to ablate or suppress adult neurogenesis. Although effective at suppressing adult neurogenesis, x-irradiation or anti-mitotic drugs are not specific for adult-born neurons. They target all dividing cells, may alter the neurovascular niche important for adult neurogenesis, and induce neural inflammation. The side effects intrinsic to these methods may be confounding factors contributing to inconsistent behavior results. Indeed, two studies using similar protocols for AraC administration to the lateral ventricle to suppress adult neurogenesis yielded opposite conclusions concerning a role for adult neurogenesis in long-term olfactory memory (Breton-Provencher et al., 2009; Sultan et al., 2010). Studies using traditional knockout of genes important for neurogenesis are also useful, but their interpretations are limited by widespread abnormalities of brain structure or compensatory effects elicited during development. Transgenic expression of a lethal gene, such as diphtheria toxin or thymidine kinase (Imayoshi

et al., 2008; Singer et al., 2009), to kill adult-born neurons is more specific to adult neural stem/progenitor cells. However, large amounts of cell death in the SVZ-RMS-OB axis may interfere with normal olfactory function. These technical issues make it difficult to establish a definitive connection between adult neurogenesis and olfactory behavior.

The existing controversy necessitates studies using more specific genetic approaches with less off-target side effects to manipulate adult neurogenesis. Clearly, identification of a signaling molecule that is required for adult neurogenesis which is only expressed in neurogenic regions of the brain, coupled with a transgenic mouse engineered to temporally and spatially delete that gene only in adult neurogenic regions would be a powerful tool to evaluate the relationship between adult neurogenesis and olfaction. This study identifies *erk5* as such a candidate gene. Here we report that in the adult mouse brain, ERK5 MAP kinase is selectively expressed in the neurogenic regions including the SVZ, along the RMS, and in the center of the granular cell layer of the OB where adult-born neurons exit the RMS. Furthermore, conditional deletion of the *erk5* gene specifically in the neurogenic regions attenuated SVZ/OB neurogenesis *in vivo*. These data suggest an important role for ERK5 in the regulation of adult neurogenesis along the SVZ/RMS/OB axis.

We demonstrate that *erk5* deletion specifically in neurogenic regions of the adult mouse brain did not affect olfactory discrimination in the habituation/dishabituation assay. However, ERK5 icKO mice forget a previously exposed odor much faster and have reduced short-term olfactory memory compared with control littermates. Furthermore, ERK5 icKO mice exhibit reduced detection sensitivity to 1-octanol, a chemically defined odor, at a lower concentration. These results support the findings by Breton-Provencher et al. (Breton-Provencher et al., 2009).

Although adult neurogenesis has been implicated in regulating pheromone-based animal behaviors in mice, such as mating, paternal recognition, and male-male aggression (Mak et al., 2007; Mak and Weiss, 2010; Sakamoto et al., 2011), it is not known whether adult neurogenesis influences the detection sensitivity of pheromones. We report here that ERK5 icKO mice exhibit reduced detection sensitivity to 2-heptanone, a mouse pheromone (Novotny et al., 1986; Leinders-Zufall et al., 2000; Trinh and Storm, 2003), to farnesene, a synthetic, aggression-evoking pheromone (Novotny et al., 1990; Wang et al., 2004), and to TMT, an innate fear- and alarm-inducing pheromone (Fendt et al., 2005; Kobayakawa et al., 2007; Sakamoto et al., 2011). Furthermore, unlike control animals, ERK5 icKO male mice do not show preference to urine from normal over ovariectomized females. Following ovariectomy, circulating estradiol in rats is undetectable within 24 hours (Woolley and McEwen, 1993). It is known that urine samples collected from ovariectomized female mice contain lower levels of protein and lipids than those from normal females (Achiraman et al., 2011). Furthermore, certain fatty acids, including tridecanoic, palmitic and oleic acids, are present in the urine of normal but not ovariectomized female mice (Achiraman et al., 2011). These decreases in circulating estrogen and biochemical constituents in the urine may cause and/or be an indicator of subtle differences in pheromones/odorants present in urine collected from ovariectomized vs. normal female animals. Together, these data suggest that adult neurogenesis in mice plays a critical role in their ability to detect pheromones in low concentrations. Since pheromones are likely present only in low abundance in their normal living environment, adult neurogenesis regulation of pheromone detection may underlie a variety of pheromone-based animal behaviors in mice.

Although several studies have evaluated the impact of adult neurogenesis on long-term memory retention (days) of odor-cued associative olfactory learning (Lazarini et al., 2009; Sultan et al., 2010; Kageyama et al., 2012), adult neurogenesis has not been implicated in the acquisition of this learning task (Sultan et al., 2010). We demonstrate here that ERK5 icKO mice

do not learn nearly as well as control mice to associate a specific mouse urine sample with a food reward. These data provide the first evidence that adult neurogenesis in the OB may be critical for the acquisition of odor-cued associative olfactory learning, a process that is independent of the hippocampus (Kaut and Bunsey, 2001; Kaut et al., 2003; Jonasson et al., 2004; Sultan et al., 2010).

In summary, our data identify ERK5 MAP kinase as a novel signaling pathway regulating adult neurogenesis in the SVZ/OB. This is the first study utilizing genetic approaches to conditionally delete a specific gene selectively in the neurogenic regions of the adult brain to demonstrate a causal relationship between adult neurogenesis and several distinct forms of olfactory behavior, including detection sensitivity to odorants and pheromones, short-term olfactory memory, as well as acquisition of odor-cued associative olfactory learning.

Chapter 6

Comparative Behavioral Analysis between Genotype and Drug Control Animals

INTRODUCTION

Throughout this dissertation research, the majority of experimental results presented directly compared ERK5 icKO mice with a genotype control cohort. The decision to use a genotype control group lies in the fact that this provides the most stringent control for ERK5 icKO animals since the only variable affecting controls and ERK5 icKO mice is the administration of tamoxifen with all other genetic differences and experimental manipulations held constant. Despite this argument, many publications use only one conrol group when assessing behavioral differences and merely mention that a separate control group had been tested and similar results were obtained (Bergami et al., 2008; Zhang et al., 2008a; Imayoshi et al., 2009; Gao et al., 2011; Guo et al., 2011; Sahay et al., 2011; Sakamoto et al., 2011; Amiri et al.).

With many limitations restricting even the most robust experimental designs due to many variables including: financial stability, resource scarcity, time pressures, etc., it has generally been accepted that one control group is often times adequate. Because intrinsic genotypic and phenotypic differences exist when cross breeding animals for laboratory research, limitations should not be the primary factor in selecting a control group. Since we were able to perform many of our behavioral experiments using three separate control groups, we now present a comparative analysis across drug and genotype control animals. Our aim in this study was to assess if any obvious differences existed between the controls and sought to justify the use of one control group versus another.

MATERIALS AND METHODS

Animals

Nestin-CreER^{TM} (Kuo et al., 2006) mice and ERK5$^{loxP/loxP}$ (Wang et al., 2005) mice were crossed to yield Nestin-CreER^{TM}/ERK5$^{loxP/+}$ animals. Nestin-CreER^{TM}/ERK5$^{loxP/+}$ mice were further crossed with ERK5$^{loxP/loxP}$ mice to yield homozygous Nestin-CreER^{TM}/ERK5$^{lox/P/loxP}$ animals, which were used for experimental breeding. All animal experiments were performed with identically treated and handled littermate controls. Animals were housed under standard conditions (12 h light/dark cycle) with food and water provided *ad libitum* except where indicated. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Reagents

The following primary antibody and dilution was used for immunohistochemistry: rabbit polyclonal ERK5 antibody (1:500) was generated previously (Cavanaugh et al., 2001) and affinity purified using recombinant MBP-ERK5 protein.

Immunohistochemistry (IHC)

Mice were perfused intracardially with ice cold solutions of 20 ml PBS followed by 20 ml 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were harvested and post-fixed in 4% PFA/PBS overnight at 4°C, followed by 30% (w/v) sucrose in PBS solution at 4°C until brains sunk. Immediately after sucrose embedding, brains were frozen at -80°C until IHC processing. IHC was performed on 30 µm-thick coronal brain sections using a free-floating antibody staining method. Briefly, brain sections were washed 4 x 10 min with 0.1 M, pH 7.4 PBS, followed by 3 x 10 min with PBST (PBS + 0.25% Triton X-100). Brain slices were then incubated in blocking buffer (PBST + 0.1% bovine serum album (BSA) + 10% normal serum) for

>2 h at room temperature. Where IHC for BrdU visualization was required, brain sections were washed 4 x 10 min with 0.1 M, pH 7.4 PBS, then subjected to HCl treatment by sequential incubation in water for 5 min, ice-cold 1 N HCl for 10 min, 2 N HCl for 30 min at 37°C, and finally neutralized by rinsing 2 x 5 min in 0.5 M borate buffer (pH 8.5). This was followed by PBST washes and blocking as stated above. Brain sections were incubated with primary antibodies for 48–60 h at 4°C on a platform shaker. Brain sections were washed in PBST 4 x 10 min after primary antibody incubation and incubated with secondary antibodies conjugated with Alexa Fluor dyes (1:500 dilution, Invitrogen) in blocking buffer overnight at 4°C on a platform shaker. Brain sections were then washed 4 x 10 min in PBST and incubated with 2.5 µg/mL Hoechst 33342 (Invitrogen) for 30 min and washed 3 x 5 min with PBST. Unless otherwise stated, all IHC procedures were carried out at room temperature. Brain sections were then mounted on gelatin-coated Superfrost plus slides (VWR) with anti-fade Aqua Poly/Mount (Polysciences).

Confocal imaging and analysis

All images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with numerical aperture (NA) 0.75, 20X lens, NA 1.3, 40X oil lens, or NA 1.35, 60X oil immersion lens. Optical Z-sections (0.5–1 µm) were collected and processed using ImageJ software (NIH). Images were uniformly adjusted for color, brightness, and contrast with Adobe Photoshop CS4 (Adobe Systems, Inc).

Behavior Assays

Open field test and habituation

Mice were placed in a 10”(W) x 10”(D) x 16”(H) TruScan Photo Beam Tracking arena with clear sidewalls and infrared beams spaced 0.6” apart providing a spatial resolution of 0.3” (Coulbourn). Mice were not pre-habituated to the arena to permit baseline locomotor activity and anxiety level analysis. Twenty minutes of free exploratory time were allotted per mouse on day

one for the open field test. Data was collected and analyzed using TruScan 2.02 software (Coulbourn). To analyze habituation in the open field, mice were placed in the same arena 24 h and 48 h later for 20 min each.

Novel object recognition

Mice were placed into a 10”(W) x 10”(D) x 16”(H) contextual arena with clear sidewalls (Coulbourn) containing 2 objects (A and B) made of plastic material for a 5 min training session and then returned to their home cages. For short-term memory retention, mice were placed back into the same arena one hour later with one of the previous objects (i.e. A) and a novel object (C) for a 5-min testing session. For longer term memory retention at 24 h or 48 h, a different cohort of mice were trained as above except that training lasted 10 min and testing was performed either 24 h or 48 h later, respectively, during a 10-min testing session. A different set of objects was used for the 24 h and 48 h memory tests. Exploratory activity of each object was recorded for both training and testing sessions and analyzed by experimenters blinded to genotype and treatment group.

Standard cued and contextual fear conditioning and contextual fear extinction

Mice were placed in a 10”(W) x 10”(D) x 16”(H) square-shaped arena fitted with a metal grid shock floor (Coulbourn). On the day of training, each mouse was placed in the training context (with striped wallpaper) and allowed to freely explore for 2 min. A 90 dB tone, the conditioned stimulus (CS), was then presented for 30 s. During the final 2 s of tone presentation, a 0.7 mA foot shock, the unconditioned stimulus (US), was delivered. CS and US were delivered automatically using tone generator and shocker controlled by TruScan software (Coulbourn). Mice were then returned to their home cages. Twenty-four hours later, contextual fear conditioning test was performed in the training room, where mice were placed in the same context arena without any foot shock for 2 min. Two hours after the contextual test, mice were

subjected to a cued test. Mice were placed in a novel context (triangular shaped arena with solid grey plastic walls) in a different room and allowed to freely explore for 2 min. The CS (tone) was then presented for 2 min. Two hours after the cued test, mice were subjected to a control test in which they were placed in another novel context (hexagonal shaped arena with clear plastic walls) in a third room for 2 min with no presentation of either tone or foot shock. Freezing behavior was recorded manually every 5 s for each of the 2 min assessment periods for the three tests. Freezing behavior was defined as lack of bodily movement with all 4 paws remaining stationary on the floor except normal respiration. Data was collected and analyzed with experimenters blinded to genotype and treatment group.

One day following cued and contextual fear conditioning tests, mice were placed in the training context without the foot shock for two extinction trials each day, with a 4 h inter-trial interval. Each extinction trial lasted a total of 10 min and freezing behavior was recorded during the final 2 min of each trial. One day after the completion of the 8 d fear extinction trials, mice were placed in a novel context (trapezoid shaped arena with solid grey plastic walls) and freezing behavior was recorded for 2 min.

Modified cued and contextual fear conditioning using weaker electric shock

The same training and testing setup for cued and contextual fear conditioning was used as described above except that animals were subjected to 1-3 successive foot shocks, 0.3 mA, 2 s each and separated by 2 min inter-shock intervals.

Morris water maze assay

This was done as described with slight modifications (Wu et al., 1995; Zhang et al., 2008b). Mice were placed in a circular pool of water at room temperature (25°C) (1.2 meter diameter, 25 cm deep). The water was made opaque using non-toxic white paint. Three extra-

maze cues, different in shape and size were uniformly spaced and placed on the wall surrounding the water tank. A small escape platform (13 cm x 8 cm) made of clear plexiglass was submerged just below the surface of the water and maintained in a fixed location for the entire training session. To begin a trial or test, mice were randomly started in drop zones, facing the wall, in any of the 3 quadrants without the platform. A total of 32 trials (4 trials per day for 8 consecutive days) were performed during the training session. Mice were allowed to swim for 40 s to find the platform, or guided to the platform after 40 s of the allotted maximum swim time was reached. Each trial ended after mice were allowed to stay on the escape platform for 15 s. A probe test was performed 24 h after the last training trial in which the escape platform was removed and mice were allowed to swim for 60 s in search of the escape platform. Reversal training ensued 24 h after the probe test for a total of 28 trials (4 trials per day for 7 consecutive days) where the escape platform was placed in the opposite quadrant from the initial training session. A reversal probe test was performed 24 h after the last trial of reversal training. Five hours after the reversal probe test, a visible platform test consisting of 4 trials was performed where a visible platform was placed above the water surface in a new quadrant other than the initial or reversal quadrants and mice were allowed to swim to locate the visible platform. In all sessions and tests, mice were allowed an inter-trial interval of 30 min. All data were collected using ANYmaze software (San Diego Instruments) and analyzed offline with the experimenter blinded to treatment and genotypes.

Pattern separation assay

This was measured by the delayed-non-matching-to-place test using the 8-arm radial maze (Clelland et al., 2009; Guo et al., 2011). Mice were food restricted 4 d prior to beginning the assay until 85-90% of free-feeding body weight was reached. Each mouse was subjected to 4 trials per day for 5 consecutive days, with an inter-trial interval of 30 min. Each trial was divided into two phases, a training phase and a choice phase. During the training phase, 6 of 8

arms of the radial arm maze were blocked, leaving the sample arm and start arm open, separated by 90-degrees from each other. Food reward was placed at the end of the sample arm. Mice were placed in the maze facing the end of the start arm and allowed to freely explore for 3 min and then guided to the sample arm unless they retrieved the food reward from the sample arm before the 3 min cutoff time. Mice were allowed to eat for 60 s in the sample arm before being placed back in their home cage for approximately 30 s, during which time the maze was rotated 45 degrees and the arms cleaned with 5% acetic acid to minimize odor reference. The choice phase test was conducted immediately after cleaning, during which new sample and start arms were opened and the original arms were blocked, but the relative location of the arms during the training phase remained fixed. Additionally, a test arm was opened and separated by either 1-arm (separation 2) or 3-arms (separation 4) from the new sample arm. Each mouse was subjected to the same total number of separation 2 or 4 tests, although the two separations were presented randomly in sequence each day. Food reward was placed at the end of the test arm during the choice phase. Mice were placed in the maze facing the end of the start arm and allowed to make a choice in the maze. When a mouse entered the test arm, it was scored as a correct choice and the mouse was allowed to eat for 60 s before being removed from the maze. When a mouse entered the sample arm, re-entered the start arm, or did not make a choice within 3 min, it was scored as an incorrect choice. On day 1 and day 2, mice that made an incorrect choice were allowed to self-correct and explore the maze for a maximum of 3 min before being guided to the test arm and allowed to eat for 60 s. On days 3-5, mice that made an incorrect choice were removed from the maze immediately and not allowed access to food reward. Correct or incorrect choices during the choice phase were scored manually with the experimenter blinded to experimental conditions.

Passive avoidance and remote memory assay

A light/dark chamber (Coulbourn) with a guillotine trap door separating the two chambers was used. A 60 W light bulb was placed over the opening of the light chamber, while the dark chamber was completely covered with black film. Mice were placed in the light chamber with the trap door closed and allowed to freely explore for 1 min. After which, the trap door was opened and the latency to cross over was recorded. Once mice crossed over into the dark chamber, the trap door was closed immediately followed by delivery of a mild foot shock (0.7 mA, 2 s); mice then remained in the dark chamber for an additional 1 min before being removed and placed in their home cage. Twenty-four hours later, mice were placed in the light chamber with the trap door closed and allowed to freely explore for 1 min. After 1 min, the trap door was opened and latency to cross over to the dark chamber was recorded with a maximum crossover latency cutoff of 5 min. For remote memory test, mice were tested 21 d post-training using the same procedure as the 24 h test.

Olfactory behavior assays

Male mice were used for behavior assays. Mice were individually housed and handled at least 4 d before olfactory behavior assays and remained singly caged throughout all olfaction assays. With the exception of the TMT-based innate fear assay, all other olfactory behavior assays were conducted in mouse home cages. For all cotton-tip based behavior assays, cotton swabs dipped in vehicle control (mineral oil or water) or odorant solution were suspended from the wire top of the animal's home cage with the cotton tips 8 cm above the cage floor. The duration of animals' sniffing of the cotton swab was recorded. The sniffing was defined as animals' noses approaching to and within 1 cm distance to the swabs. Odorants and corresponding concentrations used in various olfaction tests are listed in Table 1, Chapter 5.

Olfactory habituation/dishabituation test with chemical odorants

This was conducted as described (Zou et al., 2012). Briefly, naïve animals were pre-trained with mineral oil-laced cotton swabs for four presentations (60 s each, 2 min intervals) to ensure that subsequent exposure to an odorant-laced cotton swab did not elicit a response due to novelty. The odor habituation/dishabituation test was then performed by presenting citralva, IAA, and ethyl vanillin sequentially, with four presentations for each odorant (60 s each presentation, 2 min intervals). A significant decrease in the number of investigations during subsequent presentations of the same odorant indicates odor recognition and habituation. An increase in investigation of a new odorant indicates dishabituation.

Olfactory habituation/dishabituation test with diluted mouse urine

This was conducted as described above using diluted (1:50) normal female mouse urine, ovariectomized female mouse urine, male mouse urine, pregnant female mouse urine, and lactating female mouse urine, sequentially, with four presentations of each urine sample (60 s each presentation, 2 min intervals). A significant decrease in total investigation time during subsequent presentations of the same urine sample indicates recognition and habituation. An increase in investigation of the next, new urine sample indicates dishabituation.

Olfactory short-term memory

This was performed as described with modifications (Breton-Provencher et al., 2009). Mice were presented with a cotton swab laced with the same odorant during two different 5 min sessions separated by 30, 240, 300, or 360 min intervals. Odorant detection and investigation of the cotton swab was recorded for each 5 min session. A different odor was used for each interval time point, but only one time interval was tested on each day for each mouse to avoid cross interference of olfactory detection and memory. A significant decrease in total investigation time of the cotton swab during the 2nd presentation of the odorant suggests

olfactory memory for the 1st presentation of the same odorant (Rochefort et al., 2002; Mechawar et al., 2004; Breton-Provencher et al., 2009).

Odor detection threshold

This was performed as described with modifications (Breton-Provencher et al., 2009). Mice were presented with 2 cotton swabs simultaneously; one laced with vehicle control and the other laced with a specific odorant or pheromone as indicated. The relative location of the two cotton swabs was randomly switched between presentations to avoid spatial learning. One session was performed per day per concentration, with increasing concentrations each day. Each session lasted 3 min and total investigation time of each cotton swab was recorded during the entire 3 min session. Data are presented as the mean ratio between the time spent investigating the odor and the total sniffing of both cotton swabs (percent sniffing duration). A 50% sniffing duration indicates no detection of the odorant because the animals spent an equal amount of time investigating both cotton swabs. Sniffing durations greater than 50% of the time indicates that animals detected the odorant.

To measure detection sensitivity for TMT, the above assay was performed with slight modifications. Since TMT is a known fear-inducing odor component of fox feces (Fendt et al., 2005), we mixed it with odorants that mice are not averse to (1 mM 2-heptanone, 1-octanol, or R-terpinen-4-ol). Mice were then presented with 2 cotton swabs as above, one laced with just the odorant and the other laced with the odorant plus increasing concentrations of TMT. When mice detect TMT, they avoided this cotton swab; thus a sniffing duration less than 50% indicates that the animal detected the fear odor.

TMT-based innate fear assay

This was conducted in a 3-chamber testing apparatus as described (Hacquemand et al.; Buron et al., 2007). Briefly, the apparatus (60 cm x 22 cm x 12 cm) consisted of three 20 cm x 22 cm x 12 cm partitions. Dividers between chambers had a small opening to allow mice to cross freely between the three chambers. Mice were placed in the middle chamber and a small plastic dish was placed at the far end of each of the other two chambers; mice were allowed to habituate in the apparatus for 15 min. Following habituation, the two plastic dishes were retrieved and a filter paper (2 cm x 2 cm) containing 5 µl of water or 5 mM TMT was placed into each of the two dishes in a randomized manner between mice. The dishes were then placed back into the apparatus at their original location; the duration of investigation to each chamber and freezing behavior were recorded during a 5-min test session with the experimenter blind to treatment conditions. Avoidance of the TMT chamber and increased freezing behavior are indicative of innate fear responses.

Olfactory preference

Mice were presented simultaneously with two cotton swabs laced with undiluted mouse urine (10 µL). Mouse urine was collected from group-housed mice (n = 4–5 per cage) and pooled over a period of 2 weeks to minimize day-to-day fluctuations in basal pheromone and urine amount. Total investigation time of each cotton swab was recorded during a 2 min session. Data presented are percent sniffing duration for each odorant or urine relative to the total sniffing of both cotton swabs. A 50% sniffing duration indicates no preference for either urine.

Sand-digging based odor-cued associative olfactory learning

Following olfactory preference assay, mice were food restricted to maintain 85-90% of free-feeding body weight for 4–5 d prior to the beginning and throughout the entirety of

associative olfactory discrimination assay, which was performed as previously described using the same apparatus, pre-training, and training protocols (Zou et al., 2012). Briefly, after mice learned to associate sand digging with a food reward at the bottom of the dish, mice were presented with 2 sand dishes in each trial, for 4 trials per block, and 2 blocks per day. One dish contained 100 μL of a urine sample associated with a food reward, while the other contained 100 μL of another urine sample (both urine samples were diluted 1:50) without food reward. To avoid spatial learning, the two dishes were placed on either the left or right side randomly as long as each dish was placed on each side twice per block but no more than three consecutive times in each day. Scoring for correct or incorrect choice was based entirely on the animal's first dig, either in the urine dish with the food reward (correct) or in the urine dish without the food reward (incorrect). Mice that dug in the incorrect dish were not allowed to self-correct. Two pairs of urine samples were used in this assay: 1) ovariectomized (enforced with food reward) vs. normal female urine; 2) pregnant (enforced with food reward) vs. lactating female urine.

Statistical analysis

All data are expressed as mean ± standard error of the means (s.e.m.) from at least 2 independent experiments ($n \geq 10$ for each treatment group/genotype) for *in vivo* behavior experiments. Repeated measures ANOVA was used to analyze data for all water maze tests (except the probe test), fear extinction assays, olfactory habituation, pheromone habituation, and pheromone-cued associative olfactory learning assays. One-Way ANOVA with *Fisher's LSD* post-hoc analysis (Alpha=0.05) was used to analyze all other behavior data. n.s. not significant ($p>0.05$); *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

RESULTS

ERK5 protein is expressed at similar levels along the SGZ in both drug and genotype control animals

Brains from tamoxifen treated ERK5$^{loxP/loxP}$ (Control B) and vehicle treated Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ (Control C) animals were processed using a free-floating immunohistochemistry protocol. ERK5 expression was detected using an affinity-purified ERK5 antibody and found to be comparably expressed along the SGZ of the dentate gyrus in both Control B and Control C animals (Fig. 6.1). Since vehicle treated ERK5$^{loxP/loxP}$ (Control A) animals are essentially like wild-type animals, we sought not to examine this control group for ERK5 expression in the adult mouse brain. However, we did include all three control groups in all subsequent behavior assays.

No overt motor deficits or deficits in control animals' ability to habituate to a novel environment were detected

Control animals were exposed to the open field chamber to examine any overt motor behavior deficits for a period of 20 min. This was repeated 2 more times on the subsequent 2 days, for a total of 3 trials to determine if their ability to habituate to a novel environment was affected. All mice performed similarly in the open field habituation assay (Fig. 6.2).

Control animals perform similarly in the novel object recognition assay 1 h, 24 h, or 48 h after a single training session

Similar to data presented in Fig. 3.5, all control mice were able to remember the familiar toy at both 1 h and 24 h after their independent training sessions (Fig. 6.3A and B) Unlike ERK5 icKO mice however, all control animals were able to discriminate between a familiar and novel toy 48 h after initial training (Fig. 6.3C). This data suggests that the longer term memory deficit observed in ERK5 icKO mice is likely not due to any off target side effects of tamoxifen.

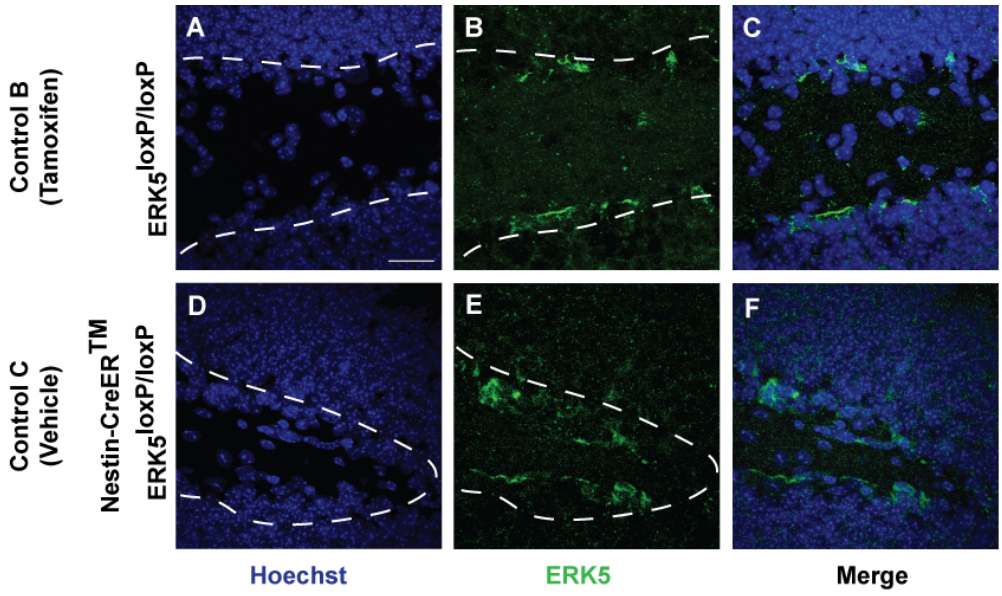


Figure 6.1. No differences in ERK5 expression between Control B and Control C mice along the SGZ. Brain slices from ERK5$^{loxP/loxP}$ mice treated with tamoxifen (Control B, A–C) and Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice treated with vehicle (Control C, D–F) were immunostained for ERK5 expression. Scale bar in A represents 25 μm and applies to all images. Dashed lines outline SGZ layer of the dentate gyrus.

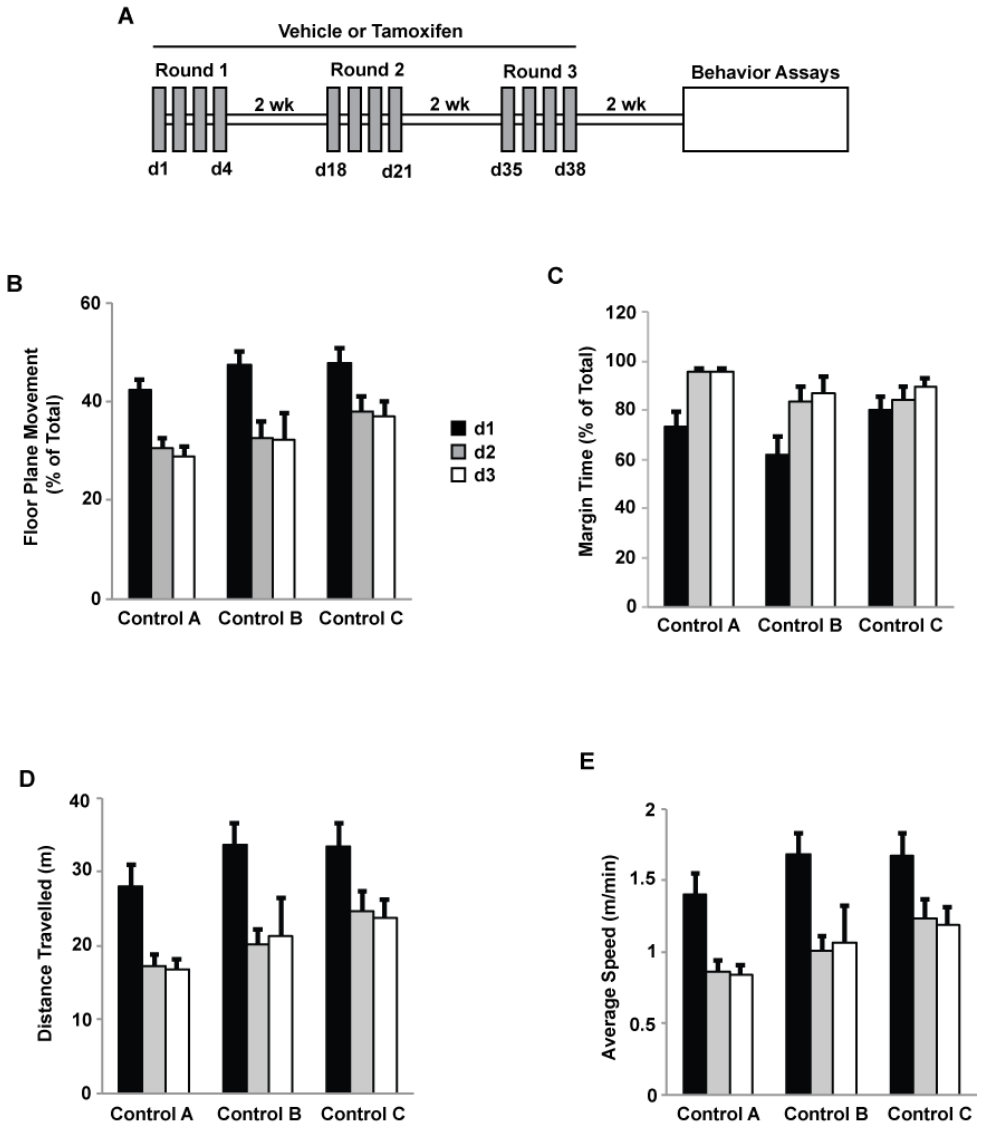


Figure 6.2. Baseline motor activity and habituation, monitored via the open field activity test, is not affected across all control mice. (A) Schematic illustration of the experimental design. Tamoxifen or vehicle was administered to 12-week old ERK5$^{loxP/loxP}$ or Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice orally, 4 d in each cycle, for a total of 3 cycles with 2-week inter-cycle intervals. Animals were subjected to behavior assays 2 weeks after the last dose of tamoxifen. (B) Total floor plane movement on day 1 (d1), d2, and d3. (C) Total time spent along the margins of the open field. (D) Total distance traveled. (E) Average traveling speed. Data represent mean of two independent experiments with n ≥ 10 animals per treatment group per experiment.

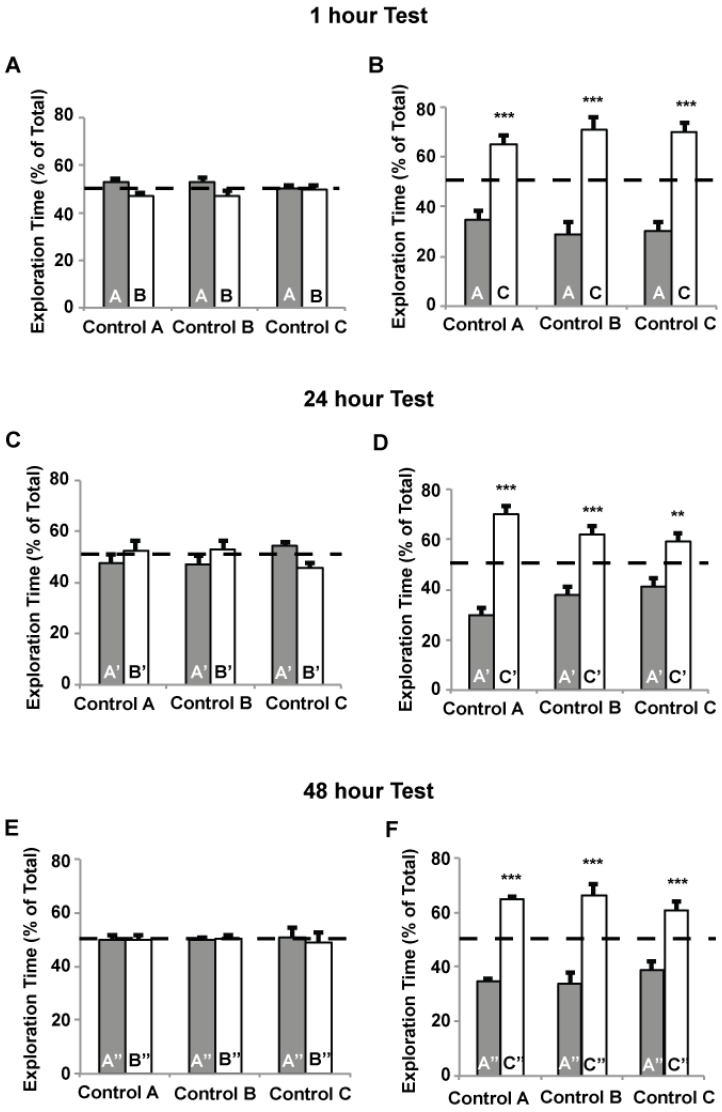


Figure 6.3. All control animals perform similarly in the novel object recognition assay across all 3 time intervals. (A, B) Memory for novel object 1 h after training is not affected. During the training session, mice were presented with 2 objects, A and B, for 5 min and the percent of time spent exploring each object was quantified (A). During the test session, conducted 1 h later, mice were presented again with 2 objects, A (familiar) and C (novel), for 5 min and the percent of time spent exploring each object was quantified (B). (C, D) Memory for novel object 24 h after training is not affected. A new cohort of mice was used. Training session was conducted for 10 min with objects A' and B' and exploration time was quantified (C). Test session was conducted 24 h later with objects A' and C' for 10 min (D). (E, F) No memory deficits were observed for novel object recognition assay 48 h after training. After the 24 h test, animals were trained with objects A'' and B'' for 10 min (E). Test session with presentations of objects A'' and C'' was performed 48 h later (F"). Data represent mean exploration time from three independent experiments with n ≥ 8 animals per treatment group per experiment. **, $p<0.01$; ***, $p<0.001$.

Performance in the contextual fear extinction assay is comparable across all control animals

Using the standard fear conditioning assay (1X 0.7 mA, 2 s shock), all control mice were able to associate the shocking context 24 h after training (Fig. 6.4B). This effect was context specific since all mice failed to freeze when exposed to a novel context (Fig. 6.4C). Additionally, all control mice performed similarly in the cued-associated fear conditioning assay 24 h after training (Fig. 6.4D). Contextual fear extinction was performed by re-exposing all animals to the original shocking context for 8 consecutive days after training. No deficits were observed across the 8 d extinction paradigm; this effect was context specific since all mice also failed to freeze when exposed to a novel context not previously associated with an aversive stimuli (Fig. 6.4E and F).

Control animals all perform similarly in the more challenging form of contextual fear memory assay

To determine if a more challenging paradigm of the standard contextual fear memory assay affected control animals, we reduced the foot shock from 0.7 mA to 0.3 mA. Using this lower shock protocol, we had to shock mice up to 3 times to elicit a similar freezing response as a single 0.7 mA foot shock (Fig. 3.7B). All control animals froze similar amounts when re-exposed to the shocking context 24 h later (Fig. 6.5B); and this effect was context specific since none of the control animals froze when exposed to a novel context (Fig. 6.5C). We additionally assessed cued-fear conditioning and found no differences among control animals when tested 24 h later (Fig. 6.5D).

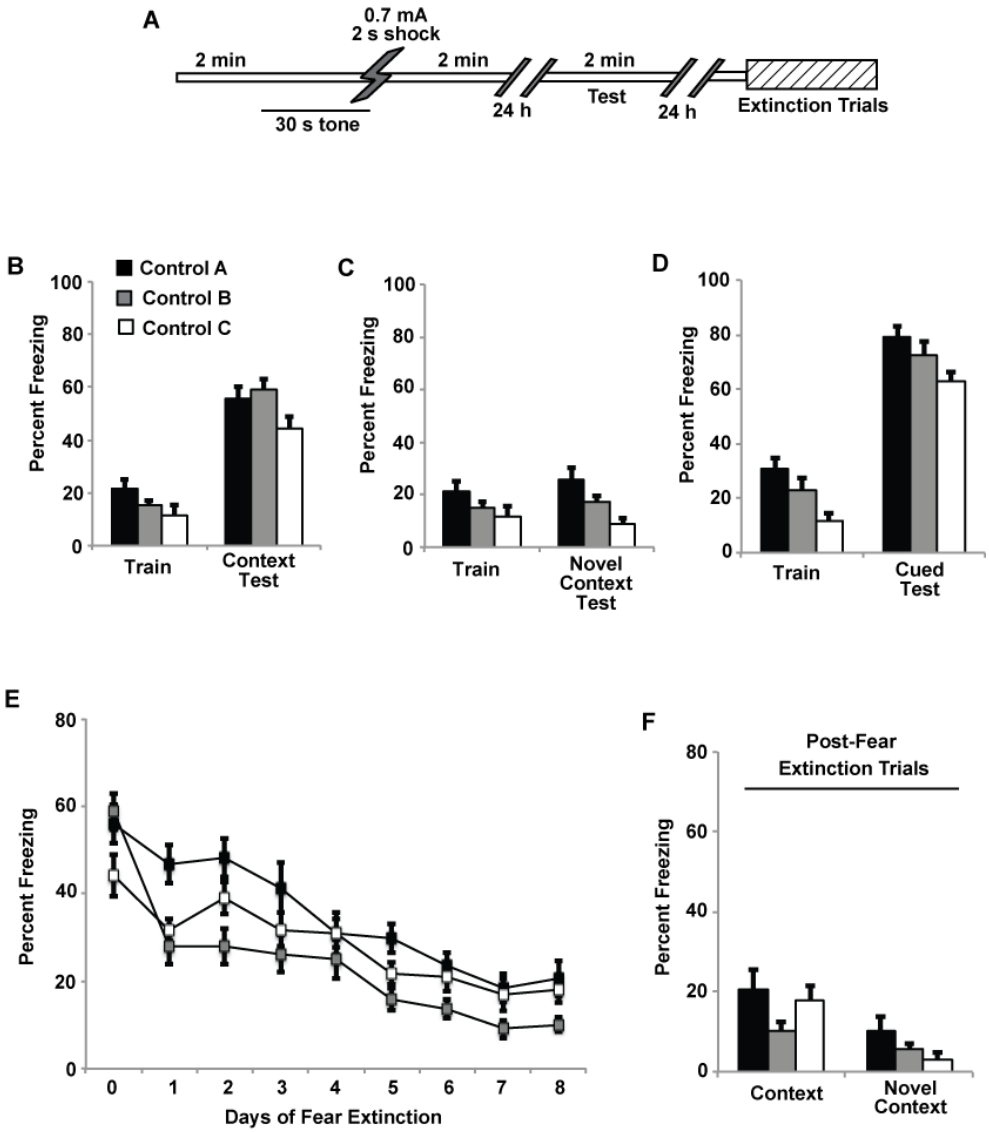


Figure 6.4. No differences between control animals in contextual fear memory extinction. (A) Schematic illustration of the standard fear conditioning paradigm where animals were subjected to one 0.7 mA, 2 s foot shock. (B) All control mice have similar levels of contextual fear memory 24 h post-training. (C) None of the control mice froze when placed in a novel context 24 h after training. (D) All control animals showed similar cued-fear response 24 h post-training. (E) Fear extinction was similar across all controls. Day 0: the day of contextual fear testing. Day 1: the first day animals were subjected to fear extinction training. (F) The freezing response following fear extinction trials was context specific because animals also did not freeze in a novel context on Day 9, one day after the completion of the fear extinction trials. Data are mean ± (s.e.m.) from two independent experiments with n ≥ 8 animals per treatment group per experiment.

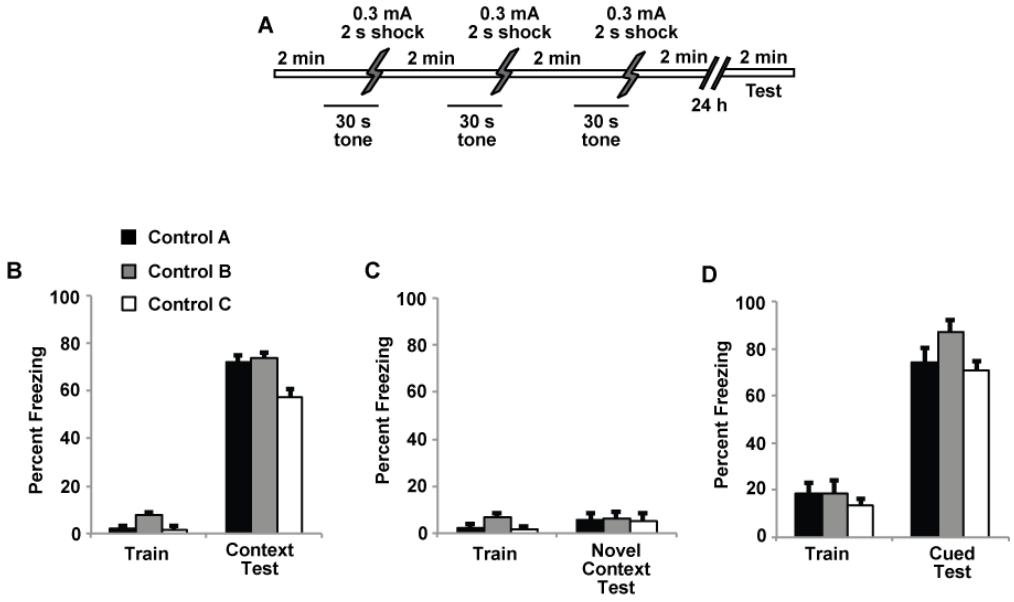


Figure 6.5. Contextual fear memory was not impaired when control animals were trained with weaker electric foot shocks. (A) Schematic illustration of the modified cued-contextual fear conditioning paradigm using 0.3 mA shock intensity delivered up to 3 times. Animals were subjected to 3 foot-shocks (0.3 mA, 2 s each), with 2 min inter-shock intervals. (B) When placed into the same shocking context 24 h later, contextual fear memory was similar in all mice. (C) The fear memory was context specific since animals did not freeze when placed into a novel context 24 h after training. (D) Memory for the hippocampus-independent, auditory-cued fear conditioning measured 24 h after training was unaffected in all control mice.

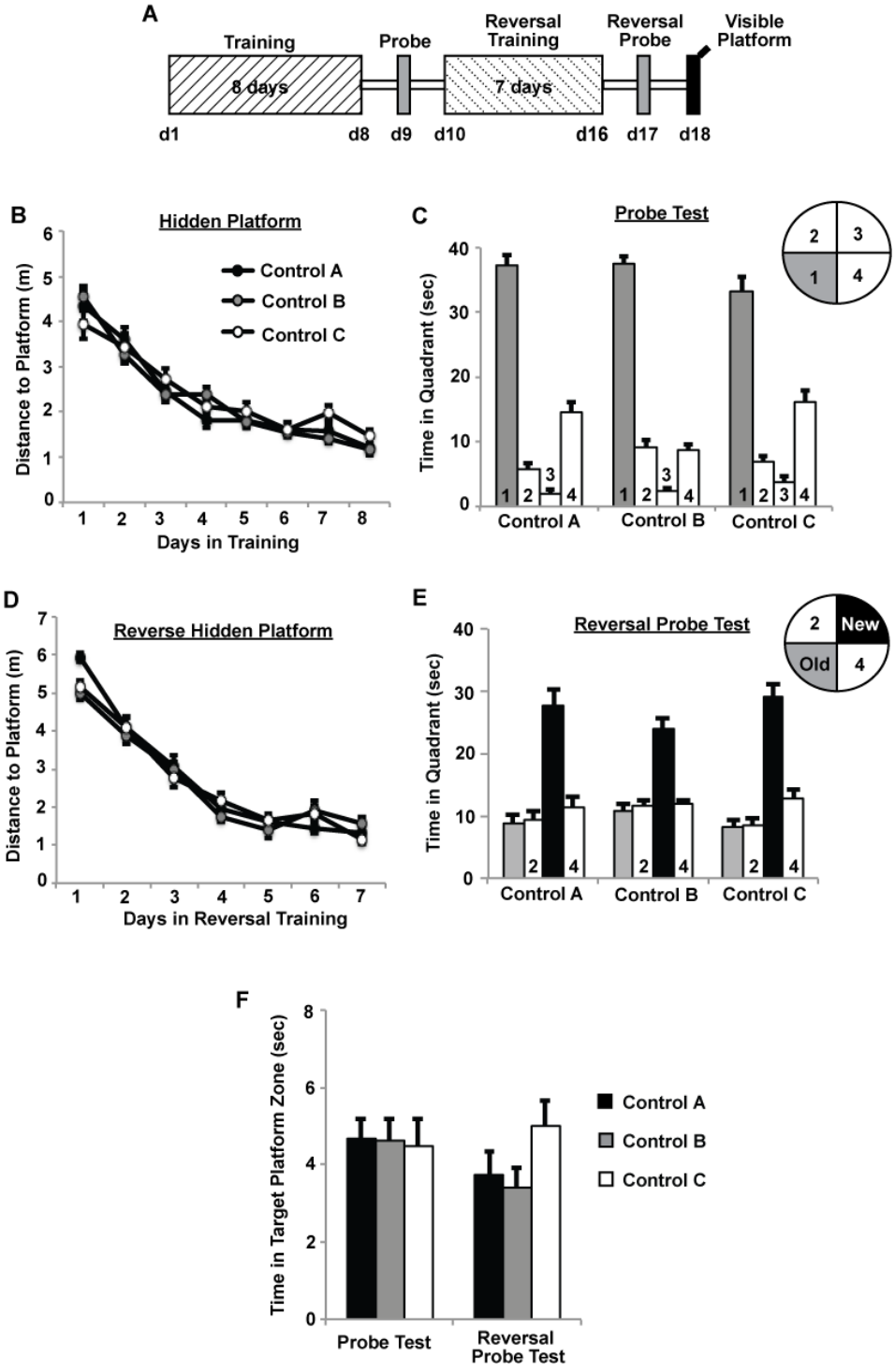
No differences were observed between control animals in two distinct spatial memory assays

Mice were trained in the Morris water maze assay as depicted (Fig. 6.6A), and all mice were able to find the hidden platform after an 8 d training protocol (Fig. 6.6B). One day following the hidden platform assay, mice were exposed to a probe test to assess their ability to spatially learn and remember the location of the hidden platform. No differences were observed across all control animals in the probe test (Fig. 6.6C). We then placed the hidden platform in the opposite quadrant from the initial location and found no differences between mice in their ability to actively re-learn a new location of the hidden platform over the course of a 7 d training paradigm (Fig. 6.6D). Testing mice in the reversal probe test, we found no differences in their ability discriminate between the previous location of the hidden platform (quadrant 1) from the new location of the hidden platform (quadrant 3, Fig. 6.6E). Additionally, all mice had no specific spatial memory deficits during the probe and reversal probe tests. All mice spent the same amount of time searching for the hidden platform in the virtual target platform zone (Fig. 6.6F).

To assess if any differences existed in their swimming ability, we assessed their total swim distance and average swim speed during the probe test. No differences existed between all control animals (Fig. 6.7A and B). Additionally, we wanted to evaluate the animals' motivation to escape water and found no differences during the visible platform test (Fig. 6.7C).

An additional spatial memory assay, the radial arm maze (RAM), was used to determine if any deficits existed between control animals. The RAM has been commonly used to assess the involvement of adult-born neurons in spatial learning and memory. We incorporated this assay as previously performed by others (Farioli-Vecchioli et al., 2008; Clelland et al., 2009; Creer et al., 2010) and found no differences in their ability to spatially discriminate between a more difficult separation 2 and an easier separation 4 (Fig. 6.8).

Figure 6.6. No differences between control animals in the Morris water maze assay. (A) Schematic depiction of the experimental design. Mice were subjected to 8 d of training (4 sessions/day, 30 min inter-session interval, from d1 to d8) in the hidden platform water maze. A probe test was conducted 24 h later. Mice were then subjected to 7 d of reversal training (4 sessions/day, 30 min intersession interval) where the escape hidden platform was relocated to the opposite quadrant of the water maze. This was followed by reversal probe test and visible platform test. (B) Target (in quadrant 1) acquisition in the hidden platform, quantified as swim distance to platform, was similar across all control animals. (C) All mice spent similar amounts of time in the virtual target quadrant in the probe test. (D) Swim distance to escape in the reversal hidden platform training where the hidden platform was moved to quadrant 3. All mice swam similar distances, suggesting no impairment in their ability to learn newer spatial information. (E) All control mice spent significantly more time in the new virtual target quadrant 3 than in the previous virtual target quadrant 1 during the reversal probe test. (F) All mice spent similar amounts of time in the virtual target platform zone during the probe and reversal probe tests.


A
Training
8 days
Probe
Reversal Training
7 days
Reversal Probe
Visible Platform
d1
d8
d9
d10
d16
d17
d18
B
Hidden Platform
Control A
Control B
Control C
Distance to Platform (m)
Days in Training
C
Probe Test
Time in Quadrant (sec)
Control A
Control B
Control C
D
Reverse Hidden Platform
Distance to Platform (m)
Days in Reversal Training
E
Reversal Probe Test
New
Old
Time in Quadrant (sec)
Control A
Control B
Control C
F
Time in Target Platform Zone (sec)
Control A
Control B
Control C
Probe Test
Reversal Probe Test

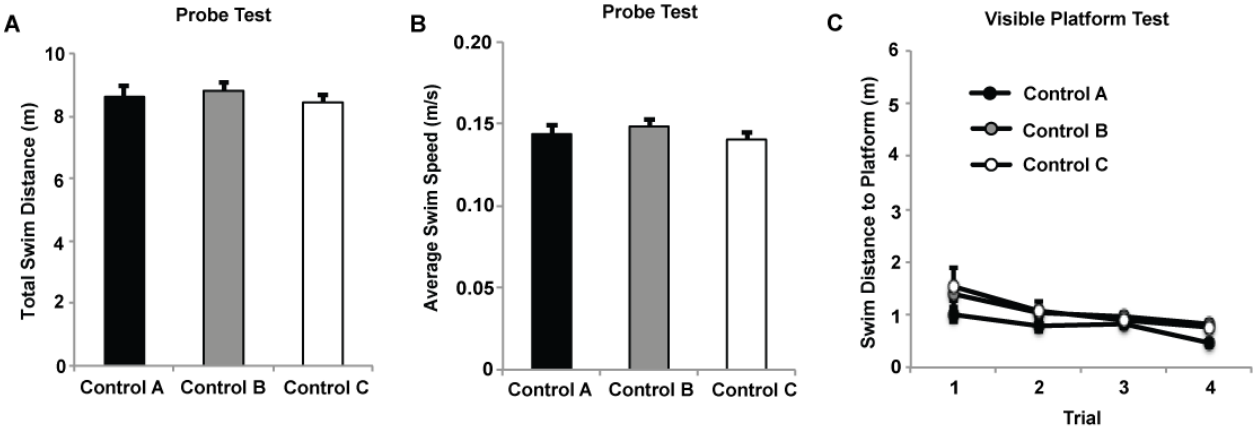


Figure 6.7. All control mice perform similarly in their ability to swim and are equally motivated to escape water in the Morris water maze assay. (A) Total swim distance was measured during the 60 s period of the probe test. (B) Average swim speed during the 60 s probe test. (C) Swim distance to platform during the visible platform test. Data represent mean from two independent experiments with n ≥ 8 animals per treatment group per experiment.

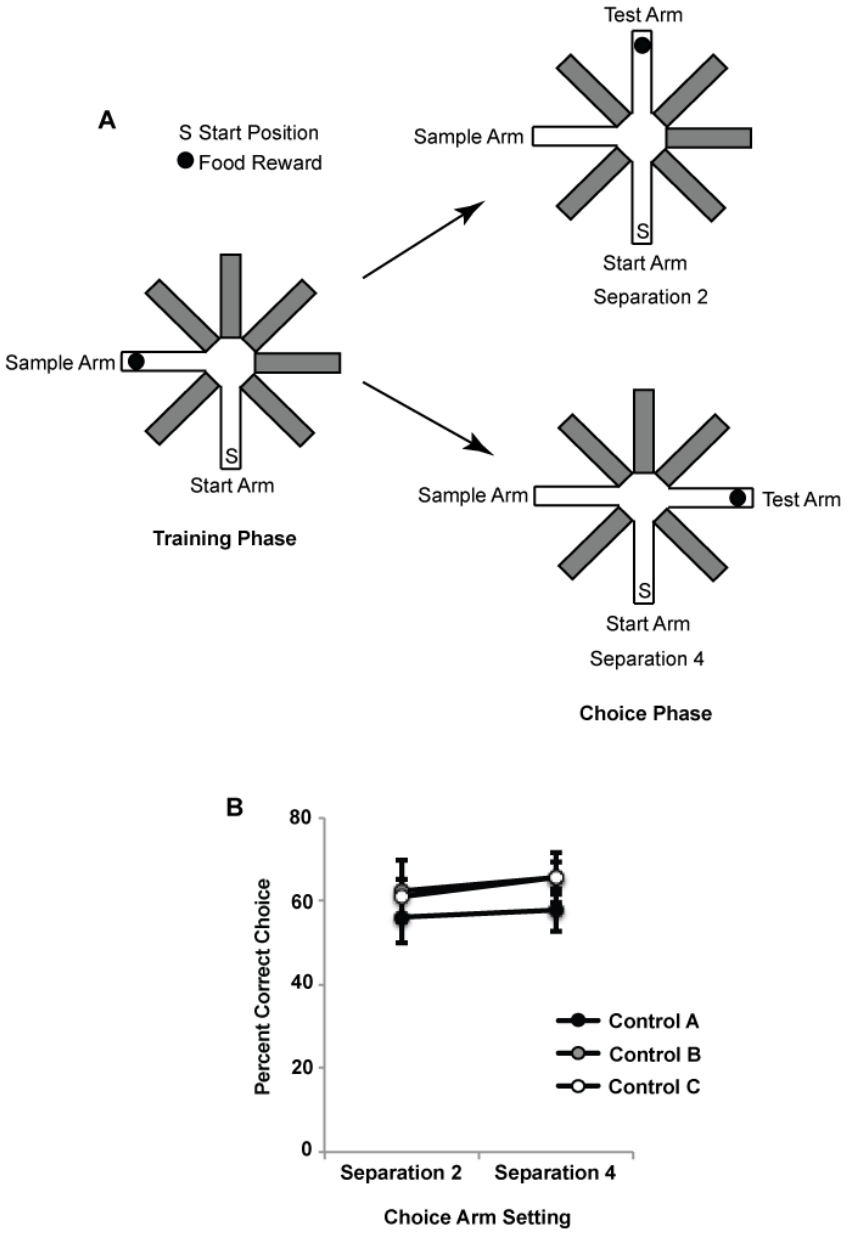


Figure 6.8. Control animals perform similarly in the radial arm maze. (A) Schematic illustration of the experimental design. (B) All mice made similar amounts of correct choices in both the easier (separation 4) and more difficult (separation 2) pattern separations. Data are from the last day of a 5 d-experimental paradigm and represent mean ± (s.e.m.) with n ≥ 10 per treatment group.

Control animals do not have any remote memory deficits assessed 21 d later in the passive avoidance assay

Using the light/dark apparatus to perform the passive avoidance assay, we introduced mice to the light side of the testing chamber. Following habituation, a small trap door was opened to allow free exploration to the dark side of the chamber. Upon entrance into the dark side of the chamber, the trap door was immediately closed and a single 0.7 mA foot shock was delivered followed by a 1 min consolidation period in the dark chamber with the trap door closed. Crossover latency to the dark side of the chamber upon re-introduction to the light side of the chamber was used to asses memory for the aversive stimuli 24 h later for long term memory and 21 d later for remote memory (Fig. 6.9).

Assessment of olfactory behavior to discrete odorants using the olfactory habituation/dishabituation, olfactory memory, and olfactory threshold assays showed no differences between all control animals

We first tested animals for olfactory behavior in the odor habituation/dishabituation assay using three distinct odorants, citralva, isoamyl acetate (IAA), and ethyl vanillin. This assay is commonly used to detect overt odor detection deficits in mice (Trinh and Storm, 2003). All mice showed normal habituation/dishabituation and were indistinguishable among control mice (Fig. 6.10). Following the olfactory habituation/dishabituation assay, we tested animals for short-term olfactory memory (within hours). All control mice were able to recall having detected the odor during the first presentation by reduced investigation of the odorant during the second presentation (Fig. 6.11). Thus, the olfactory memory was not different between control animals.

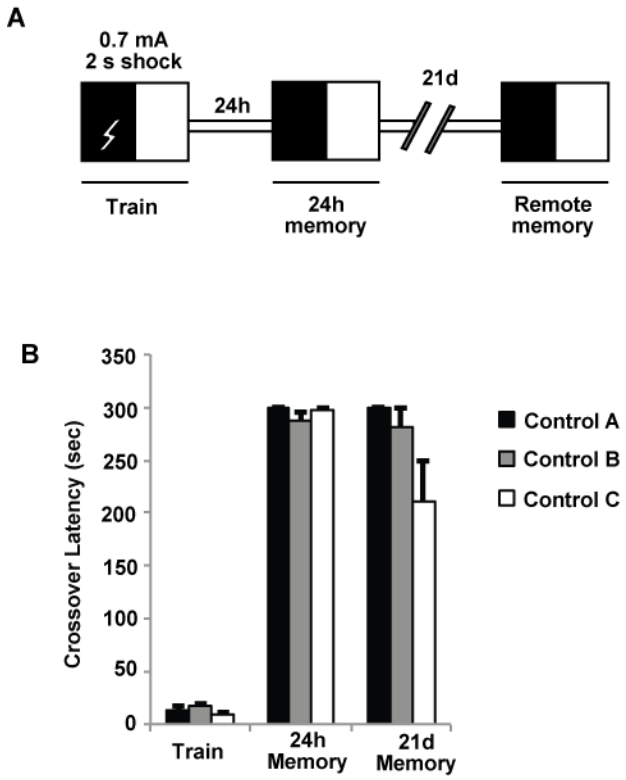


Figure 6.9. Remote memory in the passive avoidance assay is similar between control animals. Mice were trained with one 0.7 mA, 2 s foot shock in the passive avoidance assay. (A) Schematic illustration of the experimental design. (B) Crossover latency was measured 24 h or 21 d post-training. Data represent mean ± (s.e.m.) with n ≥ 10 per treatment group.

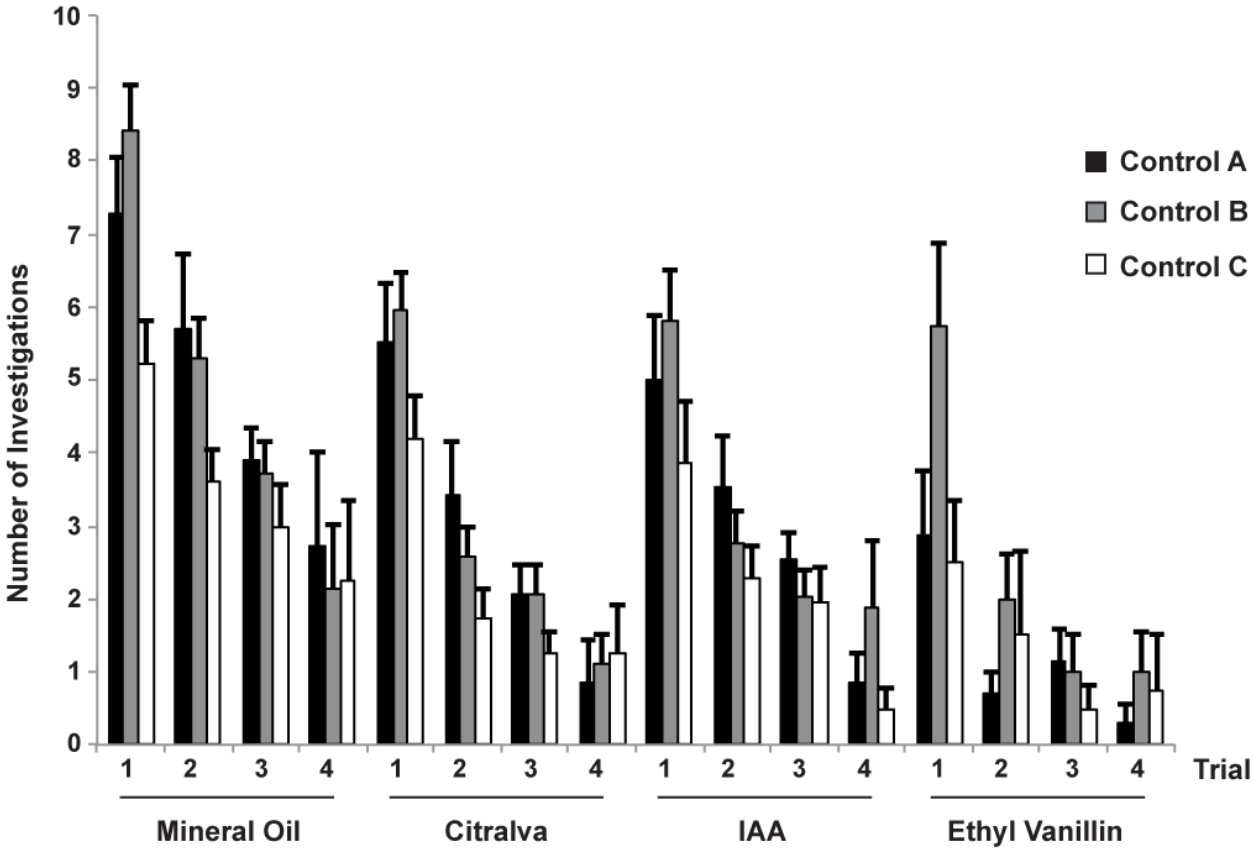


Figure 6.10. All control mice have normal olfactory detection to discrete odorants in a habituation/dishabituation assay. Naïve, adult mice were pre-trained with four presentations of mineral oil-soaked cotton swabs, then exposed to three structurally distinct odorants, citralva, isoamyl acetate (IAA), and ethyl vanillin with 4 trials per odorant. Step-wise decrease in the number of investigation during sequential presentations of the same odor followed by renewed interest in investigation of the first presentation of a new odorant suggests normal olfactory habituation/dishabituation behavior.

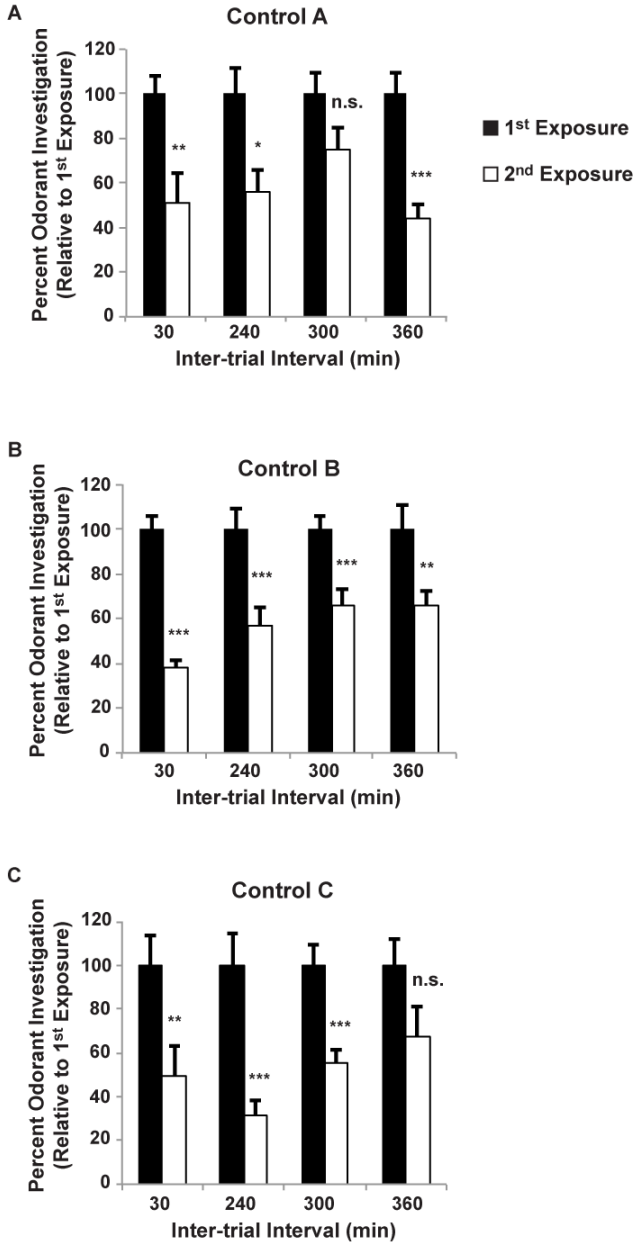


Figure 6.11. Olfactory short-term memory is in tact in all control animals. Mice were presented with cotton swabs laced with the same odorant twice at the indicated time intervals. The duration of investigation of the cotton swab was recorded for both exposure sessions and normalized to the first exposure. A decrease in investigation during the second exposure of the same odorant is suggestive of olfactory memory to the odorant. A) Short-term olfactory memory of Control A mice. B) Short-term olfactory memory of Control B mice. C) Short-term olfactory memory of Control C mice.

We next examined the sensitivity of odor detection using an olfactory threshold assay. All mice were presented with pairs of cotton swabs laced with mineral oil or increasing concentrations of 1-octanol (1-500 μM). Similarly, we also performed this assay with 2-heptanone, a mouse pheromone (Novotny et al., 1986; Leinders-Zufall et al., 2000; Trinh and Storm, 2003). There were no differences between control animals in their sensitivity to detect either 1-octanol or 2-heptanone (Fig. 6.12).

All control mice behaved similarly in their ability to detect synthetic pheromones

To examine the sensitivity of mice to farnesene, a synthetic, aggression-evoking pheromone (Novotny et al., 1990; Wang et al., 2004), we performed the olfactory detection threshold assay. All mice were able to detect farnesene at the same concentrations beginning at 50 μM and up to 500 μM (Fig. 6.13A). Additionally, Mice were also examined for their detection of TMT, a component of fox scent that is known to elicit innate fear and alarm in rodents (Fendt et al., 2005; Kobayakawa et al., 2007; Sakamoto et al., 2011). When mice detect TMT, they will typically avoid it and may exhibit freezing behavior. Most of the control animals avoided TMT-laced cotton swabs at 10 μM, however when the concentration of TMT was increased to 50 μM, all mice avoided this fox fear odor (Fig. 6.13B). Since detection and avoidance of TMT could either mean mice just disliked the pungent odor or they elicited innate fear behavior and actively chose to avoid the TMT-laced cotton swab. To distinguish between these two possibilities, we used a separate cohort of mice and a 3-chamber apparatus to directly test their intrinsic innate fear of TMT at a higher concentration. Mice were placed in the middle chamber, TMT (5 mM) and vehicle control were placed in the other two chambers, separately. The duration of investigation toward each chamber and the percent time mice froze was quantified. All control animals froze a similar amount of the time and spent less time investigating the chamber associated with TMT (Fig. 6.13C and D).

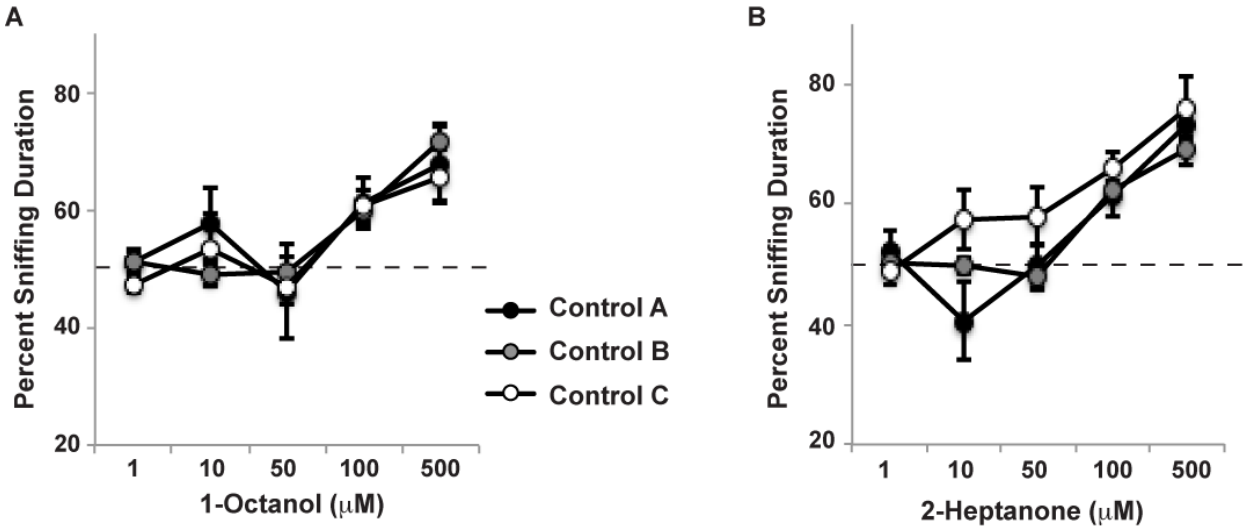


Figure 6.12. Detection sensitivity to 1-octanol and 2-heptanone is the same between control animals. Mice were presented with 2 cotton swabs, one laced with vehicle control and the other with increasing concentrations of 1-octanol or 2-heptanone. An above 50% sniffing duration (above chance) indicates detection of 1-octanol or 2-heptanone at that specific concentration. A) Dose-response of detection threshold to 1-octanol, a chemically defined odorant. B) Dose-response of detection threshold to 2-heptanone, a chemically defined mouse pheromone.

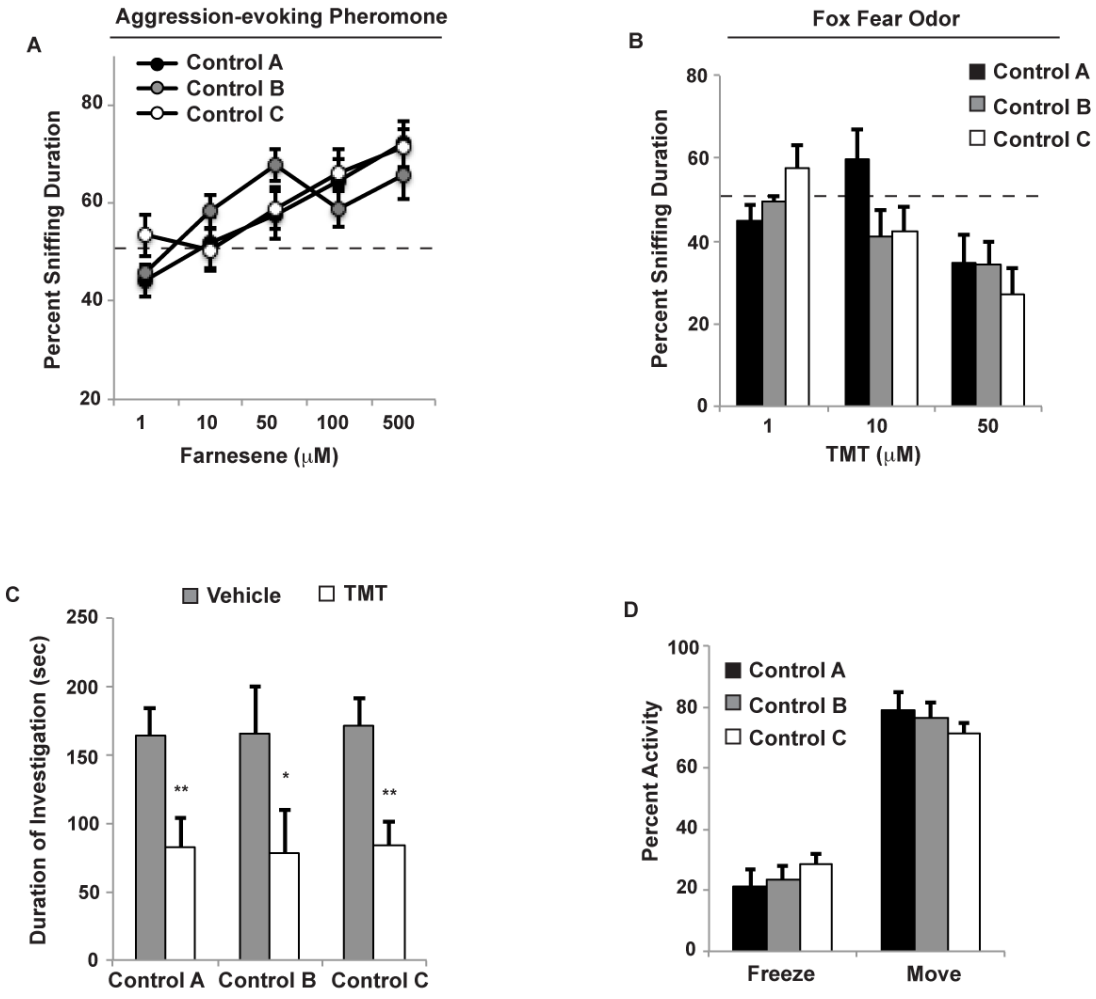


Figure 6.13. No differences exist between control animals' detection sensitivity to aggression-evoking and fear-inducing pheromones. A) Dose-response of detection threshold to farnesene, a chemically defined, aggression-evoking mouse pheromone. Mice were presented with 2 cotton swabs, one laced with vehicle control and the other with increasing concentrations of farnesene. An above 50% sniffing duration (above chance) indicates detection of farnesene. B) Dose-response detection to TMT, a fear-evoking odor found in fox feces. All mice were presented with 2 cotton swabs, one laced with vehicle control and the other with increasing concentrations of TMT. Because TMT should elicit avoidance behavior when mice detect it under normal conditions, a below 50% sniffing duration (less than chance) indicates detection of TMT. C) Animals were subjected to the innate fear assay toward TMT using a 3-chamber apparatus. The duration of investigation toward the chamber containing filter paper laced with TMT (5 mM) or vehicle control was quantified. Avoidance behavior toward the TMT chamber relative to the vehicle control chamber is a measure of TMT detection and innate fear. D) Quantification of percent time mice spent freezing, another measurement of innate fear, during the innate fear assay toward TMT using the 3-chamber apparatus.

Activity in pheromone detection, pheromone preference, and pheromone-cued associative olfactory learning are all comparable between control animals

In order to test olfactory detection of pheromones, we collected mouse urine from group-housed animals differing in sex, age, and other hormonal differences. Mouse urine was collected, pooled, and stored at -80°C until needed for behavioral assays. We first tested to see if control animals were able to clearly discriminate between different mouse urine using the olfactory habituation/dishabituation assay. All animals were clearly able to distinguish between all mouse urine samples (Fig. 6.14A).

Next, we assessed if control animals had any particular preference for pairs of mouse urine. We tested urine preference of male mice to female or male mouse urine, and as expected, male mice had a significant preference for female mouse urine over male mouse urine (Fig. 6.14B). However, when male mice were presented with pregnant or lactating female mouse urine, they did not have a preference for one over the other (Fig. 6.14C). This was clearly not the case when we tested for preference of ovariectomized female mouse urine versus normal female mouse urine. Control animals all preferred normal female mouse urine over ovariectomized female mouse urine (Fig. 6.14D).

Since mice did not have a specific preference for pregnant or lactating female mouse urine, we used this urine pair in the sand-buried food assay to assess pheromone-cued associative olfactory learning. Mice were food-restricted and trained to retrieve a food reward associated with one of the urine samples. Over the course of five training days, all mice were able to associate the food reward with pregnant female mouse urine (Fig. 6.15A). We then used the urine pair of ovariectomized and normal female mouse urine in the sand-buried food assay as well. In order to minimize intrinsic biases, we paired the food reward with ovariectomized female mouse urine since animals had a preference for normal female mouse urine. After a six

day training paradigm, all mice were able to learn to forego their intrinsic preference for normal female mouse urine and learned to associate the food reward with ovariectomized female mouse urine (Fig. 6.15B).

DISCUSSION

Many published reports using either conditional knockout mice or inducible-conditional knockout mice to study adult neurogenesis generally only include either a genotype control or a drug control (Bergami et al., 2008; Zhang et al., 2008a; Imayoshi et al., 2009; Gao et al., 2011; Guo et al., 2011; Sahay et al., 2011; Sakamoto et al., 2011; Amiri et al.). Here we sought to justify the use of just one of these controls to ensure that convincing results are not precluded by the choice of one type of control model over another. To this end, we included both a drug control (ERK5$^{loxP/loxP}$ mice treated with tamoxifen) and a genotype control (Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$ mice treated with vehicle) in our study design. In addition, we included a negative-negative control as well (ERK5$^{loxP/loxP}$ mice treated with vehicle). We used all three control groups and performed all behavior assays to determine if any underlying differences existed across genotypes and drug treatment.

The results presented in this chapter suggest that no overt differences exist across different control animals and that the choice of using either a genotype or drug control animal in an experimental design is justifiable. Despite the fact that the data presented here show no clear differences between control animals, it is ultimately the responsibility of the researcher to ensure that no differences exist in the model organism of choice. When mice or rats are used in studies, it is imperative that strain and genetic background differences are taken into consideration; no two strains are identical. In addition, the use of littermates in all studies should help to minimize inter-litter variability.

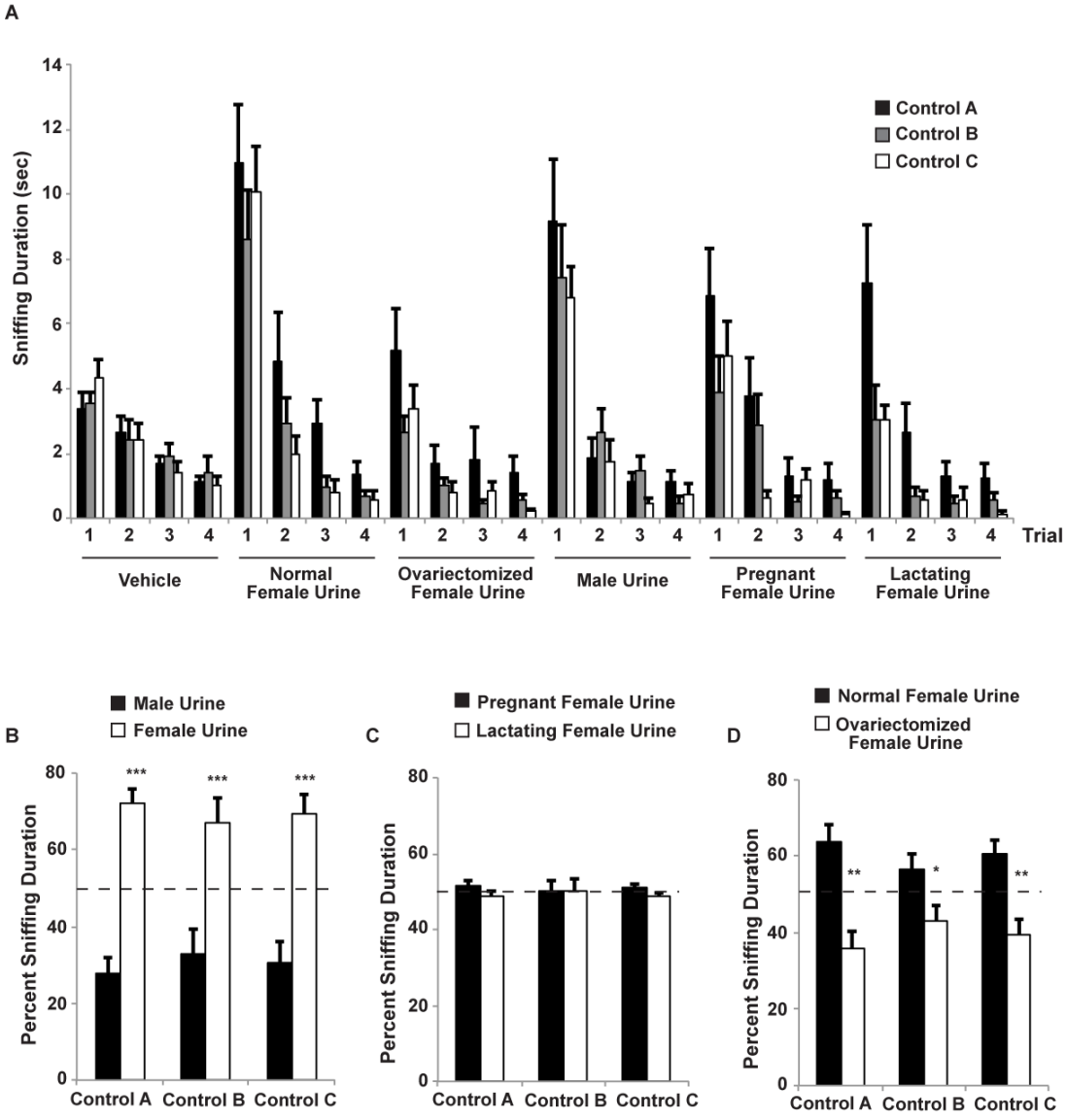


Figure 6.14. All animals were able to discriminate between distinct mouse urine samples and no differences existed in their preference to certain urine pairs. A) Male control mice were tested for their ability to discriminate between various mouse urine samples using the habituation/dishabituation assay. The duration of sniffing of the cotton swab was quantified. All mice were able to discriminate between urine samples over the course of 4 sequential presentations. B) Urine preference test between female mouse urine vs. male mouse urine, presented simultaneously with cotton swabs laced with female or male mouse urine. The duration of sniffing toward each cotton swab was quantified. All control animals showed a significant preference for sniffing toward female mouse urine vs. male mouse urine. C) Urine preference test between pregnant and lactating female mouse urine. D) Urine preference test between ovariectomized female mouse urine and age-matched, normal adult female mouse urine.

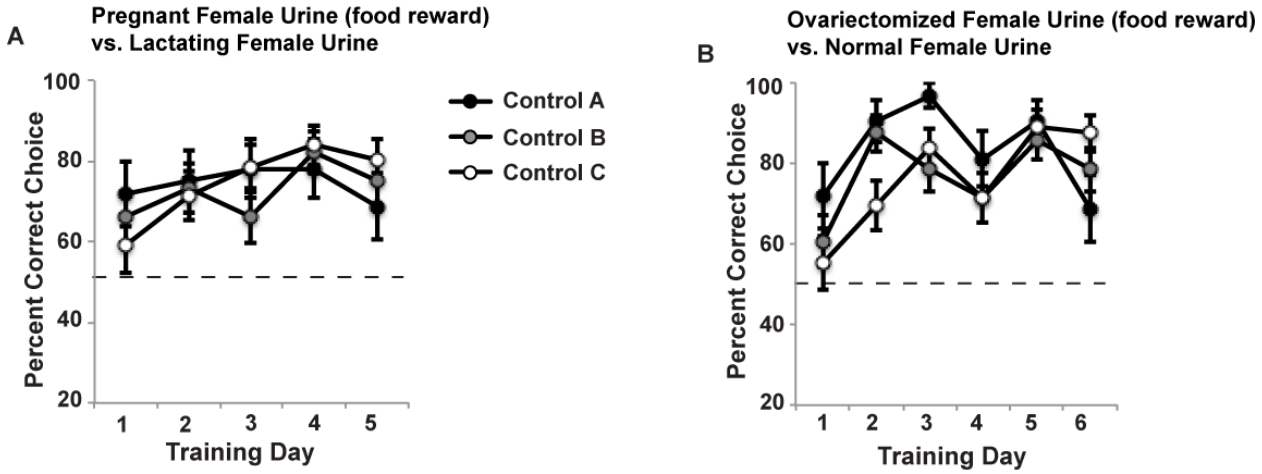


Figure 6.15. All control animals performed similarly in the acquisition of a sand digging-based, odor-cued associative learning task. A) Control animals learned to associate a food reward with pregnant female mouse over a 5-day training course. B) Association of a food reward with ovariectomized female mouse urine over a 6-day training course.

To account for many of the aforementioned variables, in our study design, we used littermates from the same genetic background and strain of mice. Because behavioral assays necessitate an $n \geq 7$ animals per group to minimize inter-animal variability, we combined littermates of not more than 2 weeks apart in birth dates. This allowed a larger cohort of animals to be used to perform our studies while maintaining a closeness in age when treating mice with vehicle or tamoxifen and performing all behavioral assays. We housed all animals identically under standard animal housing conditions and handled them identically before and during behavioral assays. By minimizing the number of variables in our study design, we feel confident that, in our specific case, the use of either drug or genotype control animals would not impact any of our behavioral results.

Chapter 7

Unpublished Data

INTRODUCTION

Throughout this dissertation, it has been highlighted that ERK5 MAPK is a critical signaling pathway in the regulation of adult neurogenesis. We provide evidence suggesting a linear pathway by which ERK5 activation via NT3 could elicit a downstream effect through Neurog2. Despite this evidence, signaling pathways are rarely linear and can be influenced via multiple inputs resulting in an exponential number of downstream effects ranging from cellular proliferation, cell-fate determination, differentiation, apoptosis, or cell survival to name a few. More work in this area is needed to fully elucidate a molecular pathway involving ERK5 and how it regulates adult neurogenesis in both the dentate gyrus and along the SVZ-RMS-OB axis.

Results presented thus far point to a functional role of ERK5 in the adult neurogenic regions, the SGZ of the dentate gryus and the SVZ along the lateral ventricles. Evidence here suggests the involvement of ERK5 in hippocampus-dependent learning and memory tasks as well as several olfactory behavioral assays. Despite the conclusions drawn in this dissertation, there are many other behavioral implications of adult neurogenesis as well. For example, reports have suggested an involvement of adult-born neurons in anxiety, other olfactory detection assays, and mating behavior (Shors et al., 2002; Stowers et al., 2002; Shingo et al., 2003; Magavi et al., 2005; Mak et al., 2007; Bergami et al., 2008; Larsen et al., 2008; Li et al., 2008; Imayoshi et al., 2009; Lazarini et al., 2009; Revest et al., 2009; Akers et al., 2010; Arenkiel, 2010; Larsen and Grattan, 2010; Okun et al., 2010; Oboti et al., 2011; Sakamoto et al., 2011; Snyder et al., 2011). In addition, exploring the possibility to increase adult neurogenesis by external factors such as environmental enrichment and/or free access to running wheels has been heavily studied (van Praag et al., 1999; Brown et al., 2003; Faherty et al., 2005; Meshi et

al., 2006; Creer et al., 2010; Sahay et al., 2011). Despite the ability to increase the overall number and survival of adult-born neurons, molecular mechanisms regulating this process are still unclear.

Adult neurogenesis is continuing to gain much interest due to the potential therapeutic implications from the ability to continuously generate new neurons. Understanding the regulatory mechanisms governing the genesis of new neurons within, and potentially outside of, the neurogenic niches is central to its future applications for the treatment of many neurological diseases. The use of adult neural stem cells for therapy remains a long-standing goal in this field of research. Endeavors toward delineating how ERK5 MAPK plays a role in regulating adult neurogenesis has led to many additional experimental results not presented in the previous chapters. This chapter is dedicated to those results that were overshadowed by more complete story lines and present possible avenues for future research directions at both the molecular and the behavioral levels.

MATERIALS AND METHODS

Animals

Nestin-CreER^{TM} (Kuo et al., 2006) mice and ERK5$^{loxP/loxP}$ (Wang et al., 2005) mice were crossed to yield Nestin-CreER^{TM}/ERK5$^{loxP/+}$ animals. Nestin-CreER^{TM}/ERK5$^{loxP/+}$ mice were further crossed with ERK5$^{loxP/loxP}$ mice to yield homozygous Nestin-CreER^{TM}/ERK5$^{lox/P/loxP}$ animals, which were used for experimental breeding. To confirm the specificity of tamoxifen-induced, Nestin-CreER^{TM}-mediated recombination in ERK5^{+} cells, a separate cohort of Nestin-CreER^{TM}/ERK5$^{lox/P/loxP}$ mice were crossed with R26-YFP reporter mice where Cre-mediated recombination removes a transcriptional STOP to allow YFP expression (Srinivas et al., 2001). This mouse strain permitted the simultaneous deletion of ERK5 and expression of YFP in Nestin^{+} adult neural stem cells upon tamoxifen treatment. All animal experiments were

performed with identically treated and handled littermate controls. Animals were housed under standard conditions (12 h light/dark cycle) with food and water provided *ad libitum* except where indicated. All experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

BrdU and Tamoxifen administration

Mice were treated with 100 mg/kg BrdU (Sigma) by intraperitoneal (IP) injection 5 times (every 2 h for 10 h) in one day followed by sacrifice 4 or 6 weeks later to identify BrdU-retaining, adult-born cells. An additional cohort of mice were injected with BrdU once and sacrificed 2 h later to identify $BrdU^+$ proliferating cells. Tamoxifen (Sigma) was made fresh daily and dissolved in 2% glacial acetic acid in corn oil solution (Sigma). To activate Cre-mediated recombination, 5 mg of pre-warmed tamoxifen was administered orally to 10-12 week-old male mice daily for 7 d or once per day for 4 d in each cycle, for 3 cycles with 2-week inter-cycle intervals.

Immunohistochemistry (IHC)

Brains were post-fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C after standard intracardial perfusion procedures. Brains were then placed in 30% (w/v) sucrose in PBS at 4°C until brains sunk and immediately frozen at -80°C. IHC was performed on 30 µm-thick coronal brain sections using a free-floating antibody staining method as described (Pan et al., 2012a).

Immunocytochemistry

Cells were fixed in PBS containing 4% PFA and 4% sucrose at room temperature for 30 min. Fixed cells were washed 3 x 5 min in PBS, 5 min in 1% SDS, and washed again 3 x 5 min in PBS. Cells were then incubated in blocking buffer consisting of 5% bovine serum albumin (BSA) in PBST (PBS + 0.1% Triton X-100) for 2 h, followed by incubation with primary

antibodies overnight at 4°C. Cells were then washed 3 x 10 min in PBST, followed by incubation with secondary antibodies at 1:5,000 dilution (Alexa Fluor-488) or 1:2,000 dilution (Alexa Fluor-594) for 2 h in blocking buffer. Cells were then washed 3 x 10 min in PBST followed by a 10 min incubation in Hoechst 33342 for nuclei visualization and a final wash of 10 min in PBST prior to mounting onto slides using anti-fade Aqua Poly/Mount solution. Unless otherwise stated, all steps were carried out at room temperature.

Reagents

The following primary antibodies and dilutions were used for immunohistochemistry: rat monoclonal anti-BrdU (1:500, AbD Serotec); mouse monoclonal antibodies against Ki67 (1:500, Leica), NeuN (1:500, Millipore), Calretinin (1:200, Abcam) and GFP (1:500, Invitrogen); goat polyclonal antibody against DCX (1:200, Santa Cruz Biotech Inc.); and rabbit polyclonal antibody against GFP (1:500, Invitrogen). Rabbit polyclonal ERK5 antibody (1:500 dilution) was generated previously (Cavanaugh et al., 2001) and affinity purified using recombinant MBP-ERK5 protein. The following primary antibodies and dilutions were used for immunocytochemistry: mouse monoclonal antibodies against Nestin (1:500, Developmental Studies Hybridoma Bank) and β-III Tubulin (1:500, Promega); rabbit polyclonal antibody against GFP (1:5,000, Invitrogen). The following primary antibodies and dilutions were used for Western blot analysis: rabbit polyclonal ERK5 antiserum (1:1,000) and mouse monoclonal β-actin antibody (1:10,000, Sigma). Secondary antibodies were rabbit polyclonal Horse Radish Peroxidase (HRP) antibody (1:10,000, Calbiochem) and mouse monoclonal HRP antibody (1:20,000, Calbiochem).

Confocal imaging and analysis

All images were captured with an Olympus Fluoview-1000 laser scanning confocal microscope with numerical aperture (NA) 0.75, 20X lens or NA 1.3, 40X oil immersion lens. Optical Z-sections (0.5–1 μm) were collected and processed using ImageJ software (NIH). Images were uniformly adjusted for color, brightness, and contrast with Adobe Photoshop CS4 (Adobe Systems Inc).

Quantification of immunostained cells

Greater than 100 immunopositive cells per coverslip per experiment were quantified using an inverted fluorescence microscope (Leitz DMIRB, Leica) with a 40X objective (Leica) following immunocytochemistry. A modified optical fractionator method was used as an unbiased stereological method for obtaining an estimation of total cell counts per SGZ following immunohistochemistry (West et al., 1991; Kempermann et al., 1997b; Malberg et al., 2000). The method for *in vivo* cellular quantification and co-localization analysis per SGZ was as described (Pan et al., 2012a).

Size measurement of dentate gyrus

Volumetric measurement of the dentate gyrus was conducted using Cavalieri Estimator probe of Stereo Investigator software (MBF Biosciences). Every eighth serial coronal section was counterstained with Hoechst 33342 and measurements were conducted on all stained nuclei of the dentate gyrus.

SGZ-derived adult neural progenitor cell (aNPCs) cultures

Primary cell cultures were prepared as described (Bull and Bartlett, 2005; Rietze and Reynolds, 2006). Briefly, tissue samples containing the dentate gyrus were micro-dissected and enzymatically digested with 0.1% trypsin-EDTA (Gibco) for 7 min at 37°C followed by incubation

with equal volume of 0.014% trypsin inhibitor (Gibco). Tissue samples were then spun down and resuspended in culture media consisting of DMEM/F12 (Gibco), 1X N2 supplement (Invitrogen), 1X B27 supplement without retinoic acid (Gibco), 100 U/mL penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), 2 µg/mL heparin (Sigma), 20 ng/mL EGF (EMD Chemicals), and 10 ng/mL bFGF (Millipore). Culture medium for adult neural progenitor cells always contain EGF and bFGF unless otherwise specified. Tissue was mechanically triturated and filtered through a 40 µm cell sieve and plated in petri dishes and cultured for 10–14 d until neurospheres are formed. Growth factors were replenished every 3 d during this period. Following primary passage, neurospheres were isolated, dissociated into single-cell suspension enzymatically and mechanically, and replated at low density and cultured for secondary neurosphere formation. Spheres collected from secondary passage were dissociated and plated as a monolayer culture on poly-D-lysine/laminin- (BD Biosciences) or poly-L-ornithine/fibronectin- (BD Biosciences) coated aclar coverslips (Electron Microscopy Sciences) for experiments.

Viral transduction of aNPCs

Adult neural progenitor cells were plated as a monolayer culture on poly-D-lysine/laminin- or poly-L-ornithine/fibronectin-coated aclar coverslips in 24-well plates at a density of 1 x 10^5 cells per well in culture media. Twenty-four hours after plating, protamine sulfate was added to culture media at a final concentration of 8 µg/mL and 6-8 µL of 1 x 10^7 IU/mL lentiviruses were added to each well and allowed to transduce cells for 24 h. Following transduction, culture media was changed and cells were cultured for an additional 5 d before being processed for immunocytochemistry.

Lentiviral MEF2C transfer construct (pRRL-cPPT-CMV-MEF2C-PRE-SIN-IRES-EGFP)

Flag-MEF2C cDNA sequence was inserted into a multiple cloning site of lentiviral transfer vector pRRL-cPPT-CMV-X-PRE-SIN-IRES-EGFP, described in (Liu et al., 2006), upstream from the internal IRES-directed marker protein eGFP (enhanced green fluorescent protein). High-titer lentiviral stocks were produced as described (Liu et al., 2006).

Stereotaxic surgery

Stereotaxic procedure was performed on adult C57/BL6 male mice (8-10 weeks old, Charles River Laboratories) as described (Wong et al., 2000; Athos and Storm, 2001). Mice were anaesthetized by IP injection (21–23 µl/g body weight) of ketamine (7.0 mg/mL) and xylazine (0.44 mg/mL) dissolved in 0.9% bacteriostatic saline (Hospira, Inc.). One microliter of retrovirus (10^9-10^{10} IU/mL) was injected at a rate of 0.25 mL/min bilaterally into the dentate gyrus with the following coordinates relative to Bregma: 1.65 mm posterior, ±1.62 mm medial-lateral, 2.30 mm ventral.

Behavior Assays

Running wheel setup

This was performed as described (Creer et al., 2010). We housed mice in their home cages with free access to a running wheel. A small disc magnet was attached to the rim of the running wheel to permit data acquisition of various running metrics recorded by a small bicycle computer (Cateye). Runners were allowed free access to the running wheel for 4 weeks and data were collected and analyzed each day for the entire 4 week period.

Delay approach to first investigation

Videos were recorded for olfactory habituation and novel object recognition assays. Quantification of the delay from first investigation of either the cotton swab or novel object was performed offline by an experimenter blinded to the genotype and treatment group.

Social recognition assay

This was performed as previously described (Garelick et al., 2009). Male mice were singly housed and exposed to ovariectomized females for 1 min and the total investigation time of the female was quantified. Following an inter-trial interval of 10 min, the same ovariectomized female was placed back in the cage 2 more times for a total of 3 sessions. Following the final session, a novel ovariectomized female mouse was placed in the cage and the total investigation time was quantified.

Elevated plus maze

An elevated plus maze apparatus was used consisting of per arm measurements of (12" x 2" x 15.25"); closed arms had additional sidewalls measuring 6" from the arm platform. To measure basal anxiety levels, mice were not pre-habituated to the apparatus. Mice were placed in the center of the apparatus and allowed to freely explore for 5 min. All data were collected electronically using ANYmaze software (Sand Diego Instruments) and analyzed with the experimenter blinded to genotype and treatment conditions.

Olfactory preference for chemically defined odorants

Mice were presented simultaneously with two cotton swabs laced with 100 µL of different odorant pairs. Total investigation time of each cotton swab was recorded during a 2 min session. Data presented are percent sniffing duration for each odorant or urine relative to the

total sniffing of both cotton swabs. A 50% sniffing duration indicates no preference for either urine.

Olfactory preference of mouse urine

Mice were presented simultaneously with two cotton swabs laced with undiluted mouse urine (10 µL). Mouse urine was collected from group-housed mice (n = 4–5 per cage) and pooled over a period of 2 weeks to minimize day-to-day fluctuations in basal pheromone and urine amount. Total investigation time of each cotton swab was recorded during a 2 min session. Data presented are percent sniffing duration for each odorant or urine relative to the total sniffing of both cotton swabs. A 50% sniffing duration indicates no preference for either urine.

Olfactory discrimination acquisition using odorant pairs

Following olfactory preference assay, mice were food restricted to maintain 85-90% of free-feeding body weight for 4-5 d prior to beginning and throughout the entirety of the assay. This was performed as previously described (Zou et al., 2012) using the same apparatus, pre-training and training protocols. After mice learned to associate sand digging with a food reward at the bottom of the dish, mice were presented 2 sand dishes. One containing 100 µL of an odorant associated with a food reward, while the other contained 100 µL of another odorant without food reward. Odorant pairs were established based on lack of preference for either odorant in the olfactory preference assay. Animals were subjected to this assay for 4 trials per block and 2 blocks per day to learn to associate a particular odorant with a food reward. Scoring for correct or incorrect choice was based entirely on the animal's first dig, either in the odorant dish with the food reward (correct) or in the odorant dish without the food reward (incorrect). Mice that dug in the incorrect dish were not allowed to self-correct during the 5 or 6 d training

period. After each day of training, mice were fed a pre-determined amount of food to maintain 85-90% free-feeding body weight.

Olfactory discrimination with mix ratios of structurally similar odorants

One day following olfactory discrimination acquisition between structurally similar odorants (S/R-limonene and S/R-carvone), olfactory discrimination of mixtures of enantiomer odorants was performed. This assay was performed as the olfactory discrimination assay except the food reward-associated odorant was mixed with increasing concentrations of the non-food reward-associated odorant. Only one mix ratio was presented per day. The number of correct choices and incorrect choices were scored as described above.

Male-female sexual behavior assay

Ovariectomized females were injected with 20 µg estradiol and 500 µg progesterone per 20 g body weight to artificially resemble the pro-estrus cycle. Mice were injected with estradiol 2 d prior to beginning the assay, while progesterone was injected 5 h prior on the day of behavior testing. Artificial pro-estrus ovariectomized female mice were introduced into the male animal's home cage and sexual behavior was video recorded for the entire 30 min session and quantified offline.

Male-male aggression behavior assay

Normal male mice (intruder) were introduced to single-caged experimental animals (resident). Resident males were single-caged for at least 7 d prior to beginning the assay and permitted to odorize and establish dominance of the home cage. One intruder male mouse was placed in the home cage of a single resident male and aggressive behavior was video recorded for 15 min and quantified offline.

Electroolfactogram (EOG) measurement

EOG studies in the main olfactory epithelium were performed as described (Wong et al., 2000; Trinh and Storm, 2003). Briefly, awake mice were sacrificed by cervical dislocation and skulls were cut in half along the midline. The septal cartilage was removed to expose the olfactory turbinates. Olfactory turbinates from both sides were used for recordings. The EOG was recorded with an agar- and saline- filled glass microelectrode in contact with the apical surface of the main olfactory epithelium in the open circuit configuration. Using an automated four-way slider valve, odorants were puffed onto the exposed epithelia for 0.2 s. Traces were captured and digitized using a Digidata 1,200A (Axon Instruments, Union City, California). The traces were lowpass filtered at 30 Hz and sampled at 100 Hz.

RESULTS

Confirmation of affinity-purified ERK5 antibody specificity to endogenous ERK5 protein

Brain sections from wild-type adult animals were sectioned and processed for IHC. Affinity purified polyclonal rabbit anti-ERK5 antibody was used to identify ERK5 expressing cells in the brain. Pre-absorbed ERK5 antibody was used to evaluate specificity of ERK5 antibody to its corresponding antigen. No ERK5 staining was detected using pre-absorbed ERK5 antibody, while $ERK5^{+}$ cells were clearly detected using affinity-ERK5 antibody alone (Fig. 7.1).

Adult neural progenitor cells isolated from the dentate gyrus express ERK5

To confirm the proliferative nature of aNPCs isolated form the dentate gyrus, dissociated primary cells were plated at low cell density and neurosphere formation was examined 2 weeks later. Following initial passage of neurospheres, we dissociated primary neurospheres into a single-cell suspension and again plated at low density to permit neurosphere formation. Following secondary neurosphere formation, 2 individual neurospheres were dissociated and

processed for Western blot. ERK5 protein was abundantly expressed in DG-derived aNPCs suggesting a role of ERK5 in aNPC proliferation (Fig. 7.2)

Dominant-negative myocyte enhancer factor 2 C (MEF2C) expression reduces neurogenesis *in vitro*

MEF2C is a known substrate of ERK5 in embryonic cortical progenitor cells (Liu et al., 2003a), however, its involvement in aNPCs is unknown. To begin preliminary investigation of MEF2C as a downstream substrate of ERK5 in DG-derived aNPCs, we dissociated neurospheres and cells were plated as a monolayer on PDL-coated aclar coverslips. Cells were then infected with lentiviruses expressing GFP control or dominant negative (dn-) MEF2C for 24 h. Following infection, media was refreshed and cells were maintained in culture media for an additional 5 d. Immunostaining of aNPCs infected with GFP control or dnMEF2C showed a decrease in the number of β-III Tubulin$^+$ neurons while a concomitant increase in the number of nestin$^+$ neural stem cells (Fig. 7.3). This result suggests a possible role of MEF2C in regulating adult neurogenesis *in vitro*.

Overexpression of constitutive active MEK5 does not affect neuronal migration but increases the population of cells expressing neuronal markers

Using the same stereotaxic method introduced in Chapter 2, we took brain sections and analyzed the migration of control GFP-infected and constitutive active (ca-) MEK5-infected 2 week old adult-born neurons. Our preliminary data shows that expression of caMEK5 does not overtly affect migration of adult neurons into the granule cell layer of the dentate gyrus (Fig. 7.4).

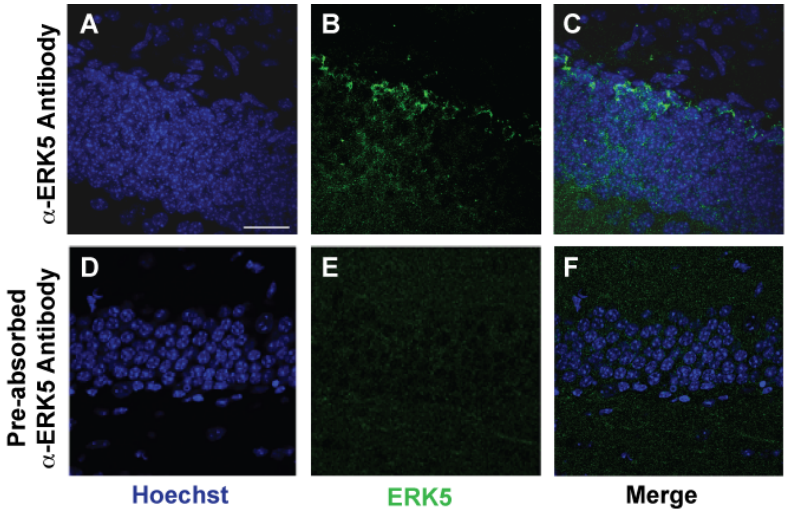


Figure 7.1. Specificity of affinity-purified ERK5 antibody directed against the C-terminal domain of ERK5 protein. A–C) Slices from adult mouse brains were incubated with the affinity-purified, polyclonal antibody against ERK5 or D–F) the same antibody pre-absorbed with purified recombinant C-terminal fragment of ERK5 protein. Hoechst staining was used to visualize all cell nuclei. Scale bar in A represents 25 μm and applies to all images.

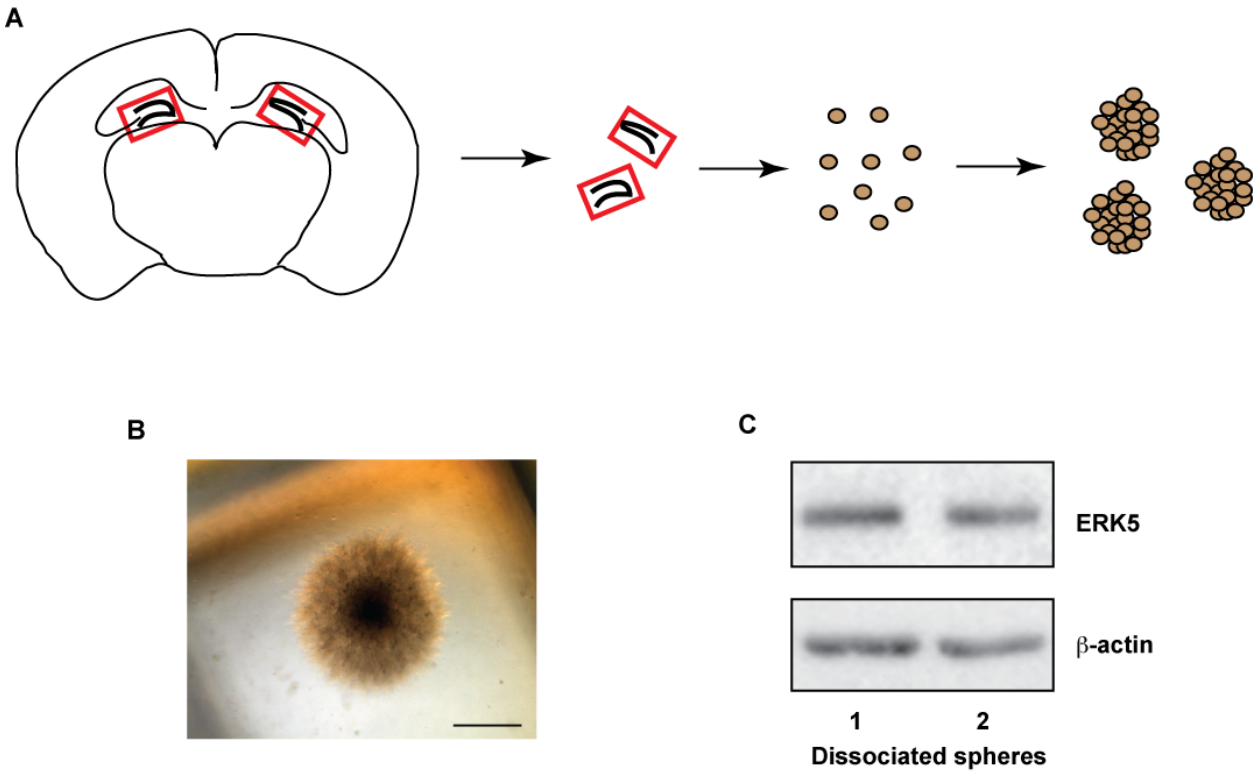


Figure 7.2. Murine adult neural progenitor cells derived from the dentate gyrus express ERK5 protein. A) Schematic illustration of the micro-dissection process to isolate adult hippocampal neural progenitor cells from the dentate gyrus. B) Pictomicrograph of a neurosphere isolated from the dentate gyrus. C) Western analysis of ERK5 protein expression in two individually dissociated neuropheres. β-actin was used as a loading control. Scale bar represents 0.5 mm.

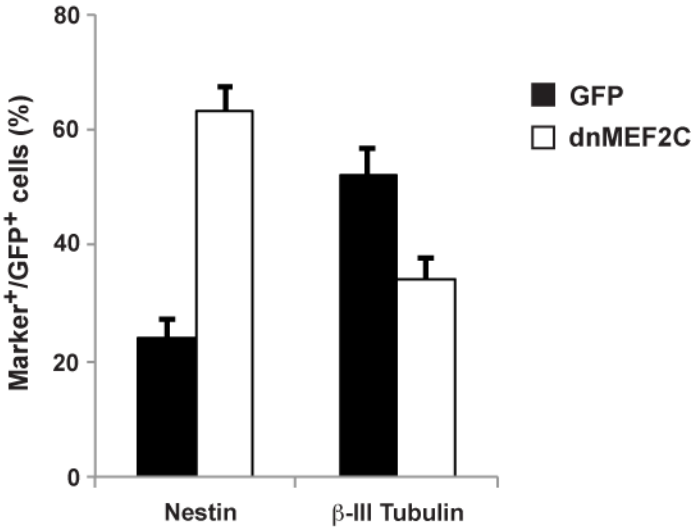


Figure 7.3. Dominant negative MEF2C inhibits neurogenesis in DG-derived aNPCs. Lentiviruses expressing GFP control or dnMEF2C were used to infect aNPCs for 24 h. Immunostaining of aNPCs 5 d post-infection shows a significant increase in the number of Nestin$^+$ neural stem cells and a concomitant decrease in β-III Tubulin$^+$ neurons.

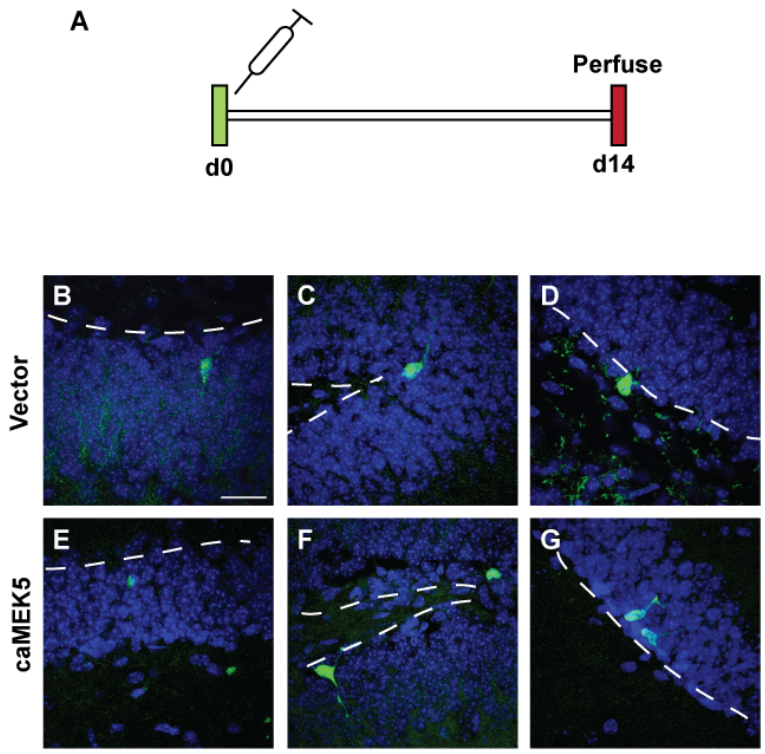


Figure 7.4. Endogenous activation of ERK5 via stereotaxic delivery of retroviral vectors does not alter migration of infected cells. A) Schematic illustration of the experimental design. B–D) Three individual representative images of vector-infected cells 2 weeks following stereotaxic injection. E–G) Three individual representative images of caMEK5-infected cells 2 weeks following stereotaxic injection. Scale bar in B represents 25 μm and applies to all images. Dashed lines outline SGZ layer of the dentate gyrus.

Although an enhanced neuronal differentiation affect was previously reported (Pan et al., 2012b), we wanted to further examine other neuronal markers to determine if caMEK5 had an effect on other populations of 2 week old neurons. Brain sections were processed for immunohistochemistry and stained for proliferating cell marker, Ki67, transiently amplifying progenitor cell or newborn neuron marker, DCX, and a newborn immature neuron marker, calretinin (Fig. 7.5). Due to processing difficulties, brain sections stained for Ki67 in caMEK5-injected mice were not able to yield results. However, we were able to obtain data for DCX and calretinin markers. Although there was a slight increase in the number of DCX^+ and $calretinin^+$ cells in the dentate gyrus, the results were not significant at this 2 week time point.

Deletion of *erk5* causes an increase of proliferating cells and overall apoptotic cell marker activated caspase 3

Following *erk5* gene deletion we wanted to determine if the overall area or volume of the dentate gyrus was affected. Using the Cavalieri estimator probe, it was determined that no differences existed between control and ERK5 icKO mice (Fig. 7.6). We additionally processed brains 3 weeks following tamoxifen administration and stained them with proliferating cell markers Ki67 and BrdU following a 2 h pulse. The total number of BrdU+ and Ki67+ cells were significantly increased in ERK5 icKO mice (Fig. 7.7). These results suggest that although no overall effects are found in the dentate gyrus, ERK5 is indeed involved in the regulation of aNPCs, specifically upregulating the proliferation of aNPCs in the SGZ following *erk5* deletion.

To further examine the molecular effect of aNPCs in ERK5 icKO mice, we stained brain sections of mice sacrificed 4 weeks and 6 weeks following BrdU administration. Total numbers of BrdU, DCX, and calretinin were not different between control and ERK5 icKO mice (Fig. 7.8A); however, a difference was observed in the total BrdU population of cells expressing DCX or calretinin (Fig. 7.8B). These results suggest that although the total population of neuronal

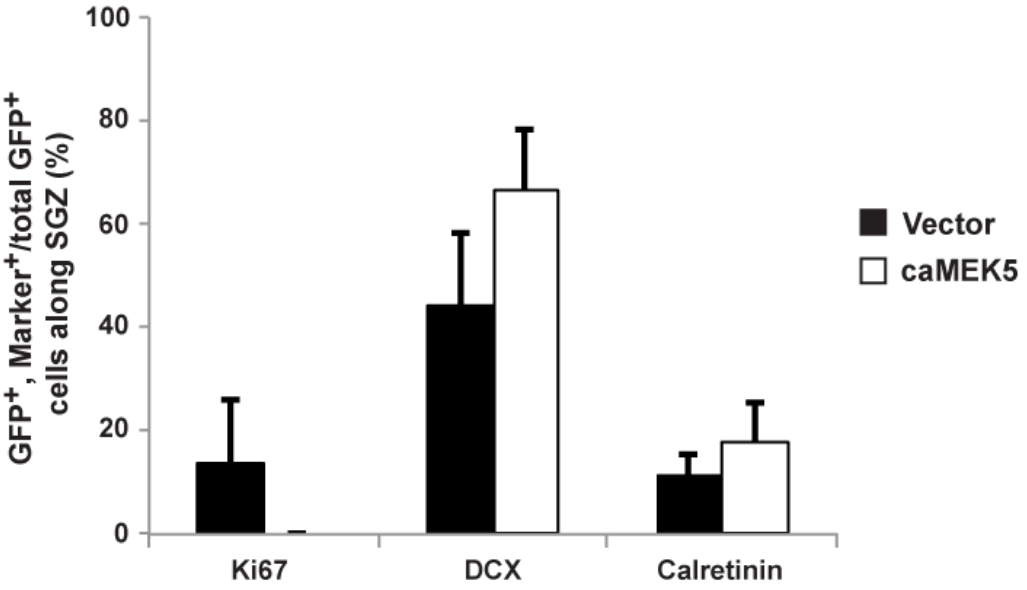


Figure 7.5. Ectopic activation of ERK5 promotes SGZ neurogenesis *in vivo*. Retroviral eGFP control (vector) or caMEK5-IRES-eGFP alone were stereotaxically injected into the dentate gyrus of 8-10 week old mice. Mice were sacrificed 2 weeks later and brain sections immunostained for GFP (green) and proliferating cell marker Ki67, transiently amplifying progenitor cell or newborn neuron maker DCX, or immature neuron marker calretinin. The percentage of GFP^+ and $marker^+$ co-labeled cells along the SGZ was quantified. Expression of caMEK5 alone increases the number of DCX^+ and $calretinin^+$ cells in total GFP^+ population.

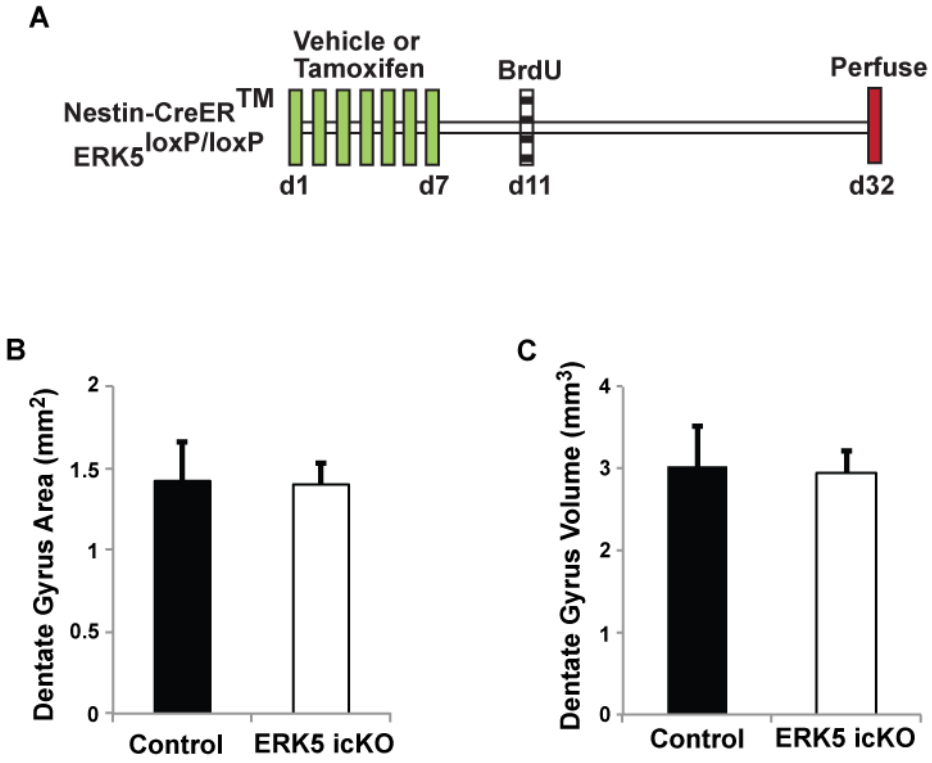


Figure 7.6. Deletion of *erk5* along the SGZ does not affect overall area or volume of the dentate gyrus. Mice were dosed with tamoxifen to induce cre-mediated recombination of the *erk5* gene. Mice were sacrificed 3 weeks following BrdU administration and brains were stained with Hoechst 33342 to visualize all cell nuclei. A) Schematic illustration of the experimental design. B) Area measurement of dentate gyrus. C) Volume measurement of the dentate gyrus.

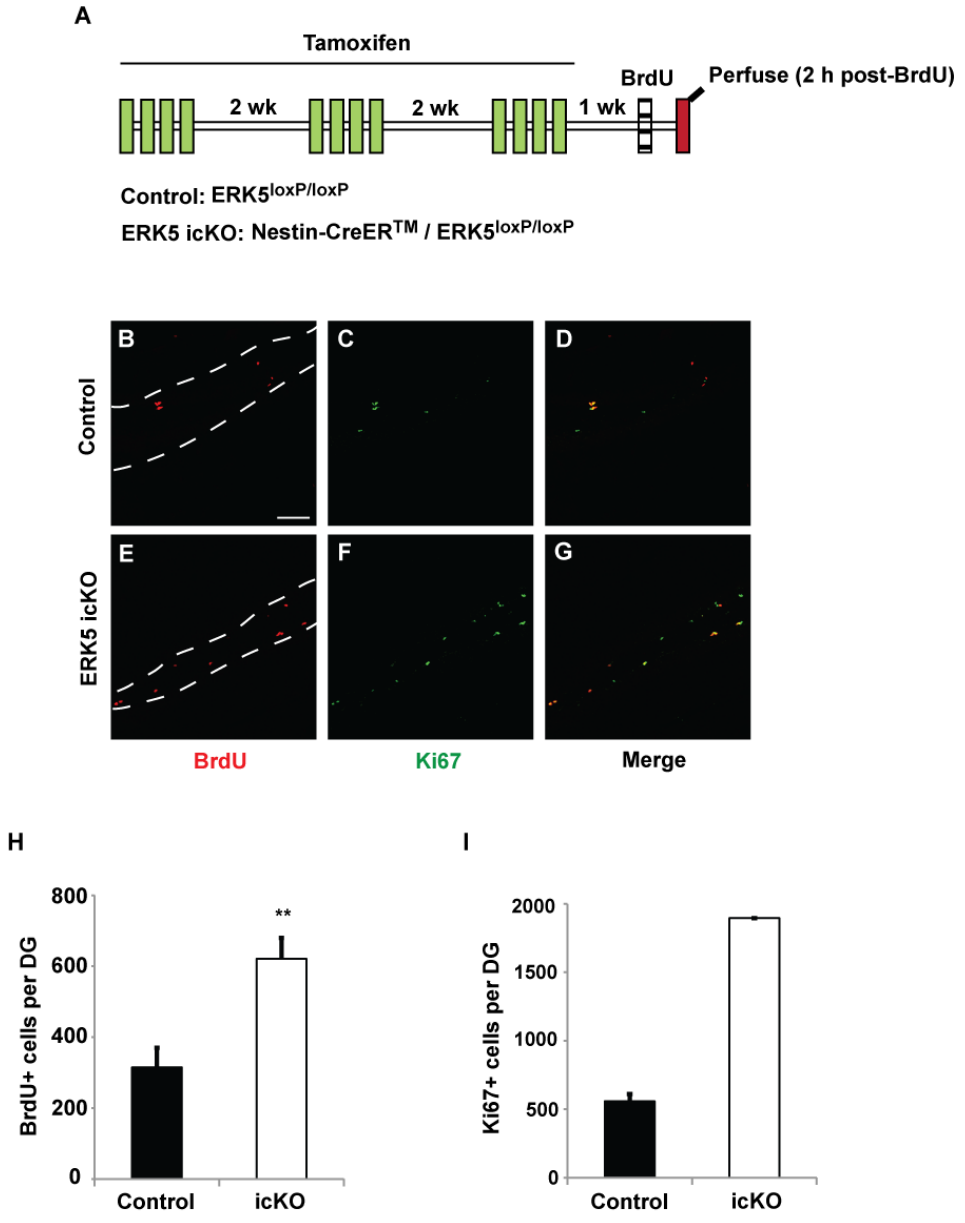


Figure 7.7. Increased proliferating cell markers along the SGZ in ERK5 icKO mice. Mice were dosed with tamoxifen to induce cre-mediated recombination of the *erk5* gene and brains processed by IHC 2 h after BrdU administration. A) Schematic illustration of the experimental design. B–D) Brain sections from control animals stained with proliferation markers BrdU and Ki67. E–G) Brain sections from ERK5 icKO mice stained with BrdU and Ki67. H) Quantification of total $BrdU^+$ cells along the SGZ per dentate gyrus. I) Quantification of total $Ki67^+$ cells along the SGZ per dentate gyrus. Dashed lines outline the SGZ. Scale bar in B represents 100 μm and applies to all images.

cells is not affected, adult neurogenesis is at 4 weeks post-BrdU. Lastly, we examined total apoptotic cells in the dentate gyrus and observed a difference in the number of cells expressing activated caspase 3 between control and ERK5 icKO mice at 4 weeks post-BrdU (Fig. 7.8C). Our final time point of analysis was 6 weeks following BrdU and we found no differences in the total number of $BrdU^+$ cells or the number of $BrdU^+$, $NeuN^+$ adult-born neurons (Fig. 7.9). These results suggest that although a significant difference existed in the number of $BrdU^+$, $NeuN^+$ cells at 4 weeks following BrdU administration (Pan et al., 2012b), perhaps a refractory period where increased proliferation resulted in equalizing the number of newborn neurons at 6 weeks post-BrdU.

ERK5 icKO mice with R26-YFP reporter can be a useful tool to examine molecular and cellular effects of *erk5* gene deletion *in vivo*

We crossed our Nestin-$CreER^{TM}$/$ERK5^{loxP/loxP}$ mice with the R26-YFP reporter strain mouse to obtain Nestin-$CreER^{TM}$/$ERK5^{loxP/loxP}$/R26-$YFP^{loxP/loxP}$ mice. Dosing these animals with vehicle control or tamoxifen initiates cre-mediated recombination of genes flanked by loxP sites. Our results demonstrate a reduction in ERK5 staining along both neurogenic regions with an abundance of YFP^+ expressing cells when mice were treated with tamoxifen (Fig. 7.10). Additionally, no leakiness of cre-recombinase, resulting in non-specific YFP expression, was observed when mice were dosed with vehicle control. These results suggest that leakiness of cre-recombinase is below our level of detection by IHC, and that we can use these animals for future experiments toward understanding the molecular and cellular consequence of cells lacking ERK5 *in vivo*.

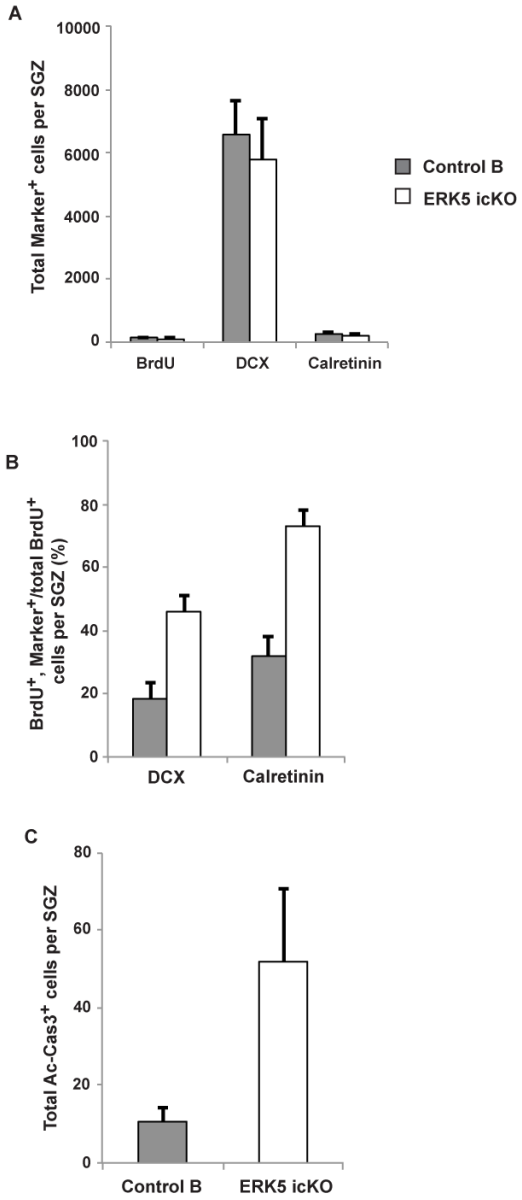


Figure 7.8. Increased overall apoptosis following *erk5* deletion in the SGZ. Mice were dosed with tamoxifen to induce cre-mediated recombination of the *erk5* gene and brains processed by IHC 4 weeks after BrdU administration. A) Quantification of total $BrdU^+$, DCX^+, and $calretinin^+$ cells along the SGZ. B) Quantification of total BrdU, marker double positive cells among total $BrdU^+$ cells along the SGZ. C) Quantification of total activated caspase 3 $(Ac\text{-}Cas3)^+$ cells along the SGZ.

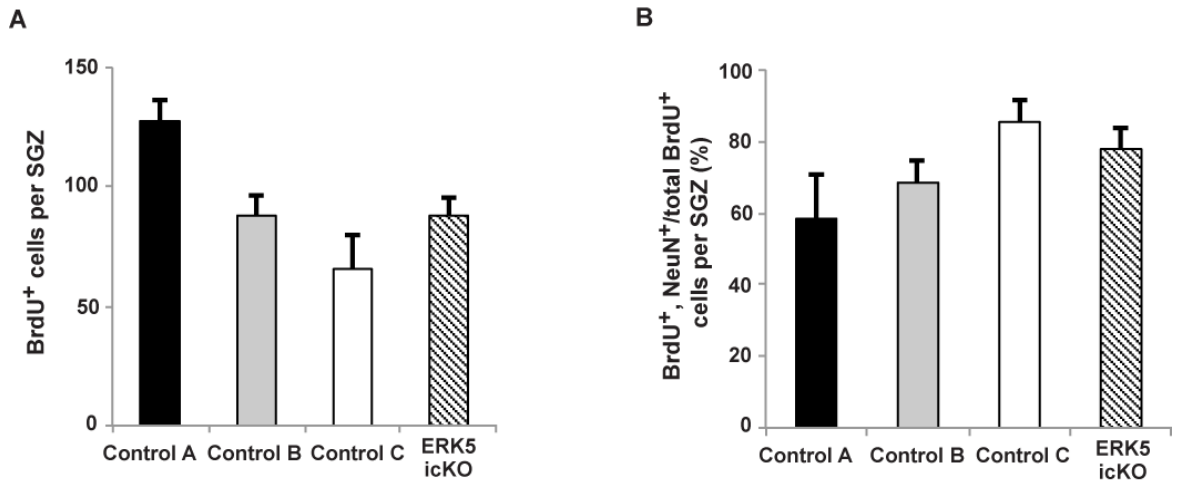


Figure 7.9. ERK5 icKO mice do not display any differences in total BrdU$^+$ or BrdU, NeuN double-positive cells 6 weeks following BrdU administration along the SGZ. A) Quantification of total BrdU$^+$ cells along the SGZ. B) Quantification of total BrdU, NeuN double-positive cells among total BrdU$^+$ cells along the SGZ.

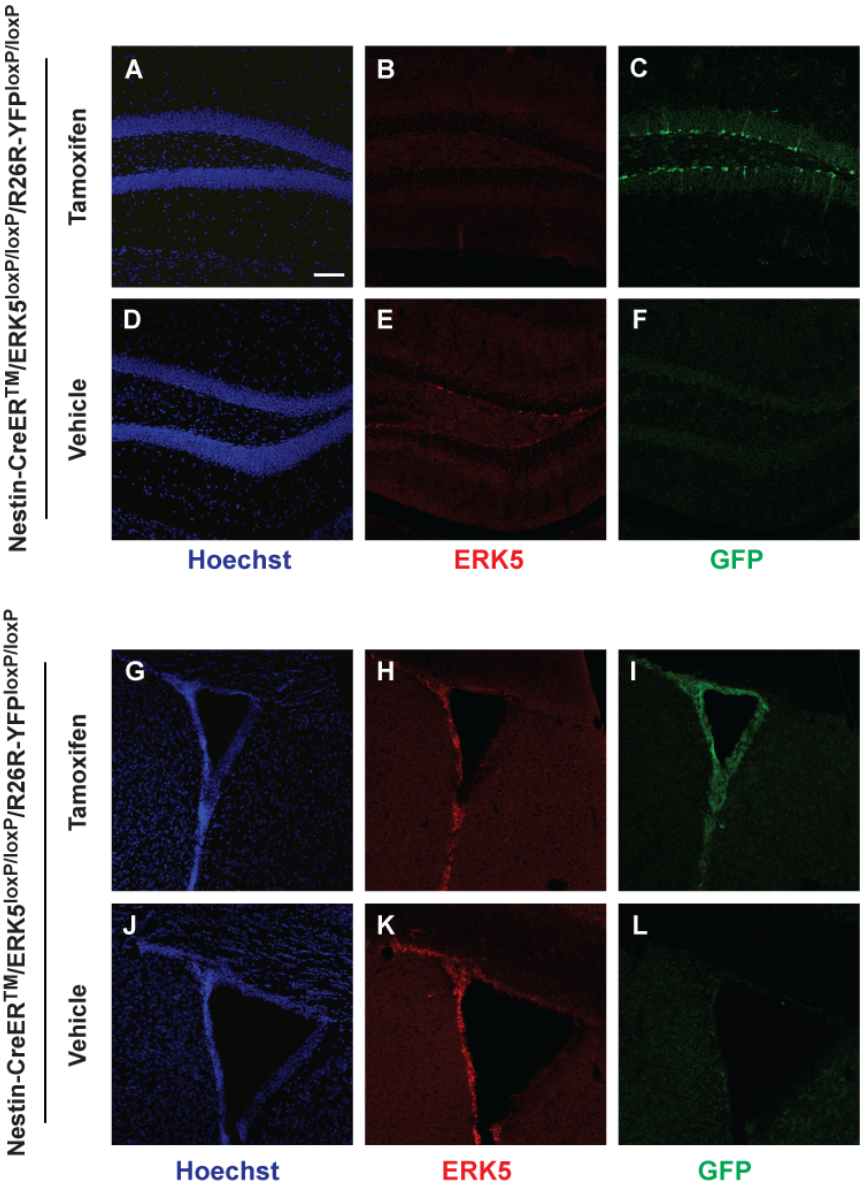


Figure 7.10. No leaky cre is observed in vehicle treated Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$/R26-YFP$^{loxP/loxP}$ mice. A–C) Representative image of tamoxifen treated animals showing reduced ERK5 immunostaining (red) and abundant GFP^{+} cells (green) along the SGZ. D–F) Brain sections from vehicle treated animals shows ERK5 but no GFP staining along the SGZ. G-I) Brain sections containing the lateral ventricles showing reduced ERK5 staining but induced GFP staining in tamoxifen treated animals. J–L) Vehicle treated control animals show ERK5 staining but no GFP staining. Hoechst was used as a counterstain to visualize all cell nuclei. Scale bar in A represents 25 μm and applies to all images.

ERK5 icKO mice lack motivation to run or investigate novel subjects and display an anxiety phenotype in the elevated plus maze assay

As a consequence of our experiments to determine if running-induced adult neurogenesis is blocked by *erk5* deletion, we permitted mice free access to running wheels for 4 weeks in their home cages. Our preliminary results examining just the running metrics when mice are allowed to run show a decreased trend in interest or motivation to run (Fig. 7.11). This result precludes any differences we would likely observe at the cellular level and requires further analyses as to possible reasons why ERK5 icKO and Control C mice are less motivated or interested in running. Albeit, the results presented here are preliminary with a small cohort of mice per genotype (n=3), it is still interesting to suggest a lack of motivation phenotype without normal physiological levels of adult neurogenesis.

Examining motivation using three other behavioral assays, we found that ERK5 icKO mice, and to some degree, control C mice have a longer delay to their first investigation of a novel cotton swab during the olfactory habituation assay (Fig. 7.12A). Offline analysis of delayed approach to the first investigation of either toy A or toy B in the novel object recognition assay also demonstrates a trend toward increased delay to first investigation in ERK5 icKO mice (Fig. 7.12B). Lastly, we compared the total investigation time of control B and ERK5 icKO mice in the social recognition assay. This was performed as previously described (Garelick et al., 2009), where ovariectomized female mice were placed into the home cages of singly-housed male mice for 2 min intervals. The same female was placed in the cage 2 more times following a 10 min inter-trial interval for a total of 3 exposures. Following the 3rd exposure, a novel ovariectomized female mouse was placed in the cage and the duration of investigation of the novel female was quantified. Reduced interest in continuous exposure to a familiar female mouse should be followed by renewed interest upon introduction of a novel female mouse.

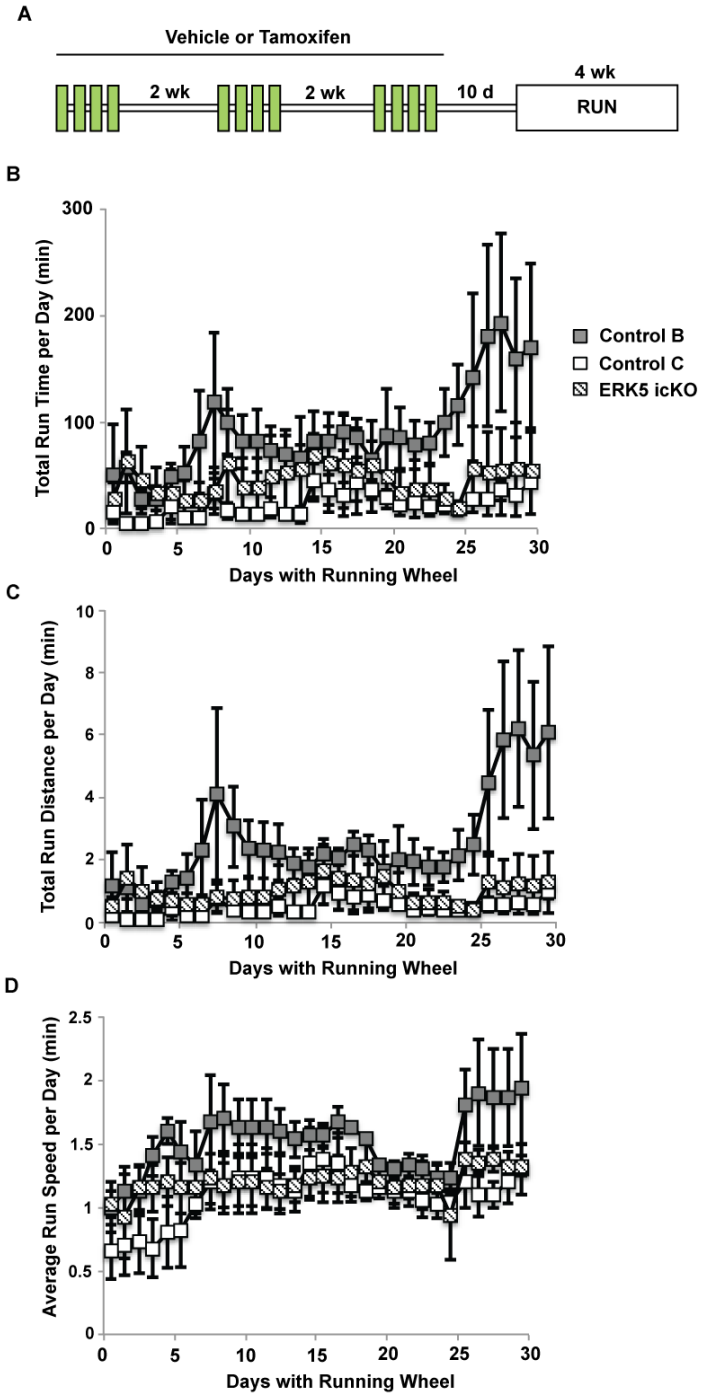


Figure 7.11. ERK5 icKO mice may lack motivation to run when allowed free access to a running wheel. A) Schematic illustration of the experimental design. B) Quantification of total run time per day. C) Quantification of total run distance per day. D) Average speed of running on the running wheel per day.

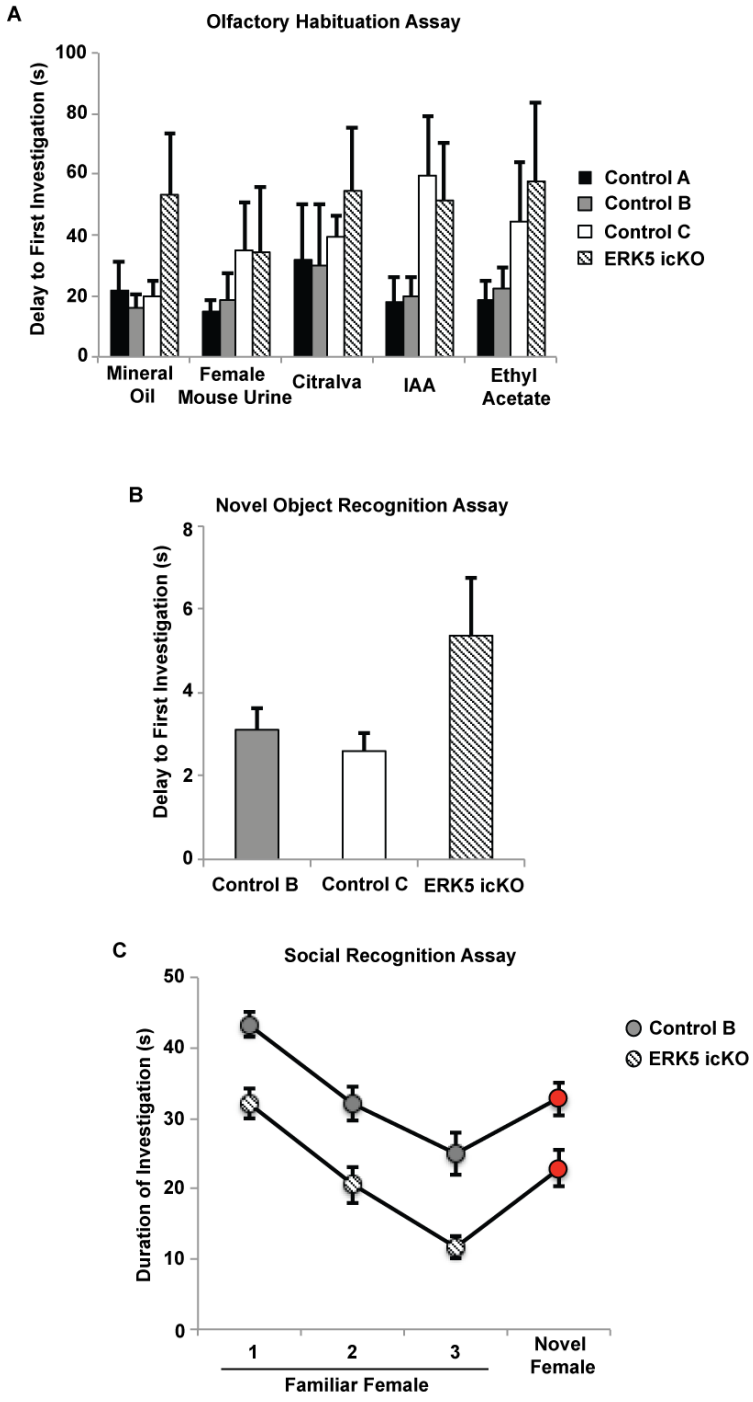


Figure 7.12. ERK5 icKO mice may lack motivation to investigate novel subjects. A) Offline analysis of delayed approach to the first presentation of a cotton swab in the olfactory habituation assay. B) Offline quantification of delayed approach to either toy A or toy B in the training session of the novel object recognition assay. C) Quantification of total investigation time when presented with familiar or novel ovariectomized female mice.

On average, ERK5 icKO mice were less interested in or motivated to investigate the female mice regardless of whether it was the first introduction, last introduction, or novel introduction of ovariectomized females (Fig. 7.12C). Taken together, these results suggest a possible role of adult neurogenesis in mediating motivation or interest to explore novel subjects.

An additional phenotype we wanted to examine was whether reduced levels of adult neurogenesis affect basal levels of anxiety. Toward investigating this phenotype, we tested mice using the elevated plus maze assay. Mice were not pre-habituated to the maze such that the anxiety phenotype would more closely resemble basal levels of anxiety. During a 5 min free exploration session, all control animals showed no specific preference for exploring the open arms over the closed arms, though ERK5 icKO mice clearly spent more time in the closed arms (Fig. 7.13A). Analysis of closed ends exploration in the open and closed arms also showed a significant difference between controls and ERK5 icKO mice (Fig. 7.13B). Additionally, we measured the total distance traveled and the average traveling speed while mice were in the maze, and ERK5 icKO mice were more stationary and did not travel as fast as control animals (Fig. 7.13C, D). These results suggest an increased basal level of anxiety in ERK5 icKO mice since they preferred to remain in the closed arms of the maze, traveled shorter distances, and did not move as quickly as controls.

Cellular characterization of adult-born neurons along the subventricular zone

Toward investigating how ERK5 is involved in adult olfactory bulb neurogenesis, we examined the cellular effect of adult-born, $BrdU^+$ cells along the subventricular zone (SVZ). At 4 weeks post-BrdU administration, we found no clear differences in the total number of $BrdU^+$ cells nor did we discover any differences in the overall population of DCX^+ or $calretinin^+$ cells along the SVZ (Fig. 7.14A).

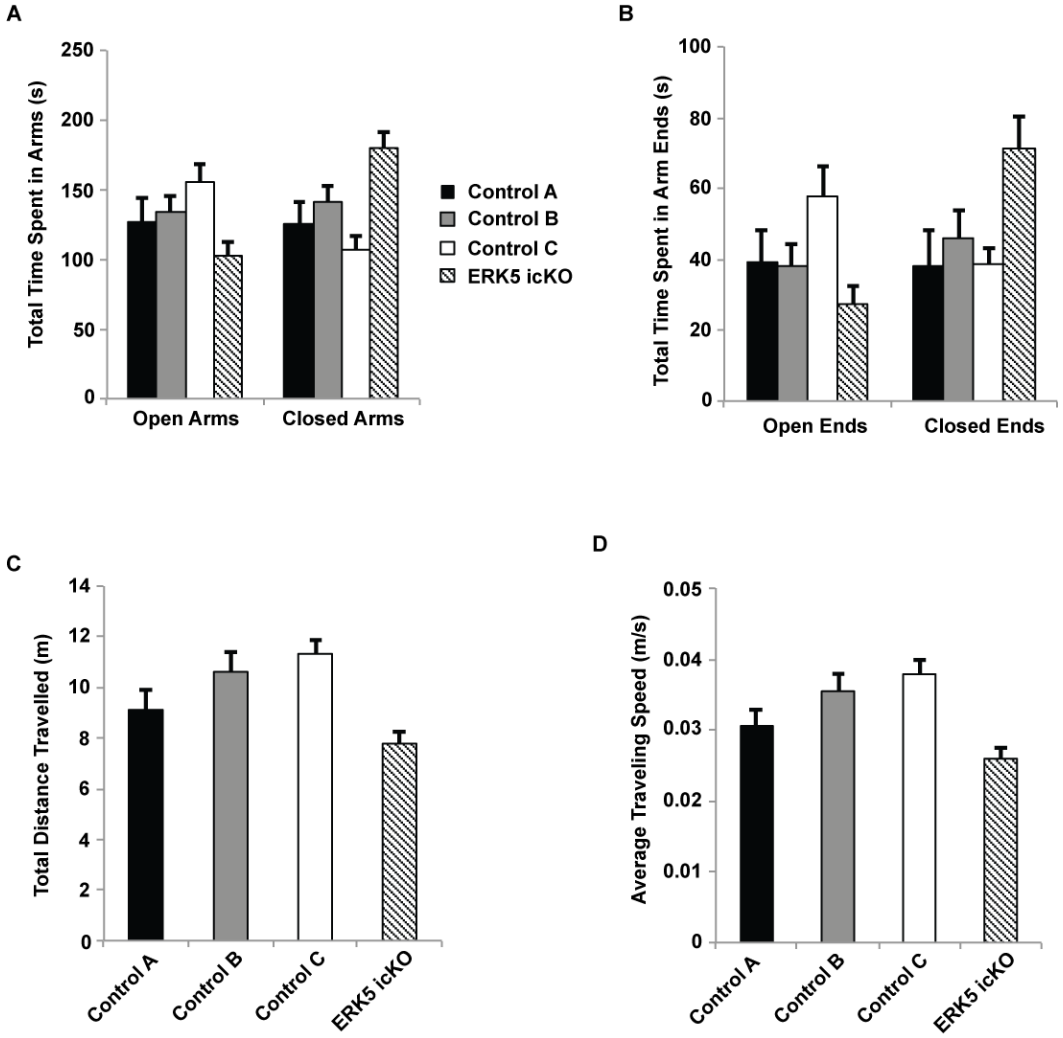


Figure 7.13. ERK5 icKO mice display increased basal anxiety in the elevated plus maze assay. Mice were placed into the center of the elevated plus maze for 5 min free exploration. A) Quantification of total time spent in the open and closed arms. B) Quantification of total time spent in the open and closed arm ends of the maze. ERK5 icKO mice spend more time in the closed arms and closed arm ends demonstrating an anxiety phenotype. C) Total distance traveled during the 5 min exploration session. D) Average traveling speed while in the maze.

We also found no differences in the cellular phenotype of $BrdU^+$ adult-born cells since a similar proportion of cells positive for BrdU were also positive for DCX or calretinin between controls and ERK5 icKO mice (Fig. 7.14B). Additionally, we examined cellular apoptosis and were able to observe a small trend of increased activated caspase 3 staining along the SVZ (Fig. 7.14C).

Adult-born neurons were characterized at an additional time point at 6 weeks post-BrdU administration. We found no differences between control animals and ERK5 icKO mice in the total number of $BrdU^+$ cells or the percentage of $BrdU^+$, $NeuN^+$ cells along the SVZ (Fig. 7.15). Together, with the 4 week BrdU data, these results suggest that further investigation into cellular effects of *erk5* deletion along the SVZ through the rostral migratory stream and into the olfactory bulb are necessary.

Olfactory discrimination is impaired in ERK5 icKO mice with mixed ratios of enantiomer odorants

Since ERK5 icKO mice displayed deficits in a number of olfactory associated behavioral assays (Chapter 5), we wanted to further explore the possibility that ERK5 icKO mice are deficient in discriminating between enantiomer odorant pairs in the sand-buried food assay. Assessment of odorant preference for one versus the other odorant showed no preferences between odorant pairs presented (Fig. 7.16). Citralva and IAA are structurally distinct odorants used as an assay control demonstrating the robustness of this assay. Pentanol and butanol are structurally similar odorants with a single carbon difference in chemical structure. S/R-limonene and S/R-carvone are enantiomer odorants. Using the sand-buried food assay as previously described (Zou et al., 2012); we found no differences between controls and ERK5 icKO mice in their ability to learn to discriminate between structurally distinct, structurally similar, or enantiomer odorants to retrieve a food reward (Fig. 7.17A–D).

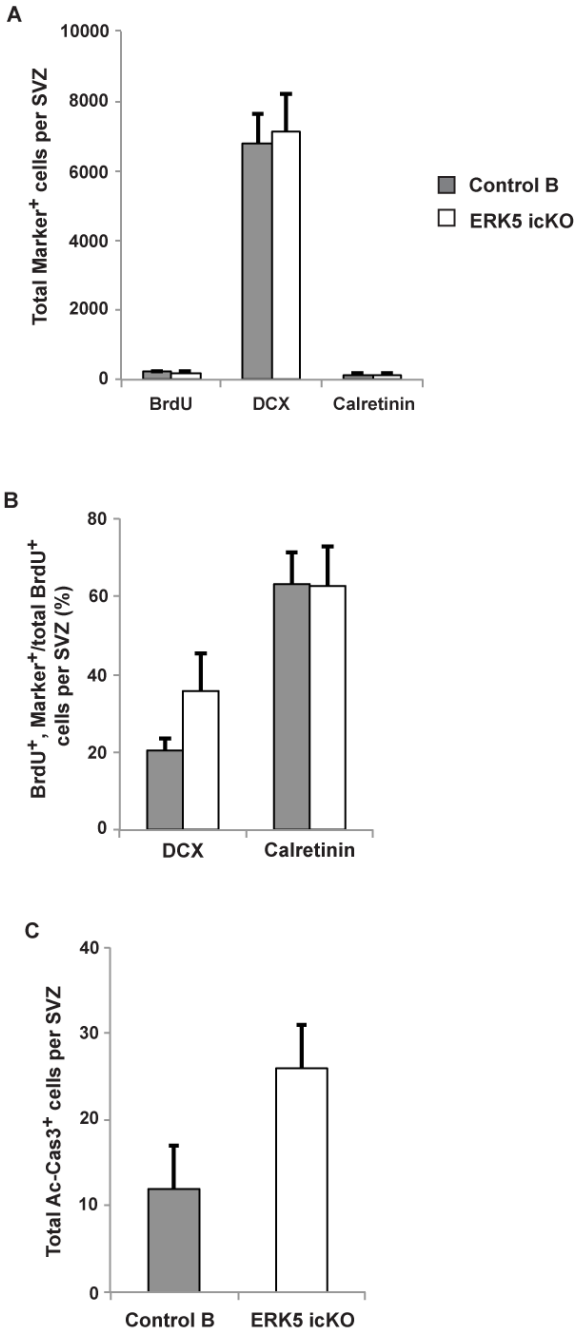


Figure 7.14. Increased overall apoptosis following *erk5* deletion in the SVZ. Mice were dosed with tamoxifen to induce cre-mediated recombination of the *erk5* gene and brains processed by IHC 4 weeks after BrdU administration. A) Quantification of total $BrdU^+$, DCX^+, and $calretinin^+$ cells along the SVZ. B) Quantification of total BrdU, marker double positive cells among total $BrdU^+$ cells along the SVZ. C) Quantification of total activated caspase 3 $(Ac\text{-}Cas3)^+$ cells along the SVZ.

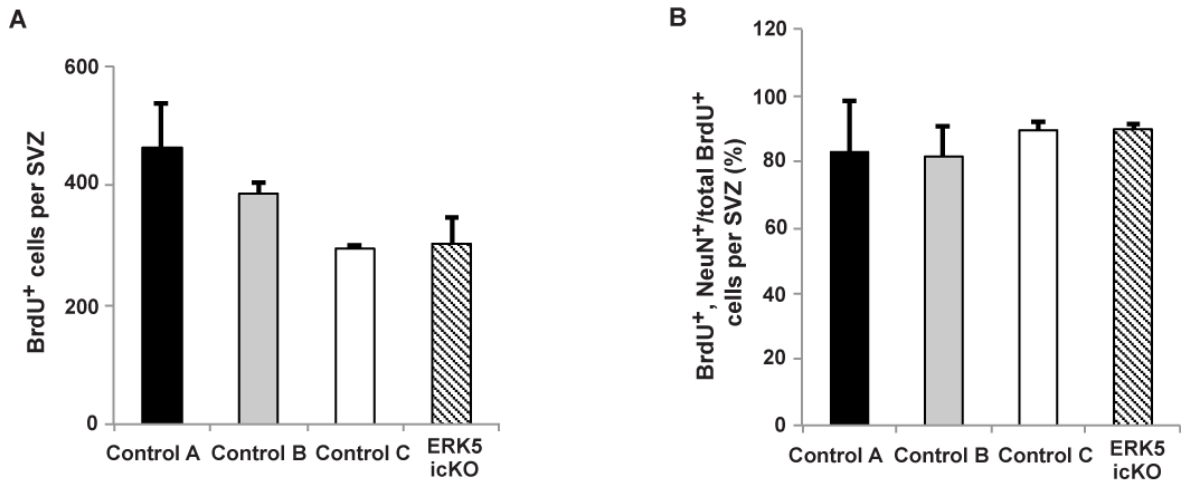


Figure 7.15. ERK5 icKO mice do not display any differences in total BrdU$^+$ or BrdU, NeuN double-positive cells 6 weeks following BrdU administration along the SVZ. A) Quantification of total BrdU$^+$ cells along the SVZ. B) Quantification of total BrdU, NeuN double positive cells among total BrdU$^+$ cells along the SVZ.

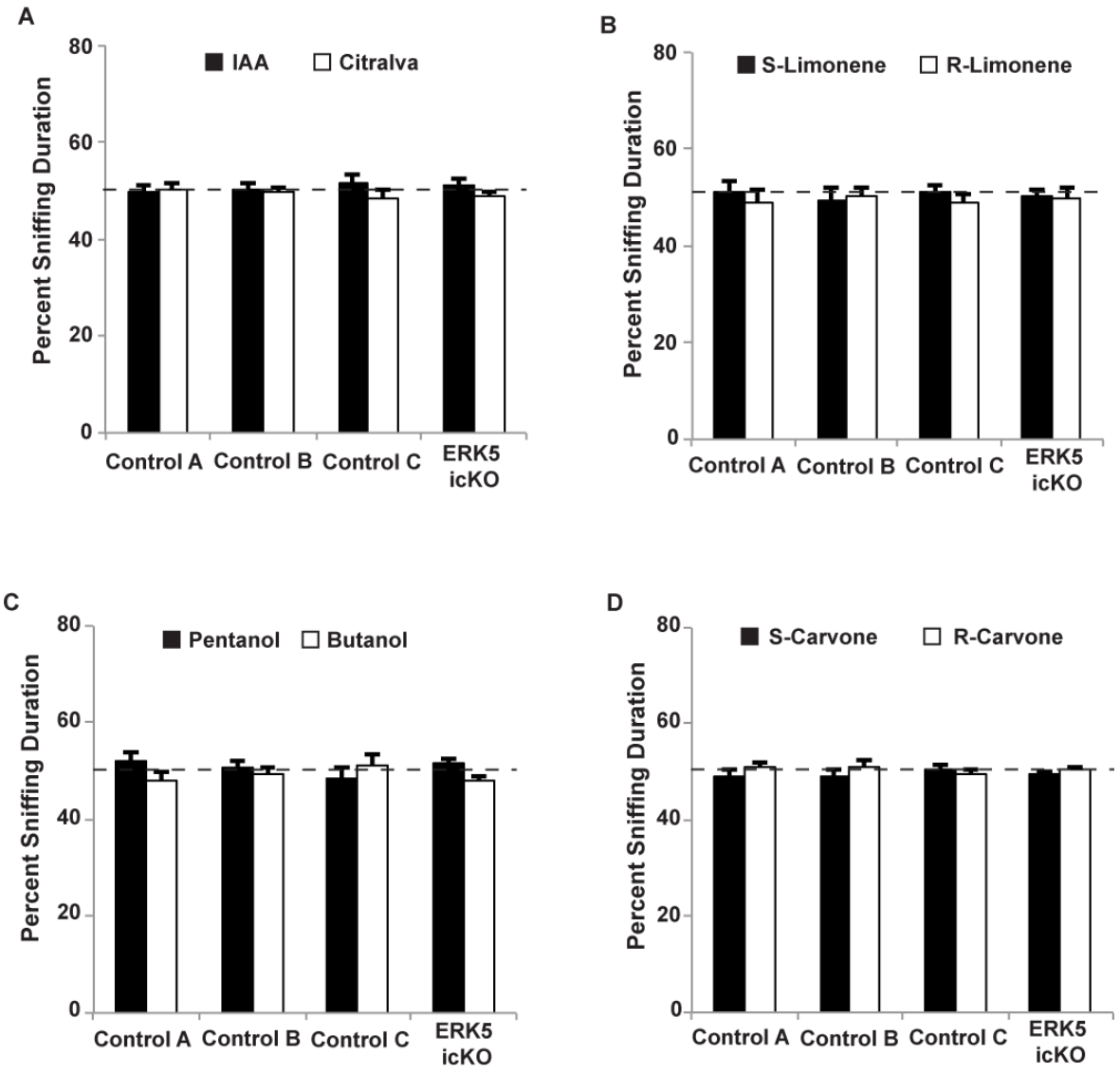


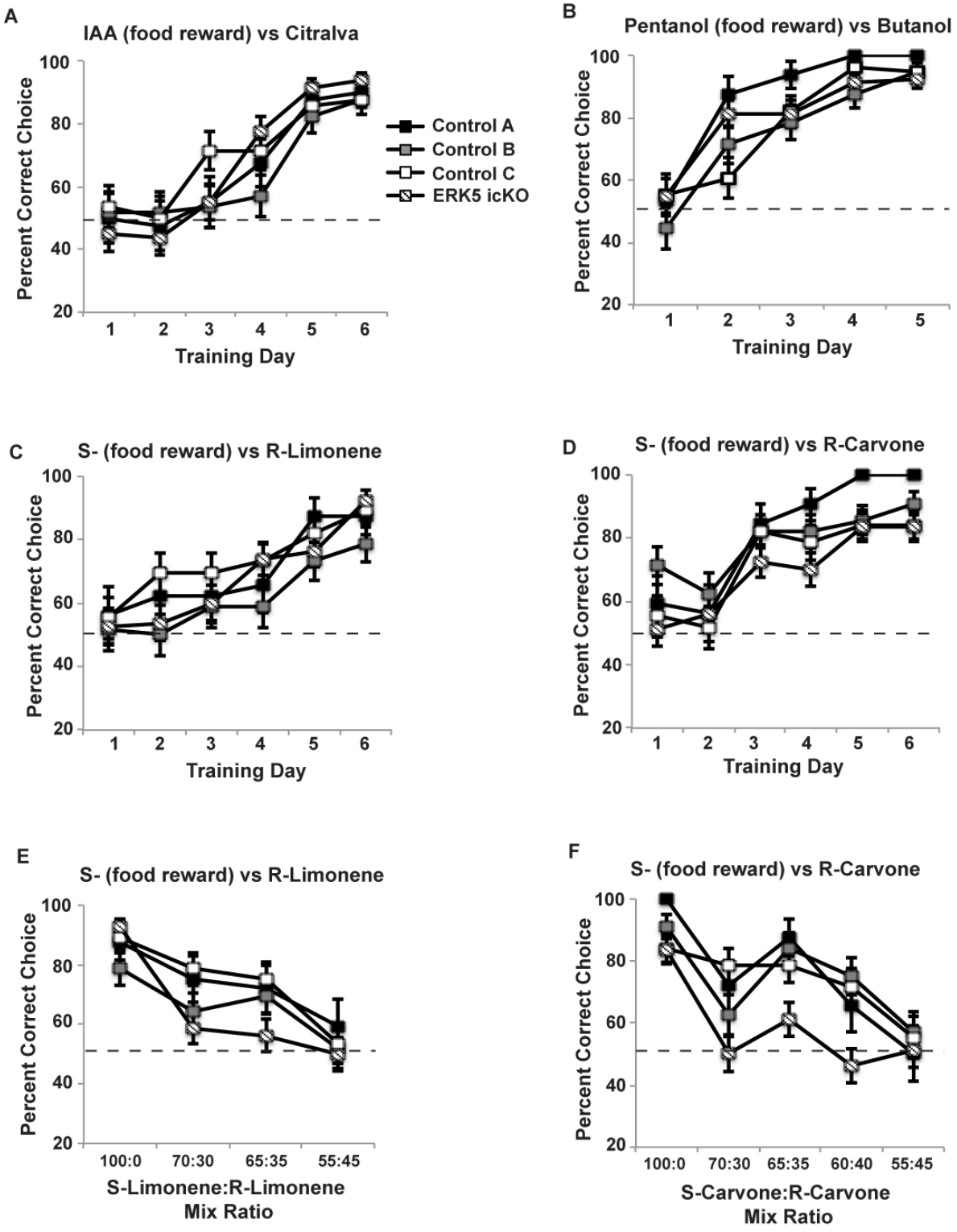
Figure 7.16. No odorant preferences exist between structurally distinct, structurally similar, or enantiomer odorant pairs. Quantification of total percent time spent sniffing cotton swabs laced with A) citralva or IAA, B) S-limonene or R-limonene, C) pentanol or butanol, D) S-carvone or R-carvone.

To explore this phenotype further, we made this assay more challenging by mixing enantiomer odorants at different ratios and found that ERK5 icKO mice, in general, had a more difficult time discriminating between the food reward-associated odorant mixture and the non-associated mixture (Fig. 7.17E, F). This was particularly true when odorants were presented at 70:30, 65:35, and 60:40 mix ratios. Both controls and ERK5 icKO mice were not able to distinguish between odorant mixtures at a 55:45 ratio.

Male mice prefer senior and juvenile female mouse urine to normal adult female urine

Adult olfactory bulb neurogenesis has been implicated to play a possible role in mating behavior (Shingo et al., 2003; Mak et al., 2007; Larsen et al., 2008). We examined this possibility by performing the olfactory preference assay using urine collected from group house female mice. Senior females were designated as 1.5 years or older, adult females were designated as 6 months old, and juvenile females were designated as 3-4 weeks old. All ages are relative to time of urine collection. Urine were collected from group housed animals, pooled and stored at -80°C until needed. Different cohorts of senior, adult, and juvenile female mouse urine were used to minimize habituation to any one cohort of urine. Mice were presented with 2 cotton swabs, each laced with 10 µL of undiluted female mouse urine. Preference for one age of female mouse urine to the other was quantified and expressed as the total percent of sniffing duration. All mice preferred senior female urine over adult female urine, juvenile female urine over senior female urine, and juvenile female urine over adult female urine (Fig. 7.18). These results suggest that mice typically do not care for sexually mature adult female mouse urine; however, the results are preliminary. This opens an avenue for exploring why male mice are more interested in senior and juvenile female mouse urine. One possibility is that protein components of mouse urine could differ with age (Pawluski et al., 2009); and that the preference of senior and juvenile female mouse urine over adult mouse urine is more a result of diverse protein components rather than a preference for non-sexually mature adult female mice.

Figure 7.17. ERK5 icKO mice are unable to discriminate between enantiomer mixtures of odorants. A) ERK5 icKO mice were able to discriminate between structurally distinct odorants IAA and citralva as well as control mice. B) ERK5 icKO mice were also able to discriminate between structurally similar odorants pentanol and butanol. Further testing their ability to discriminate between enantiomers of odorants, ERK5 icKO mice were presented with pairs of odorants S/R-limonene and S/R-carvone. D, E) All mice were able to discriminate between the enantiomer odorants. F) When mixtures of enantiomers were presented to ERK5 icKO mice, a significant deficit in the ability to discriminate between the S/R-limonene mixtures was observed at 70:30 and 65:35 ratios. G) ERK5 icKO mice were not able to discriminate between enantiomer mixture S/R-carvone at ratios of 70:30, 65:35, nor 60:40. Dashed lines indicate chance.


A
IAA (food reward) vs Citralva
Percent Correct Choice
Training Day
Control A
Control B
Control C
ERK5 icKO
B
Pentanol (food reward) vs Butanol
C
S- (food reward) vs R-Limonene
D
S- (food reward) vs R-Carvone
E
S- (food reward) vs R-Limonene
100:0
70:30
65:35
55:45
S-Limonene:R-Limonene
Mix Ratio
F
S- (food reward) vs R-Carvone
60:40
S-Carvone:R-Carvone
Mix Ratio

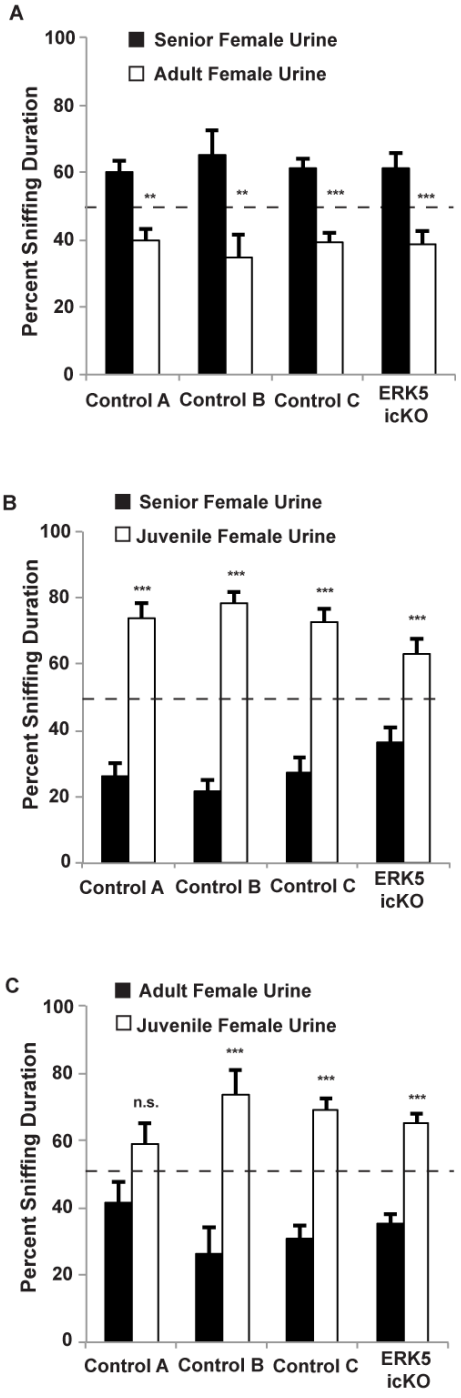


Figure 7.18. All mice prefer senior and juvenile female mouse urine over normal adult female mouse urine. Quantification of total percent sniffing duration of A) senior and adult female mouse urine, B) senior and juvenile female mouse urine, C) adult and juvenile female mouse urine. Dashed lines indicate chance.

Male ERK5 icKO mice are similar to control mice in social behaviors

Since all male animals were able to distinguish between distinct types of female mouse urine, we additionally wanted to assess whether ERK5 icKO mice displayed any overt social behavior deficits. First, we introduced artificially induced pro-estrus ovariectomized female mice to single-caged male mice and quantified the latency to first mount, total duration of mounting and total number of mounts during a 30 min testing session (Fig. 7.19A–C). No differences were observed between control animals and ERK5 icKO mice in sexual behavior. Following this assay, we performed the male aggression assay by introducing normal male mice to our single-caged experimental animals. By permitting free activity during the 15 min testing session, we quantified the latency to first attack, total duration of attack and total number of attacks (Fig. 7.19D–F). Similar aggression behavior was observed for all control animals and ERK5 icKO mice. Together, these data suggest no overt social behavior deficits in ERK5 icKO mice.

The main olfactory epithelium is not responsible for many of the observed olfactory deficits in ERK5 icKO mice

The possibility exists that the olfactory phenotypes we have discovered are not solely due to olfactory bulb neurogenesis and may be to due alterations in other olfactory sensory organs such as the olfactory epithelium. To determine if the olfactory threshold deficit observed in ERK5 icKO mice was due to effects in the olfactory epithelium, we performed the electroolfactogram (EOG) assay. We used a vehicle control to determine if a pressure detection deficit was apparent as well as different concentrations of structurally distinct odorants (Fig. 7.20). These results suggest that no clear differences exist between control and ERK5 icKO mice in their olfactory epithelium sensitivity to the odorants tested. It remains a possibility that the olfactory epithelium does indeed play a role in some of the olfactory behavioral deficits observed, however, that could be another avenue for future research.

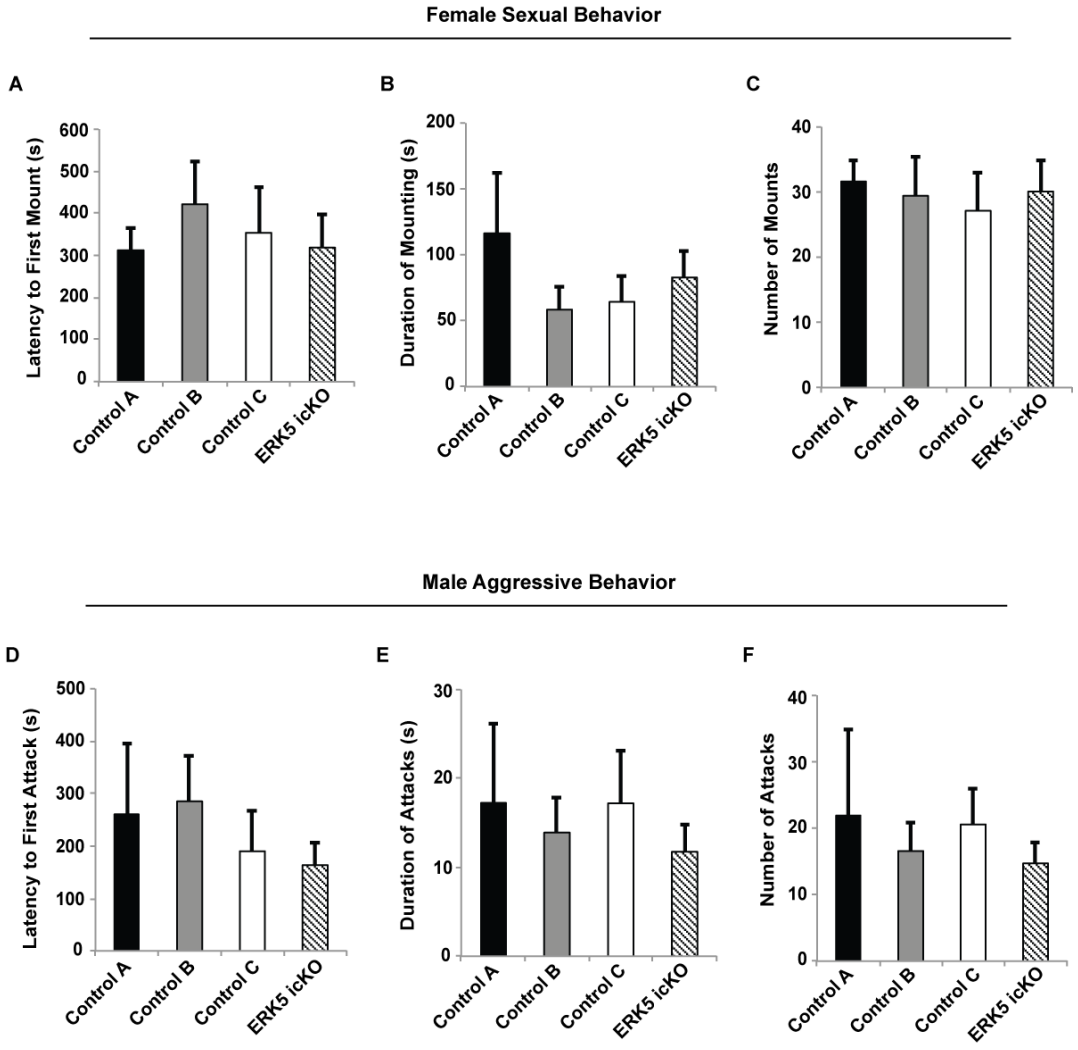


Figure 7.19. ERK5 icKO mice are not deficient in social behaviors. Male-female sexual behavior assay was performed and sexual behavior was quantified. A) Latency to first mount. B) Total duration of mounting. C) Total number of mounts during the assay. Male-male aggression behavior assay. A) Quantification of latency to first attack from resident male mice. B) Total duration of attack. C) Total number of attacks.

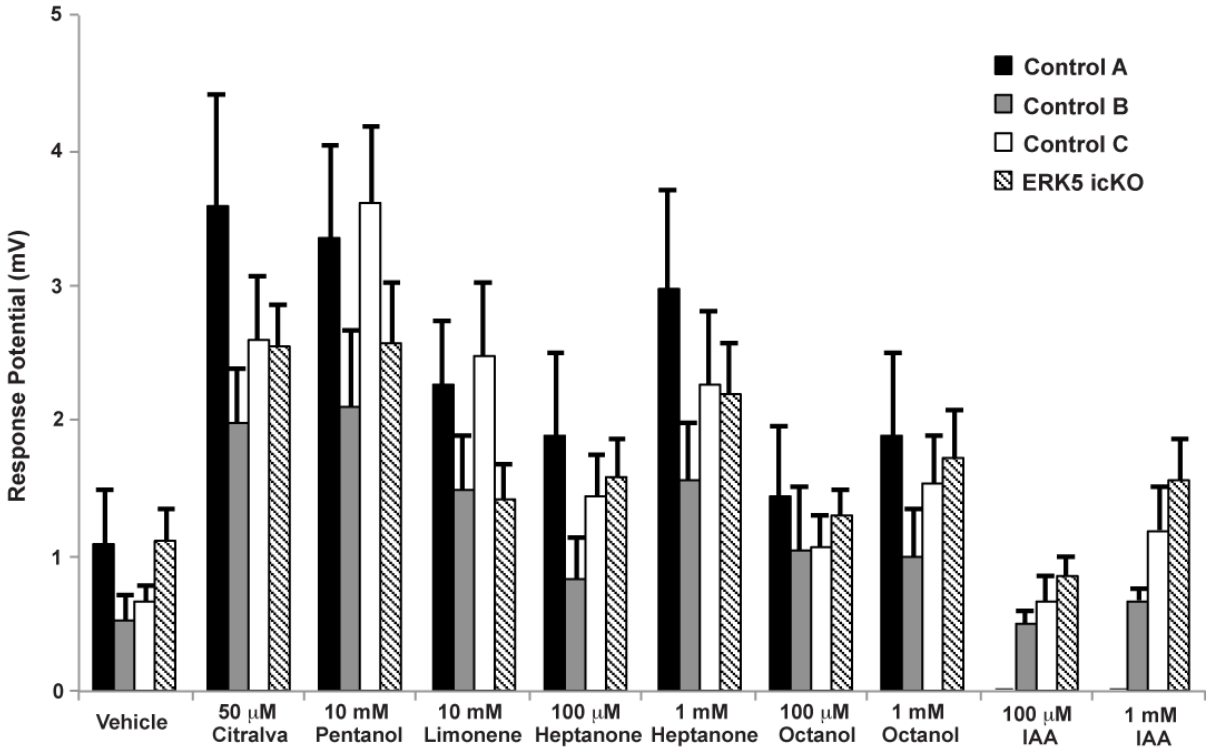


Figure 7.20. No deficits are observed in olfactory epithelium response to different odorants in ERK5 icKO mice. Measurement of response potential of the main olfactory epithelium using the electroolfactogram assay. Different concentrations of structurally distinct odorants were used and no differences were observed between control and ERK5 icKO mice.

DISCUSSION

Throughout this chapter, the data presented are preliminary and results are inconclusive without additional research. The goal of this chapter was to highlight various potential avenues to pursue to further explore the role of ERK5 in regulating adult neurogenesis in both the SGZ and the SVZ. We have developed tools to further explore the cellular and molecular outcome of deleting *erk5* in aNPCs by using Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$/R26-YFP$^{loxP/loxP}$ animals. Additionally, many behavior assays were performed and provide intriguing insights as to explore whether adult neurogenesis may play roles in regulating behaviors other than hippocampus-dependent learning and memory or olfaction.

Chapter 8

Conclusions and Future Directions

The field of adult neurogenesis is still being shaped as more discoveries continue to be reported. From efforts toward characterizing the molecular and cellular signature of adult neural stem cells to determining a functional role of adult-born neurons and everything in between, the days where scientists believed that the adult brain was stagnant and incapable of generating new neurons is now a part of history. It took many years since the initial reporting by Altman et al. (Altman and Das, 1965) for the field to accept the possibility that the adult brain is capable of generating new brain cells in the dentate gyrus. In fact, it took convincing research in songbirds (Alvarez-Buylla et al., 1988) many years later before the idea of adult neurogenesis was even considered again. As this field continues to grow and we discover new regulatory mechanisms while continuing to define functions of adult-born neurons, the possibility to use adult-generated brain cells for neurodegenerative diseases remains a distant yet achievable goal.

My contribution in the field of adult neurogenesis stemmed from the belief that extracellular signals are able to regulate the proliferation and/or differentiation of aNPCs; and from this foundation, I studied the role of ERK5 MAPK in the regulation of adult neurogenesis. We can now firmly conclude that ERK5 is a protein that is discretely and specifically expressed along both neurogenic regions and plays a regulatory role in the progression of aNPC differentiation into functional, adult-born neurons. We have mapped out a linear pathway by which ERK5 plays a role in regulating the differentiation of aNPCs *in vitro* by way of NT3 activation of ERK5 thereby exacting a downstream effect on a transcription factor, Neurog2. Although we were able determine the contribution of ERK5 signaling by NT3 through Neurog2, cellular signals are not linear in nature since other activators and effectors of ERK5 exist. To fully define ERK5 as a targetable protein permitting increased adult neurogenesis, we would

need to fully elucidate other pathways feeding into and targets of the ERK5 MAPK signaling pathway.

It is now clear that adult-born neurons play a functional role in hippocampus-dependent learning and memory as well as several olfactory behaviors. Results presented throughout this dissertation support that notion and also posit ERK5 as a novel regulatory protein necessary for maintaining physiological levels of adult neurogenesis. Indeed, we report that adult animals lacking ERK5 as a direct result of tamoxifen-induced *erk5* gene deletion have several behavioral deficits including: longer term memory for novel objects, contextual fear extinction, contextual fear acquisition using more a challenging training paradigm, spatial learning and memory, remote memory, olfactory detection threshold to both odorants and pheromones, olfactory memory, olfactory discrimination, and olfactory-cued associative learning. The behavioral consequences found in ERK5 icKO mice unearth a wealth of clues into the functional impact of adult-born neurons, but also leaves many doors open as additional avenues for further research.

In the future, it would be helpful in our understanding of how ERK5 regulates adult neurogenesis to fully identify other upstream activators and downstream effectors of the ERK5 signaling pathway. Established *in vitro* culture conditions for aNPCs and the use of shERK5 retroviral vectors permit further research along this path. We have also generated the Nestin-CreER^{TM}/ERK5$^{loxP/loxP}$/R26-YFP$^{loxP/loxP}$ reporter mouse, which now provides a powerful tool for further downstream investigation to clarify the cellular consequence(s) of *erk5* deletion in aNPCs *in vivo*. And lastly, the ERK5 icKO mice still provide their lives for our continued research into other functional roles of adult-born neurons. We are interested in possible links of adult neurogenesis to anxiety and depression, mating behavior, and basal levels of interest and/or motivation to explore novel subjects.

References

Achiraman S, Archunan G, Sankarganesh D, Rajagopal T, Rengarajan RL, Kokilavani P, Kamalakkannan S, Kannan S (2011) Biochemical analysis of female mice urine with reference to endocrine function: a key tool for estrus detection. Zoolog Sci 28:600-605.

Agasse F, Bernardino L, Kristiansen H, Christiansen SH, Ferreira R, Silva B, Grade S, Woldbye DP, Malva JO (2008) Neuropeptide Y promotes neurogenesis in murine subventricular zone. Stem Cells 26:1636-1645.

Aimone JB, Deng W, Gage FH (2011) Resolving new memories: a critical look at the dentate gyrus, adult neurogenesis, and pattern separation. Neuron 70:589-596.

Akers KG, Sakaguchi M, Arruda-Carvalho M (2010) Functional contribution of adult-generated olfactory bulb interneurons: odor discrimination versus odor memory. J Neurosci 30:4523-4525.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Alvarez-Buylla A, Lois C (1995) Neuronal stem cells in the brain of adult vertebrates. Stem Cells 13:263-272.

Alvarez-Buylla A, Garcia-Verdugo JM (2002) Neurogenesis in adult subventricular zone. J Neurosci 22:629-634.

Alvarez-Buylla A, Theelen M, Nottebohm F (1988) Birth of projection neurons in the higher vocal center of the canary forebrain before, during, and after song learning. Proc Natl Acad Sci U S A 85:8722-8726.

Amiri A, Cho W, Zhou J, Birnbaum SG, Sinton CM, McKay RM, Parada LF (2012) Pten deletion in adult hippocampal neural stem/progenitor cells causes cellular abnormalities and alters neurogenesis. J Neurosci 32:5880-5890.

Anagnostaras SG, Maren S, Fanselow MS (1999) Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. J Neurosci 19:1106-1114.

Aonurm-Helm A, Jurgenson M, Zharkovsky T, Sonn K, Berezin V, Bock E, Zharkovsky A (2008) Depression-like behaviour in neural cell adhesion molecule (NCAM)-deficient mice and its reversal by an NCAM-derived peptide, FGL. Eur J Neurosci 28:1618-1628.

Arenkiel BR (2010) Adult neurogenesis supports short-term olfactory memory. J Neurophysiol 103:2935-2937.

Arruda-Carvalho M, Sakaguchi M, Akers KG, Josselyn SA, Frankland PW (2011) Posttraining ablation of adult-generated neurons degrades previously acquired memories. J Neurosci 31:15113-15127.

Athos J, Storm DR (2001) High precision stereotaxic surgery in mice. In: Current protocols in neuroscience.

Athos J, Impey S, Pineda VV, Chen X, Storm DR (2002) Hippocampal CRE-mediated gene expression is required for contextual memory formation. Nat Neurosci 5:1119-1120.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. Nature Neurosci 1:602-609.

Balordi F, Fishell G (2007a) Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. J Neurosci 27:5936-5947.

Balordi F, Fishell G (2007b) Mosaic removal of hedgehog signaling in the adult SVZ reveals that the residual wild-type stem cells have a limited capacity for self-renewal. J Neurosci 27:14248-14259.

Balu DT, Lucki I (2009) Adult hippocampal neurogenesis: regulation, functional implications, and contribution to disease pathology. Neurosci Biobehav Rev 33:232-252.

Bardy C, Pallotto M (2010) What Happens to Olfaction without Adult Neurogenesis? Front Neurosci 4:24.

Belvindrah R, Lazarini F, Lledo PM (2009) Postnatal neurogenesis: from neuroblast migration to neuronal integration. Rev Neurosci 20:331-346.

Bergami M, Rimondini R, Santi S, Blum R, Gotz M, Canossa M (2008) Deletion of TrkB in adult progenitors alters newborn neuron integration into hippocampal circuits and increases anxiety-like behavior. Proc Natl Acad Sci U S A 105:15570-15575.

Breton-Provencher V, Saghatelyan A (2012) Newborn neurons in the adult olfactory bulb: unique properties for specific odor behavior. Behav Brain Res 227:480-489.

Breton-Provencher V, Lemasson M, Peralta MR, 3rd, Saghatelyan A (2009) Interneurons produced in adulthood are required for the normal functioning of the olfactory bulb network and for the execution of selected olfactory behaviors. J Neurosci 29:15245-15257.

Breunig JJ, Silbereis J, Vaccarino FM, Sestan N, Rakic P (2007) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. Proc Natl Acad Sci U S A 104:20558-20563.

Breunig JJ, Sarkisian MR, Arellano JI, Morozov YM, Ayoub AE, Sojitra S, Wang B, Flavell RA, Rakic P, Town T (2008) Primary cilia regulate hippocampal neurogenesis by mediating sonic hedgehog signaling. Proc Natl Acad Sci U S A 105:13127-13132.

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. Eur J Neurosci 17:2042-2046.

Bruel-Jungerman E, Veyrac A, Dufour F, Horwood J, Laroche S, Davis S (2009) Inhibition of PI3K-Akt signaling blocks exercise-mediated enhancement of adult neurogenesis and synaptic plasticity in the dentate gyrus. PLoS One 4:e7901.

Bull ND, Bartlett PF (2005) The adult mouse hippocampal progenitor is neurogenic but not a stem cell. J Neurosci 25:10815-10821.

Buron G, Hacquemand R, Pourie G, Lucarz A, Jacquot L, Brand G (2007) Comparative behavioral effects between synthetic 2,4,5-trimethylthiazoline (TMT) and the odor of natural fox (Vulpes vulpes) feces in mice. Behav Neurosci 121:1063-1072.

Carlen M, Cassidy RM, Brismar H, Smith GA, Enquist LW, Frisen J (2002) Functional integration of adult-born neurons. Curr Biol 12:606-608.

Carleton A, Rochefort C, Morante-Oria J, Desmaisons D, Vincent JD, Gheusi G, Lledo PM (2002) Making scents of olfactory neurogenesis. J Physiol Paris 96:115-122.

Cavanaugh JE, Ham J, Hetman M, Poser S, Yan C, Xia Z (2001) Differential regulation of mitogen-activated protein kinases ERK1/2 and ERK5 by neurotrophins, neuronal activity, and cAMP in neurons. J Neurosci 21:434-443.

Chan JP, Cordeira J, Calderon GA, Iyer LK, Rios M (2008) Depletion of central BDNF in mice impedes terminal differentiation of new granule neurons in the adult hippocampus. Mol Cell Neurosci 39:372-383.

Clelland CD, Choi M, Romberg C, Clemenson GD, Jr., Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ (2009) A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325:210-213.

Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ (2010) Running enhances spatial pattern separation in mice. Proc Natl Acad Sci U S A 107:2367-2372.

Crews L, Mizuno H, Desplats P, Rockenstein E, Adame A, Patrick C, Winner B, Winkler J, Masliah E (2008) Alpha-synuclein alters Notch-1 expression and neurogenesis in mouse embryonic stem cells and in the hippocampus of transgenic mice. J Neurosci 28:4250-4260.

Cude K, Wang Y, Choi HJ, Hsuan SL, Zhang H, Wang CY, Xia Z (2007) Regulation of the G2-M cell cycle progression by the ERK5-NF{kappa}B signaling pathway. J Cell Biol 177:253-264.

Cundiff P, Liu L, Wang Y, Zou J, Pan YW, Abel G, Duan X, Ming GL, Englund C, Hevner R, Xia Z (2009) ERK5 MAP kinase regulates Neurogenin1 during cortical neurogenesis. PLoS ONE 4:e5204.

Debiec J, LeDoux JE, Nader K (2002) Cellular and systems reconsolidation in the hippocampus. Neuron 36:527-538.

Deng W, Aimone JB, Gage FH (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11:339-350.

Deng W, Saxe MD, Gallina IS, Gage FH (2009) Adult-born hippocampal dentate granule cells undergoing maturation modulate learning and memory in the brain. J Neurosci 29:13532-13542.

Denis-Donini S, Dellarole A, Crociara P, Francese MT, Bortolotto V, Quadrato G, Canonico PL, Orsetti M, Ghi P, Memo M, Bonini SA, Ferrari-Toninelli G, Grilli M (2008) Impaired adult neurogenesis associated with short-term memory defects in NF-kappaB p50-deficient mice. J Neurosci 28:3911-3919.

Di Benedetto B, Hitz C, Holter SM, Kuhn R, Vogt Weisenhorn DM, Wurst W (2007) Differential mRNA distribution of components of the ERK/MAPK signalling cascade in the adult mouse brain. J Comp Neurol 500:542-556.

Dobrossy MD, Drapeau E, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2003) Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. Mol Psychiatry 8:974-982.

Doetsch F, Hen R (2005) Young and excitable: the function of new neurons in the adult mammalian brain. Curr Opin Neurobiol 15:121-128.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703-716.

Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y, Liu XB, Yang CH, Jordan JD, Ma DK, Liu CY, Ganesan S, Cheng HJ, Ming GL, Lu B, Song H (2007) Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. Cell 130:1146-1158.

Duman RS, Monteggia LM (2006) A neurotrophic model for stress-related mood disorders. Biol Psychiatry 59:1116-1127.

Dupret D, Revest JM, Koehl M, Ichas F, De Giorgi F, Costet P, Abrous DN, Piazza PV (2008) Spatial relational memory requires hippocampal adult neurogenesis. PLoS One 3:e1959.

Dupret D, Fabre A, Dobrossy MD, Panatier A, Rodriguez JJ, Lamarque S, Lemaire V, Oliet SH, Piazza PV, Abrous DN (2007) Spatial learning depends on both the addition and removal of new hippocampal neurons. PLoS Biol 5:e214.

Ekdahl CT, Kokaia Z, Lindvall O (2009) Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience 158:1021-1029.

English JM, Vanderbilt CA, Xu S, Marcus S, Cobb MH (1995) Isolation of MEK5 and differential expression of alternatively spliced forms. J Biol Chem 270:28897-28902.

Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247-251.

Epp JR, Spritzer MD, Galea LA (2007) Hippocampus-dependent learning promotes survival of new neurons in the dentate gyrus at a specific time during cell maturation. Neuroscience 149:273-285.

Erickson KI, Voss MW, Prakash RS, Basak C, Szabo A, Chaddock L, Kim JS, Heo S, Alves H, White SM, Wojcicki TR, Mailey E, Vieira VJ, Martin SA, Pence BD, Woods JA, McAuley E, Kramer AF (2011) Exercise training increases size of hippocampus and improves memory. Proc Natl Acad Sci U S A 108:3017-3022.

Faherty CJ, Raviie Shepherd K, Herasimtschuk A, Smeyne RJ (2005) Environmental enrichment in adulthood eliminates neuronal death in experimental Parkinsonism. Brain Res Mol Brain Res 134:170-179.

Farioli-Vecchioli S, Saraulli D, Costanzi M, Pacioni S, Cina I, Aceti M, Micheli L, Bacci A, Cestari V, Tirone F (2008) The timing of differentiation of adult hippocampal neurons is crucial for spatial memory. PLoS Biol 6:e246.

Fendt M, Endres T, Lowry CA, Apfelbach R, McGregor IS (2005) TMT-induced autonomic and behavioral changes and the neural basis of its processing. Neurosci Biobehav Rev 29:1145-1156.

Finegan KG, Wang X, Lee EJ, Robinson AC, Tournier C (2009) Regulation of neuronal survival by the extracellular signal-regulated protein kinase 5. Cell Death Differ 16:674-683.

Fischer A, Radulovic M, Schrick C, Sananbenesi F, Godovac-Zimmermann J, Radulovic J (2007) Hippocampal Mek/Erk signaling mediates extinction of contextual freezing behavior. Neurobiol Learn Mem 87:149-158.

Frankland PW, Bontempi B (2005) The organization of recent and remote memories. Nat Rev Neurosci 6:119-130.

Frankland PW, Ding HK, Takahashi E, Suzuki A, Kida S, Silva AJ (2006) Stability of recent and remote contextual fear memory. Learn Mem 13:451-457.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Gage FH, Kempermann G, Palmer TD, Peterson DA, Ray J (1998) Multipotent progenitor cells in the adult dentate gyrus. J Neurobiol 36:249-266.

Gale GD, Anagnostaras SG, Godsil BP, Mitchell S, Nozawa T, Sage JR, Wiltgen B, Fanselow MS (2004) Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. J Neurosci 24:3810-3815.

Galichet C, Guillemot F, Parras CM (2008) Neurogenin 2 has an essential role in development of the dentate gyrus. Development 135:2031-2041.

Gao Z, Ure K, Ables JL, Lagace DC, Nave KA, Goebbels S, Eisch AJ, Hsieh J (2009) Neurod1 is essential for the survival and maturation of adult-born neurons. Nat Neurosci 12:1090-1092.

Gao Z, Ure K, Ding P, Nashaat M, Yuan L, Ma J, Hammer RE, Hsieh J (2011) The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells. J Neurosci 31:9772-9786.

Garelick MG, Chan GC, DiRocco DP, Storm DR (2009) Overexpression of type I adenylyl cyclase in the forebrain impairs spatial memory in aged but not young mice. J Neurosci 29:10835-10842.

Garthe A, Behr J, Kempermann G (2009) Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. PLoS One 4:e5464.

Ge S, Yang CH, Hsu KS, Ming GL, Song H (2007) A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron 54:559-566.

Gheusi G, Cremer H, McLean H, Chazal G, Vincent JD, Lledo PM (2000) Importance of newly generated neurons in the adult olfactory bulb for odor discrimination. Proc Natl Acad Sci U S A 97:1823-1828.

Girio A, Montero JC, Pandiella A, Chatterjee S (2007) Erk5 is activated and acts as a survival factor in mitosis. Cell Signal 19:1964-1972.

Goshen I, Brodsky M, Prakash R, Wallace J, Gradinaru V, Ramakrishnan C, Deisseroth K (2011) Dynamics of retrieval strategies for remote memories. Cell 147:678-689.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260-265.

Guo W, Allan AM, Zong R, Zhang L, Johnson EB, Schaller EG, Murthy AC, Goggin SL, Eisch AJ, Oostra BA, Nelson DL, Jin P, Zhao X (2011) Ablation of Fmrp in adult neural stem cells disrupts hippocampus-dependent learning. Nat Med 17:559-565.

Hacquemand R, Pourie G, Jacquot L, Brand G Postnatal exposure to synthetic predator odor (TMT) induces quantitative modification in fear-related behaviors during adulthood without change in corticosterone levels. Behav Brain Res 215:58-62.

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat Neurosci 11:277-284.

Hawkes C (2006) Olfaction in neurodegenerative disorder. Adv Otorhinolaryngol 63:133-151.

Hayashi M, Lee JD (2004) Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. J Mol Med 82:800-808.

Hayashi M, Kim SW, Imanaka-Yoshida K, Yoshida T, Abel ED, Eliceiri B, Yang Y, Ulevitch RJ, Lee JD (2004) Targeted deletion of BMK1/ERK5 in adult mice perturbs vascular integrity and leads to endothelial failure. J Clin Invest 113:1138-1148.

Hodge RD, Kowalczyk TD, Wolf SA, Encinas JM, Rippey C, Enikolopov G, Kempermann G, Hevner RF (2008) Intermediate progenitors in adult hippocampal neurogenesis: Tbr2 expression and coordinate regulation of neuronal output. J Neurosci 28:3707-3717.

Hsieh J, Eisch AJ (2010) Epigenetics, hippocampal neurogenesis, and neuropsychiatric disorders: Unraveling the genome to understand the mind. Neurobiol Dis:Epub ahead of print.

Hsieh J, Nakashima K, Kuwabara T, Mejia E, Gage FH (2004) Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. Proc Natl Acad Sci U S A 101:16659-16664.

Imayoshi I, Sakamoto M, Ohtsuka T, Kageyama R (2009) Continuous neurogenesis in the adult brain. Dev Growth Differ 51:379-386.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153-1161.

Jaholkowski P, Kiryk A, Jedynak P, Ben Abdallah NM, Knapska E, Kowalczyk A, Piechal A, Blecharz-Klin K, Figiel I, Lioudyno V, Widy-Tyszkiewicz E, Wilczynski GM, Lipp HP, Kaczmarek L, Filipkowski RK (2009) New hippocampal neurons are not obligatory for memory formation; cyclin D2 knockout mice with no adult brain neurogenesis show learning. Learn Mem 16:439-451.

Jessberger S, Clark RE, Broadbent NJ, Clemenson GD, Jr., Consiglio A, Lie DC, Squire LR, Gage FH (2009) Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. Learn Mem 16:147-154.

Jiao J, Chen DF (2008) Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem Cells 26:1221-1230.

Jonasson Z, Ballantyne JK, Baxter MG (2004) Preserved anterograde and retrograde memory of rapidly acquired olfactory discrminations after neurotoxic hippocampal lesions. Hippocampus 14:28-39.

Kageyama R, Imayoshi I, Sakamoto M (2012) The role of neurogenesis in olfaction-dependent behaviors. Behav Brain Res 227:459-463.

Kasler HG, Victoria J, Duramad O, Winoto A (2000) ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Mol Cell Biol 20:8382-8389.

Kato Y, Kravchenko VV, Tapping RI, Han JH, Ulevitch RJ, Lee JD (1997) BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. EMBO J 16:7054-7066.

Kaut KP, Bunsey MD (2001) The effects of lesions to the rat hippocampus or rhinal cortex on olfactory and spatial memory: retrograde and anterograde findings. Cogn Affect Behav Neurosci 1:270-286.

Kaut KP, Bunsey MD, Riccio DC (2003) Olfactory learning and memory impairments following lesions to the hippocampus and perirhinal-entorhinal cortex. Behav Neurosci 117:304-319.

Ke Y, Zhang EE, Hagihara K, Wu D, Pang Y, Klein R, Curran T, Ranscht B, Feng GS (2007) Deletion of Shp2 in the brain leads to defective proliferation and differentiation in neural stem cells and early postnatal lethality. Mol Cell Biol 27:6706-6717.

Kee N, Teixeira CM, Wang AH, Frankland PW (2007) Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. Nat Neurosci 10:355-362.

Kempermann G, Kuhn HG, Gage FH (1997a) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493-495.

Kempermann G, Kuhn HG, Gage FH (1997b) Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc Natl Acad Sci U S A 94:10409-10414.

Kenney AM, Widlund HR, Rowitch DH (2004) Hedgehog and PI-3 kinase signaling converge on Nmyc1 to promote cell cycle progression in cerebellar neuronal precursors. Development 131:217-228.

Kim EJ, Leung CT, Reed RR, Johnson JE (2007) In vivo analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis. J Neurosci 27:12764-12774.

Kim JJ, Fanselow MS (1992) Modality-specific retrograde amnesia of fear. Science 256:675-677.

Kim JY, Duan X, Liu CY, Jang MH, Guo JU, Pow-anpongkul N, Kang E, Song H, Ming GL (2009) DISC1 regulates new neuron development in the adult brain via modulation of AKT-mTOR signaling through KIAA1212. Neuron 63:761-773.

Kitamura T, Saitoh Y, Takashima N, Murayama A, Niibori Y, Ageta H, Sekiguchi M, Sugiyama H, Inokuchi K (2009) Adult neurogenesis modulates the hippocampus-dependent period of associative fear memory. Cell 139:814-827.

Klempin F, Kempermann G (2007) Adult hippocampal neurogenesis and aging. Eur Arch Psychiatry Clin Neurosci 257:271-280.

Kobayakawa K, Kobayakawa R, Matsumoto H, Oka Y, Imai T, Ikawa M, Okabe M, Ikeda T, Itohara S, Kikusui T, Mori K, Sakano H (2007) Innate versus learned odour processing in the mouse olfactory bulb. Nature 450:503-508.

Komitova M, Eriksson PS (2004) Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain. Neurosci Lett 369:24-27.

Kronenberg G, Harms C, Sobol RW, Cardozo-Pelaez F, Linhart H, Winter B, Balkaya M, Gertz K, Gay SB, Cox D, Eckart S, Ahmadi M, Juckel G, Kempermann G, Hellweg R, Sohr R, Hortnagl H, Wilson SH, Jaenisch R, Endres M (2008) Folate deficiency induces neurodegeneration and brain dysfunction in mice lacking uracil DNA glycosylase. J Neurosci 28:7219-7230.

Kuo CT, Mirzadeh Z, Soriano-Navarro M, Rasin M, Wang D, Shen J, Sestan N, Garcia-Verdugo J, Alvarez-Buylla A, Jan LY, Jan YN (2006) Postnatal deletion of Numb/Numblike reveals repair and remodeling capacity in the subventricular neurogenic niche. Cell 127:1253-1264.

Kuwabara T, Hsieh J, Muotri A, Yeo G, Warashina M, Lie DC, Moore L, Nakashima K, Asashima M, Gage FH (2009) Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis. Nat Neurosci 12:1097-1105.

Larsen CM, Grattan DR (2010) Prolactin-induced mitogenesis in the subventricular zone of the maternal brain during early pregnancy is essential for normal postpartum behavioral responses in the mother. Endocrinology 151:3805-3814.

Larsen CM, Kokay IC, Grattan DR (2008) Male pheromones initiate prolactin-induced neurogenesis and advance maternal behavior in female mice. Horm Behav 53:509-517.

Lazarini F, Lledo PM (2011) Is adult neurogenesis essential for olfaction? Trends Neurosci 34:20-30.

Lazarini F, Mouthon MA, Gheusi G, de Chaumont F, Olivo-Marin JC, Lamarque S, Abrous DN, Boussin FD, Lledo PM (2009) Cellular and behavioral effects of cranial irradiation of the subventricular zone in adult mice. PLoS One 4:e7017.

Lee E, Son H (2009) Adult hippocampal neurogenesis and related neurotrophic factors. BMB Rep 42:239-244.

Lee J, Seroogy KB, Mattson MP (2002) Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice. J Neurochem 80:539-547.

Lee JD, Ulevitch RJ, Han J (1995) Primary structure of BMK1: a new mammalian map kinase. Biochem Biophys Res Commun 213:715-724.

Lehmann H, Lacanilao S, Sutherland RJ (2007) Complete or partial hippocampal damage produces equivalent retrograde amnesia for remote contextual fear memories. Eur J Neurosci 25:1278-1286.

Leinders-Zufall T, Lane AP, Puche AC, Ma W, Novotny MV, Shipley MT, Zufall F (2000) Ultrasensitive pheromone detection by mammalian vomeronasal neurons. Nature 405:792-796.

Leuner B, Gould E (2010) Structural plasticity and hippocampal function. Annu Rev Psychol 61:111-140, C111-113.

Leuner B, Mendolia-Loffredo S, Kozorovitskiy Y, Samburg D, Gould E, Shors TJ (2004) Learning enhances the survival of new neurons beyond the time when the hippocampus is required for memory. J Neurosci 24:7477-7481.

Li Y, Luikart BW, Birnbaum S, Chen J, Kwon CH, Kernie SG, Bassel-Duby R, Parada LF (2008) TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 59:399-412.

Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370-1375.

Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z (2003a) ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. Proc Natl Acad Sci USA 100:8532-8537.

Liu L, Cundiff P, Abel G, Wang Y, Faigle R, Sakagami H, Xu M, Xia Z (2006) Extracellular signal-regulated kinase (ERK) 5 is necessary and sufficient to specify cortical neuronal fate. Proc Natl Acad Sci U S A 103:9697-9702.

Liu S, Wang J, Zhu D, Fu Y, Lukowiak K, Lu YM (2003b) Generation of functional inhibitory neurons in the adult rat hippocampus. J Neurosci 23:732-736.

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179-193.

Ma DK, Ponnusamy K, Song MR, Ming GL, Song H (2009) Molecular genetic analysis of FGFR1 signalling reveals distinct roles of MAPK and PLCgamma1 activation for self-renewal of adult neural stem cells. Mol Brain 2:16.

Magavi SS, Mitchell BD, Szentirmai O, Carter BS, Macklis JD (2005) Adult-born and preexisting olfactory granule neurons undergo distinct experience-dependent modifications of their olfactory responses in vivo. J Neurosci 25:10729-10739.

Mak GK, Weiss S (2010) Paternal recognition of adult offspring mediated by newly generated CNS neurons. Nat Neurosci 13:753-758.

Mak GK, Enwere EK, Gregg C, Pakarainen T, Poutanen M, Huhtaniemi I, Weiss S (2007) Male pheromone-stimulated neurogenesis in the adult female brain: possible role in mating behavior. Nat Neurosci 10:1003-1011.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

McCormick M, Tamimi R, Snider L, Asakura A, Bergstrom D, Tapscott S (1996) NeuroD2 and neuroD3: distinct expression patterns and transcriptional activation potentials within the neuroD gene family. Mol Cell Biol 16:5792-5800.

Mechawar N, Saghatelyan A, Grailhe R, Scoriels L, Gheusi G, Gabellec MM, Lledo PM, Changeux JP (2004) Nicotinic receptors regulate the survival of newborn neurons in the adult olfactory bulb. Proc Natl Acad Sci U S A 101:9822-9826.

Merkle FT, Mirzadeh Z, Alvarez-Buylla A (2007) Mosaic organization of neural stem cells in the adult brain. Science 317:381-384.

Meshi D, Drew MR, Saxe M, Ansorge MS, David D, Santarelli L, Malapani C, Moore H, Hen R (2006) Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. Nat Neurosci 9:729-731.

Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-250.

Ming GL, Song H (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70:687-702.

Mirescu C, Peters JD, Noiman L, Gould E (2006) Sleep deprivation inhibits adult neurogenesis in the hippocampus by elevating glucocorticoids. Proc Natl Acad Sci U S A 103:19170-19175.

Mirzadeh Z, Merkle FT, Soriano-Navarro M, Garcia-Verdugo JM, Alvarez-Buylla A (2008) Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. Cell Stem Cell 3:265-278.

Morimoto H, Kondoh K, Nishimoto S, Terasawa K, Nishida E (2007) Activation of a C-terminal transcriptional activation domain of ERK5 by autophosphorylation. J Biol Chem 282:35449-35456.

Nakashiba T, Buhl DL, McHugh TJ, Tonegawa S (2009) Hippocampal CA3 output is crucial for ripple-associated reactivation and consolidation of memory. Neuron 62:781-787.

Nakashiba T, Young JZ, McHugh TJ, Buhl DL, Tonegawa S (2008) Transgenic inhibition of synaptic transmission reveals role of CA3 output in hippocampal learning. Science 319:1260-1264.

Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS (1999) Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. J Neurobiol 39:569-578.

Novotny M, Harvey S, Jemiolo B (1990) Chemistry of male dominance in the house mouse, Mus domesticus. Experientia 46:109-113.

Novotny M, Jemiolo B, Harvey S, Wiesler D, Marchlewska-Koj A (1986) Adrenal-mediated endogenous metabolites inhibit puberty in female mice. Science 231:722-725.

Oboti L, Schellino R, Giachino C, Chamero P, Pyrski M, Leinders-Zufall T, Zufall F, Fasolo A, Peretto P (2011) Newborn interneurons in the accessory olfactory bulb promote mate recognition in female mice. Front Neurosci 5:113.

Ohtsuka M, Fukumitsu H, Furukawa S (2008) Neurotrophin-3 stimulates neurogenetic proliferation via the extracellular signal-regulated kinase pathway. J Neurosci Res.

Okada M, Murase K, Makino A, Nakajima M, Kaku T, Furukawa S, Furukawa Y (2008) Effects of estrogens on proliferation and differentiation of neural stem/progenitor cells. Biomed Res 29:163-170.

Okun E, Griffioen K, Barak B, Roberts NJ, Castro K, Pita MA, Cheng A, Mughal MR, Wan R, Ashery U, Mattson MP (2010) Toll-like receptor 3 inhibits memory retention and constrains adult hippocampal neurogenesis. Proc Natl Acad Sci U S A 107:15625-15630.

Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663-1672.

Ozen I, Galichet C, Watts C, Parras C, Guillemot F, Raineteau O (2007) Proliferating neuronal progenitors in the postnatal hippocampus transiently express the proneural gene Ngn2. Eur J Neurosci 25:2591-2603.

Pan YW, Chan GCK, Kuo CT, Storm DR, Xia Z (2012a) Inhibition of Adult Neurogenesis by Inducible and Targeted Deletion of ERK5 Mitogen-Activated Protein Kinase Specifically in Adult Neurogenic Regions Impairs Contextual Fear Extinction and Remote Fear Memory. J Neurosci 32:6444-6455.

Pan YW, Zou J, Wang W, Sakagami H, Garelick MG, Abel G, Kuo CT, Storm DR, Xia Z (2012b) Inducible and conditional deletion of extracellular signal-regulated kinase 5 disrupts adult hippocampal neurogenesis. J Biol Chem 287:23306.

Pawluski JL, Brummelte S, Barha CK, Crozier TM, Galea LA (2009) Effects of steroid hormones on neurogenesis in the hippocampus of the adult female rodent during the estrous cycle, pregnancy, lactation and aging. Front Neuroendocrinol 30:343-357.

Perez JA, Clinton SM, Turner CA, Watson SJ, Akil H (2009) A new role for FGF2 as an endogenous inhibitor of anxiety. J Neurosci 29:6379-6387.

Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci 22:6106-6113.

Poulos AM, Li V, Sterlace SS, Tokushige F, Ponnusamy R, Fanselow MS (2009) Persistence of fear memory across time requires the basolateral amygdala complex. Proc Natl Acad Sci U S A 106:11737-11741.

Qian X, Davis AA, Goderie SK, Temple S (1997) FGF2 concentration regulates the generation of neurons and glia from multipotent cortical stem cells. Neuron 18:81-93.

Qian X, Shen Q, Goderie SK, He W, Capela A, Davis AA, Temple S (2000) Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28:69-80.

Ramirez-Amaya V, Marrone DF, Gage FH, Worley PF, Barnes CA (2006) Integration of new neurons into functional neural networks. J Neurosci 26:12237-12241.

Revest JM, Dupret D, Koehl M, Funk-Reiter C, Grosjean N, Piazza PV, Abrous DN (2009) Adult hippocampal neurogenesis is involved in anxiety-related behaviors. Mol Psychiatry 14:959-967.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-1710.

Rietze RL, Reynolds BA (2006) Neural stem cell isolation and characterization. Methods Enzymol 419:3-23.

Rochefort C, Gheusi G, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J Neurosci 22:2679-2689.

Rolls A, Shechter R, London A, Ziv Y, Ronen A, Levy R, Schwartz M (2007) Toll-like receptors modulate adult hippocampal neurogenesis. Nat Cell Biol 9:1081-1088.

Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. Neuron 39:13-25.

Rossi C, Angelucci A, Costantin L, Braschi C, Mazzantini M, Babbini F, Fabbri ME, Tessarollo L, Maffei L, Berardi N, Caleo M (2006) Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. Eur J Neurosci 24:1850-1856.

Russo-Neustadt AA, Alejandre H, Garcia C, Ivy AS, Chen MJ (2004) Hippocampal brain-derived neurotrophic factor expression following treatment with reboxetine, citalopram, and physical exercise. Neuropsychopharmacology 29:2189-2199.

Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 472:466-470.

Sairanen M, Lucas G, Ernfors P, Castren M, Castren E (2005) Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. J Neurosci 25:1089-1094.

Sakamoto M, Imayoshi I, Ohtsuka T, Yamaguchi M, Mori K, Kageyama R (2011) Continuous neurogenesis in the adult forebrain is required for innate olfactory responses. Proc Natl Acad Sci U S A.

Samuels IS, Karlo JC, Faruzzi AN, Pickering K, Herrup K, Sweatt JD, Saitta SC, Landreth GE (2008) Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. J Neurosci 28:6983-6995.

Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR (2006) Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. Proc Natl Acad Sci U S A 103:17501-17506.

Scharfman H, Goodman J, Macleod A, Phani S, Antonelli C, Croll S (2005) Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. Exp Neurol 192:348-356.

Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429:184-187.

Shalizi A, Lehtinen M, Gaudilliere B, Donovan N, Han J, Konishi Y, Bonni A (2003) Characterization of a neurotrophin signaling mechanism that mediates neuron survival in a temporally specific pattern. J Neurosci 23:7326-7336.

Shan Q, Chan GC, Storm DR (2008) Type 1 adenylyl cyclase is essential for maintenance of remote contextual fear memory. J Neurosci 28:12864-12867.

Shapiro LA, Ng KL, Zhou QY, Ribak CE (2007) Olfactory enrichment enhances the survival of newly born cortical neurons in adult mice. Neuroreport 18:981-985.

Shen J, Xie L, Mao X, Zhou Y, Zhan R, Greenberg DA, Jin K (2008) Neurogenesis after primary intracerebral hemorrhage in adult human brain. J Cereb Blood Flow Metab 28:1460-1468.

Shingo T, Gregg C, Enwere E, Fujikawa H, Hassam R, Geary C, Cross JC, Weiss S (2003) Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science 299:117-120.

Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci 22:3251-3261.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578-584.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992) Deficient hippocampal long-term potentiation in a-calcium-calmodulin kinase II mutant mice. Science 257:201-211.

Singer BH, Jutkiewicz EM, Fuller CL, Lichtenwalner RJ, Zhang H, Velander AJ, Li X, Gnegy ME, Burant CF, Parent JM (2009) Conditional ablation and recovery of forebrain neurogenesis in the mouse. J Comp Neurol 514:567-582.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476:458-461.

Snyder JS, Choe JS, Clifford MA, Jeurling SI, Hurley P, Brown A, Kamhi JF, Cameron HA (2009) Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. J Neurosci 29:14484-14495.

Song HJ, Stevens CF, Gage FH (2002) Neural stem cells from adult hippocampus develop essential properties of functional CNS neurons. Nat Neurosci 5:438-445.

Sparks FT, Lehmann H, Hernandez K, Sutherland RJ (2011) Suppression of neurotoxic lesion-induced seizure activity: evidence for a permanent role for the hippocampus in contextual memory. PLoS One 6:e27426.

Squire LR, Zola-Morgan S (1991) The medial temporal lobe memory system. Science 253:1380-1386.

Squire LR, Alvarez P (1995) Retrograde amnesia and memory consolidation: a neurobiological perspective. Curr Opin Neurobiol 5:169-177.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1:4.

Stowers L, Holy TE, Meister M, Dulac C, Koentges G (2002) Loss of sex discrimination and male-male aggression in mice deficient for TRP2. Science 295:1493-1500.

Stranahan AM, Arumugam TV, Cutler RG, Lee K, Egan JM, Mattson MP (2008) Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. Nat Neurosci 11:309-317.

Suh H, Deng W, Gage FH (2009) Signaling in adult neurogenesis. Annu Rev Cell Dev Biol 25:253-275.

Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH (2007) In Vivo Fate Analysis Reveals the Multipotent and Self-Renewal Capacities of Sox2(+) Neural Stem Cells in the Adult Hippocampus. Cell Stem Cell 1:515-528.

Sultan S, Mandairon N, Kermen F, Garcia S, Sacquet J, Didier A (2010) Learning-dependent neurogenesis in the olfactory bulb determines long-term olfactory memory. Faseb J 24:2355-2363.

Sutherland RJ, Lehmann H (2011) Alternative conceptions of memory consolidation and the role of the hippocampus at the systems level in rodents. Curr Opin Neurobiol 21:446-451.

Sutherland RJ, O'Brien J, Lehmann H (2008) Absence of systems consolidation of fear memories after dorsal, ventral, or complete hippocampal damage. Hippocampus 18:710-718.

Takahashi J, Palmer TD, Gage FH (1999) Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J Neurobiol 38:65-81.

Taubenfeld SM, Wiig KA, Bear MF, Alberini CM (1999) A molecular correlate of memory and amnesia in the hippocampus. Nat Neurosci 2:309-310.

Taupin P (2006) Neurogenesis in the adult central nervous system. C R Biol 329:465-475.

Tavazoie M, Van der Veken L, Silva-Vargas V, Louissaint M, Colonna L, Zaidi B, Garcia-Verdugo JM, Doetsch F (2008) A specialized vascular niche for adult neural stem cells. Cell Stem Cell 3:279-288.

Thuret S, Toni N, Aigner S, Yeo GW, Gage FH (2009) Hippocampus-dependent learning is associated with adult neurogenesis in MRL/MpJ mice. Hippocampus 19:658-669.

Toni N, Teng EM, Bushong EA, Aimone JB, Zhao C, Consiglio A, van Praag H, Martone ME, Ellisman MH, Gage FH (2007) Synapse formation on neurons born in the adult hippocampus. Nat Neurosci 10:727-734.

Trinh K, Storm DR (2003) Vomeronasal organ detects odorants in absence of signaling through main olfactory epithelium. Nat Neurosci 6:519-525.

van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266-270.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034.

Wang H, Ferguson GD, Pineda VV, Cundiff PE, Storm DR (2004) Overexpression of type-1 adenylyl cyclase in mouse forebrain enhances recognition memory and LTP. Nat Neurosci 7:635-642.

Wang SH, Teixeira CM, Wheeler AL, Frankland PW (2009) The precision of remote context memories does not require the hippocampus. Nat Neurosci 12:253-255.

Wang X, Finegan KG, C. RA, Knowles L, Khosravi-Far R, Hinchliffe KA, Boot-Handford RP, Tournier C (2006a) Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress. Cell Death Differ 13:2099-2108.

Wang X, Merritt AJ, Seyfried J, Guo C, Papadakis ES, Finegan KG, Kayahara M, Dixon J, Boot-Handford RP, Cartwright EJ, Mayer U, Tournier C (2005) Targeted deletion of mek5 causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. Mol Cell Biol 25:336-345.

Wang Y, Su B, Xia Z (2006b) Brain-derived Neurotrophic Factor Activates ERK5 in Cortical Neurons via a Rap1-MEKK2 Signaling Cascade. J Biol Chem 281:35965-35974.

Warner-Schmidt JL, Duman RS (2006) Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. Hippocampus 16:239-249.

Watson FL, Heerssen HM, Bhattacharyya A, Klesse L, Lin MZ, Segal RA (2001) Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat Neurosci 4:981-988.

West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231:482-497.

Whitman MC, Greer CA (2009) Adult neurogenesis and the olfactory system. Prog Neurobiol 89:162-175.

Wiltgen BJ, Zhou M, Cai Y, Balaji J, Karlsson MG, Parivash SN, Li W, Silva AJ (2010) The hippocampus plays a selective role in the retrieval of detailed contextual memories. Curr Biol 20:1336-1344.

Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR (1999) Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron 23:787-798.

Wong ST, Trinh K, Hacker B, Chan GC, Lowe G, Gaggar A, Xia Z, Gold GH, Storm DR (2000) Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. Neuron 27:487-497.

Woolley CS, McEwen BS (1993) Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. J Comp Neurol 336:293-306.

Wu ZL, Thomas SA, Villacres EC, Xia Z, Simmons ML, Chavkin C, Palmiter RD, Storm DR (1995) Altered behavior and long-term potentiation in type I adenylyl cyclase mutant mice. Proc Natl Acad Sci U S A 92:220-224.

Yi SH, Jo AY, Park CH, Koh HC, Park RH, Suh-Kim H, Shin I, Lee YS, Kim J, Lee SH (2008) Mash1 and Neurogenin 2 Enhance Survival and Differentiation of Neural Precursor Cells After Transplantation to Rat Brains via Distinct Modes of Action. Mol Ther.

Yin JCP, Wallach JS, Del Vecchio M, Wilder EL, Zhou H, Quinn WG, Tully T (1994) Induction of a dominant negative CREB transgene specifically blocks long-term memory in drosophila. Cell 79:49-58.

Zhang CL, Zou Y, He W, Gage FH, Evans RM (2008a) A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 451:1004-1007.

Zhang M, Moon C, Chan GC, Yang L, Zheng F, Conti AC, Muglia L, Muglia LJ, Storm DR, Wang H (2008b) Ca-stimulated type 8 adenylyl cyclase is required for rapid acquisition of novel spatial information and for working/episodic-like memory. J Neurosci 28:4736-4744.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645-660.

Zhou G, Bao ZQ, Dixon JE (1995) Components of a new human protein kinase signal transduction pathway. J Biol Chem 270:12665-12669.

Zou J, Pan YW, Wang Z, Chang SY, Wang W, Wang X, Tournier C, Storm DR, Xia Z (2012) Targeted Deletion of ERK5 MAP Kinase in the Developing Nervous System Impairs Development of GABAergic Interneurons in the Main Olfactory Bulb and Behavioral Discrimination between Structurally Similar Odorants. J Neurosci 32:4118-4132.

Curriculum Vitae

Yung-Wei Pan

Education

University of Washington, PhD in Molecular and Cellular Biology 2006 – 2012

Dissertation: Regulation of Adult Neurogenesis by the ERK5 MAPK Signaling Pathway and its Functional Implications in Learning and Memory as well as Olfaction
Mentor: Zhengui Xia, PhD – Department of Environmental and Occupational Health Sciences

University of Washington Bothell, MBA 2009 – 2011

Oregon State University, BS in Chemistry, Forensic Science 2000 – 2004

Research Experience

PhD Dissertation Research, University of Washington – Seattle, WA 2007 – 2012

Research Assistant II, Oregon Health & Science University – Portland, OR 2004 – 2006

Projects: Inadvertent Lentiviral Particle Transfer post-Stem Cell Transplantation
Regulatory Mechanisms of Hematopoietic Stem Cell Homing post-transplantation
Characterization of Murine Mesenchymal Stem Cells for Tissue-directed Gene Therapy
Genetic Modification of Hematopoietic Stem Cells to treat Fanconi's Anemia
Mentor: Peter Kurre, MD – Department of Pediatric Hematology/Oncology

Student Research Assistant, Oregon State University – Corvallis, OR 2003 – 2004

Project: Dimerization of Beet Yellows Virus p6 Protein is Crucial for Viral Cell-to-Cell Infection
Mentor: Valerian V. Dolja, PhD – Department of Botany and Plant Pathology

Leadership and Community Experience

Scientist Mentor – NIH ARRA Science Education Supplement 2009 – 2012
Teaching Assistant – University of Washington Cell Biology Laboratory 2008
Scientist Mentor – Fred Hutchinson Cancer Research Center Science Education Partnership 2008
Co-founder of "Big IF" – Bi-Quarterly MCB Graduate Student Meeting 2007

Awards and Achievements

NIH F31 Pre-doctoral Fellow – Ruth L. Kirschstein National Research Service Award 2010 – 2012

NIH T32 Pre-doctoral Trainee – University of Washington Developmental Biology Training Grant 2008 – 2010

NSF Fellow Honorable Mention – National Science Foundation Graduate Research Fellowship Program 2008

Ronald E. McNair Scholar – Ronald E. McNair Post-baccalaureate Achievement Program 2003

Publications

Pan, YW, Storm, DR, Xia, Z. The Maintenance of Established Remote Contextual Fear Memory Requires ERK5 MAP Kinase and Ongoing Adult Neurogenesis in the Hippocampus. *PLoS ONE* (in press)

Pan, YW, Kuo, CT, Storm, DR, Xia, Z. Inducible and Targeted Deletion of the ERK5 MAP Kinase in Adult Neurogenic Regions Impairs Adult Neurogenesis in the Olfactory Bulb and Several Forms of Olfactory Behavior. *PLoS ONE* (in press)

Pan, YW, Zou, J, Wang, W, Sakagami, H, Garelick, MG, Abel, G, Kuo, CT, Storm, DR, Xia, Z. Inducible and Conditional Deletion of Extracellular signal-Regulated Kinase 5 Disrupts Adult Hippocampal Neurogenesis. *J Biol Chem* **287** (28) 23306-17 (2012)

Pan, YW, Chan, GC-K, Kuo, CT, Storm, DR, Xia, Z. Inhibition of Adult Neurogenesis by Inducible and Targeted Deletion of ERK5 Mitogen-Activated Protein Kinase Specifically in Adult Neurogenic Regions Impairs Contextual Fear Extinction and Remote Fear Memory. *J Neuroscience* **32** (19) 6444-55 (2012)

Zou, J, **Pan, YW**, Wang, Z, Chang, SY, Wang, W, Wang, X, Tournier, C, Storm, DR, Xia, Z. Targeted Deletion of ERK5 MAP Kinase in the Developing Nervous System Impairs Development of GABAergic Interneurons in the Main Olfactory Bulb and Behavioral Discrimination between Structurally Similar Odorants. *J Neuroscience* **32** (12) 4118-32 (2012)

Cundiff, P, Liu, L, Wang, Y, Zou, J, **Pan, YW**, Abel, G, Duan, X, Ming, GL, Englund, C, Hevner, R, Xia, Z. ERK5 MAP Kinase Regulates Neurogenin1 during Cortical Neurogenesis. *PLoS ONE* **4** (4) e5204 (2009)

Pan, YW and Kurre, P. Avoiding Lentiviral Transduction Culture Induced MSC Senescence. *J Cell and Molec Med* **13** (6) 1186-87 (2009)

Pan, YW, Scarlett, JM, Luoh, TT, Kurre, P. Prolonged Adherence of Human Immunodeficiency Virus-Derived Vector Particles to Hematopoietic Target Cells Leads to Secondary Transduction In Vitro and In Vivo. *J Virology* **81** (2) 639-49 (2007)

Peremyslov, VV, **Pan, YW**, Dolja, VV. Movement Protein of a Closterovirus Is a Type III Integral Transmembrane Protein Localized to the Endoplasmic Reticulum. *J Virology* **78** (7) 3704-09 (2004)

Pan, YW, Sakagami, H, Garelick, MG, Chan, GC-K, Wang, X, Tournier, C, Storm, DR, Xia, Z. Targeted Deletion of ERK5 MAP Kinase in Adult Neurogenic Regions Attenuates Hippocampal Adult Nuerogenesis and Hippocampus-dependent Memory Formation. *Society for Neuroscience Annual Meeting.* New Orleans, LA (October 2012)

Pan, YW, Sakagami, H, Garelick, MG, Chan, GC-K, Wang, X, Tournier, C, Storm, DR, Xia, Z. Targeted Deletion of ERK5 MAP Kinase in Adult Neurogenic Regions Attenuates Hippocampal Adult Nuerogenesis and Hippocampus-dependent Memory Formation. *University of Washington Institute of Stem Cell and Regenerative Medicine Annual Meeting.* Seattle, WA (November 2011)

Pan, YW, Cundiff, P, Liu, L, Wang, Y, Abel, G, Xia, Z. ERK5 Regulation of Neurogenesis in the Mammalian Brain. *Northwest Society for Developmental Biology Regional Meeting.* Friday Harbor, WA (March 2009)

Pan, YW, Cundiff, P, Liu, L, Wang, Y, Abel, G, Xia, Z. ERK5 Regulation of Neurogenesis in the Mammalian Brain. *University of Washington Institute of Stem Cell and Regenerative Medicine Annual Meeting.* Seattle, WA (November 2008)

Pan, YW and Xia, Z. ERK5 Regulation of Neurogenesis in the Mammalian Brain. *University of Washington Department of Pharmacology Annual Retreat.* Leavenworth, WA (September 2008)

Pan, YW and Kurre, P. Evidence for Cellular Uptake and Subsequent Release of HIV-Derived Vector Particles after Ex Vivo Transduction Culture of Hematopoietic Cells. *American Society of Hematology 48th Annual Meeting.* Orlando, FL (December 2006)

Skinner, AM, **Pan**, **YW**, Luoh, TT, Kurre, P. Induction of CXCR4 in Murine Hematopoietic Cells to Improve Progenitor Homing and Overcome the Intrinsic Stem Cell Repopulation Defect in Fancc-/- mice. *American Society of Hematology 48th Annual Meeting.* Orlando, FL (December 2006)

Pan, YW, Skinner, AM, Kurre, P. In situ Delivery of HIV-lentivector Particles to the Bone Marrow – A Novel Approach to Hematopoietic Stem Cell-directed Gene Therapy in FA. *18th Annual Fanconi Anemia Scientific Symposium.* Bethesda, MD (October 2006)

Pan, YW, Luoh, TT, Kurre, P. Genetic Modification of Murine Stromal-Derived Cells (MSC) in Suspension Culture as a Model for the Rapid Generation of Transplantable Cytoreagents. *American Society of Gene Therapy 9th Annual Meeting.* Baltimore, MD (June 2006)

Pan, YW and Kurre, P. Inadvertent Transduction of Recipient Tissues after Transplantation of Ex Vivo Lentivirally Transduced Hematopoietic Cells. *American Society of Gene Therapy 9th Annual Meeting.* Baltimore, MD (June 2006)

Peremyslov, VV, **Pan, YW**, Dolja, VV. Movement Protein of a Closterovirus Is a Type III Integral Transmembrane Protein Localized to the Endoplasmic Reticulum. *Oregon State University Ronald E. McNair Scholars Symposium.* Corvallis, OR (June 2004)