**The Dissertation Committee for Dong-oh Seo Certifies that this is the approved version of the following dissertation:**

# ADULT HIPPOCAMPAL NEUROGENESIS MODULATES FEAR LEARNING THROUGH ASSOCIATIVE AND NONASSOCIATIVE MECHANISMS

**Committee:**

Michael R. Drew, Supervisor

Michael P. Domjan

Theresa A. Jones

Boris Zemelman

Laura L. Colgin

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**Dong-oh Seo, B.B.A.; M.A.**

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# Dedication

To my family

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# ADULT HIPPOCAMPAL NEUROGENESIS MODULATES FEAR LEARNING THROUGH ASSOCIATIVE AND NONASSOCIATIVE MECHANISMS

Dong-oh Seo, Ph.D.

The University of Texas at Austin, 2015

Supervisor: Michael R. Drew

Adult hippocampal neurogenesis is believed to support hippocampus-dependent learning and emotional regulation. These putative functions of adult neurogenesis have typically been studied in isolation, and little is known about how they interact to produce adaptive behavior. We used trace fear conditioning as a model system to elucidate mechanisms through which adult hippocampal neurogenesis modulates processing of aversive experience. To achieve a specific ablation of neurogenesis, we generated transgenic mice expressing herpes simplex virus thymidine kinase (HSV-TK) under the *dcx* gene promoter, which directs expression to neural progenitors and immature neurons. Intracerebralventricular injection of the prodrug ganciclovir (GCV) caused a robust suppression of neurogenesis without suppressing gliogenesis. Neurogenesis ablation via this method (DCX-TK/GCV system) or targeted x-irradiation caused an increase in context conditioning in trace but not delay fear conditioning. The data suggest that this phenotype represents opposing effects of neurogenesis ablation on associative and nonassociative components of fear learning. Arrest of neurogenesis sensitizes mice to nonassociative effects of fear conditioning, as evidenced by increased anxiety-like behavior in the open field after (but not in the absence of) fear conditioning. In addition,

arrest of neurogenesis impairs associative trace conditioning, but this impairment can be masked by nonassociative fear. The results suggest that adult neurogenesis modulates emotional learning via two distinct but opposing mechanisms: it supports associative trace conditioning while also buffering against the generalized fear and anxiety caused by fear conditioning.

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# Chapter 1: Introduction

The dentate gyrus (DG) is one of a small number of brain regions that generate neurons in adulthood (Barker, Boonstra, & Wojtowicz, 2011; Kempermann, Jessberger, Steiner, & Kronenberg, 2004). The adult-born neurons of the DG integrate into the tri-synaptic pathway of the hippocampus, which is one of the important circuits for learning, memory, and emotional regulation (Deng, Aimone, & Gage, 2010; Snyder, Soumier, Brewer, Pickel, & Cameron, 2011; Sahay, Drew, & Hen, 2007; Dranovsky & Leonardo, 2012).

Great effort has been made to understand the function of adult neurogenesis and how it is regulated. Computational modeling and behavioral experiments using techniques to suppress adult neurogenesis have suggested that hippocampal neurogenesis contributes to learning and memory (Aimone & Gage, 2011; Denny, Burghardt, Schachter, Hen, & Drew, 2012; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002; Saxe et al., 2006; Saxe et al., 2007; Clelland et al., 2009; Deng, Saxe, Gallina, & Gage, 2009). However, many questions remain. There are studies reporting the impairment of memory tasks after the ablation of adult-born neurons, but the studies have not clearly explained why the ablation results in the impairment of memory tasks and how new neurons contribute to the specific underlying psychological processes. Furthermore, these studies usually used techniques that ablate dividing cells nonspecifically, which means non-neuronal populations are also affected. Thus, it remains unclear whether the behavioral changes were caused by ablation of neurogenesis or ablation of another adult-generated cell type, such as glia. Finally, a number of studies report different, and sometimes contradictory, effects of ablation of adult-born neurons, even in ostensibly

identical learning and memory tasks (Groves et al., 2013; Castilla-Ortega, Pedraza, Estivill-Torrus, & Santin, 2011; Castilla-Ortega et al., 2011). The divergent results suggest that the involvement of adult-born neurons in learning and behavior is modulated by variables that have yet to be identified.

One of the variables that may modulate the effect of arresting adult neurogenesis is the emotional state of subjects. There has been considerable progress in describing how adult neurogenesis contributes to mood control (Drew & Hen, 2007; Sahay et al., 2007). Suppression of hippocampal adult neurogenesis abrogates the behavioral effects of antidepressant medications (David et al., 2009; Santarelli et al., 2003; Airan et al., 2007), and impairs feedback regulation of the HPA axis (Snyder et al., 2011; Schloesser, Lehmann, Martinowich, Manji, & Herkenham, 2010; Hayashi, Takashima, Murayama, & Inokuchi, 2008). These results imply that the behavioral effects of arresting adult hippocampal neurogenesis may reflect compound perturbation of both cognition and emotional regulation. One of the central hypotheses of this dissertation is that the arrest of adult hippocampal neurogenesis not only causes perturbation of cognitive processes but also alters the emotional response to stress. This altered emotional state can mask the contribution of adult-born neurons to cognitive processes.

To test this hypothesis we selected a simple form of learning that provides a great degree of experimental control. Pavlovian fear conditioning is a paradigm whereby animals learn the relationship between an emotionally neutral conditioned stimulus (CS; e.g., a tone) and an aversive unconditioned stimulus (US; e.g. a footshock). Through the process animals come to anticipate the aversive event and display species-typical behavioral (e.g. freezing behavior) and/or physiological (e.g. changes in heart rate or

glucocorticoid secretion) responses (Pavlov, 1927; LeDoux, 1992). Thus, in this paradigm, adaptive behavior requires integration of learning and emotional processes. Variants of Pavlovian fear conditioning paradigms engage different neural substrates depending on temporal relation between conditioned stimulus (CS) and unconditioned stimulus (US). It is well established that the hippocampus is not necessary for the formation of the CS-US association in delay conditioning procedures, where the two stimuli (CS and US) overlap in time. However, the hippocampus is critical for trace conditioning, which involves a stimulus-free gap between the two stimuli (Raybuck & Lattal, 2011; Quinn, Loya, Ma, & Fanselow, 2005; Burman & Gewirtz, 2004; Knight, Cheng, Smith, Stein, & Helmstetter, 2004; Tseng, Guan, Disterhoft, & Weiss, 2004; Quinn et al., 2005). Therefore, trace conditioning is an attractive model system to study the role of hippocampal adult neurogenesis in memory.

Several studies have shown that the trace conditioning can enhance the survival of adult-born neurons (Leuner et al., 2004; Gould, Beylin, Tanapat, Reeves, & Shors, 1999a; Sisti, Glass, & Shors, 2007). However, the contribution of adult neurogenesis in trace conditioning remains ambiguous because studies investigating the requirement of adult-born neurons in trace conditioning have produced inconsistent results. Some studies have shown the impairment of trace eye-blink or fear conditioning after the arrest of adult neurogenesis and suggested that hippocampal adult neurogenesis is crucial to the association between two stimuli separated in time (Shors et al., 2002; Shors et al., 2001; Achanta, Fuss, & Martinez, 2009; Guo et al., 2011). However, some other studies have shown little effect in the ablation of adult-born neurons or no effect of the natural reduction of adult neurogenesis by aging (Achanta et al., 2009; Cuppini et al., 2006).

There is even a study showing enhanced contextual and trace cue memory after the ablation of adult-born neurons (Jaholkowski et al., 2009). The inconsistent results on the relations between trace conditioning and adult neurogenesis is representative of the functional adult neurogenesis field as a whole, which includes many studies reaching inconsistent or contradictory conclusions about the role of adult neurogenesis in behavior.

The discrepancy of the results in this field might be attributable to the differences in the trace conditioning methodology (e.g. short inter-trial interval [ITI] versus long ITI, low footshock versus high footshock), species (e.g., mice versus rats) or the method of ablation of adult neurogenesis (e.g. x-irradiation versus transgenic mice). First, to narrow down the possible variables affecting the result, we developed a new transgenic mouse model (DCX-TK), which ablated cells expressing DCX proteins that are a specific marker for newborn neurons and their progenitors (Figure 1). In this mouse model, herpes simplex virus thymidine kinase (HSV-TK) is expressed, driven by the doublecortin (DCX) promoter. When DCX-TK mice are treated with the prodrug ganciclovir (GCV), the HSV-TK activates the GCV, preventing DNA replication and causing the death of dividing cells. In *Chapter 3* we discuss the cellular characterization of these mice. We demonstrate that two-week GCV treatment in DCX-TK mice (DCX-TK/GCV) resulted in dramatic reduction of adult neurogenesis. Because this DCX-TK/GCV system produces a more specific arrest of adult neurogenesis while not affecting gliogenesis, it may constitute a major advance over previous ablation methods.

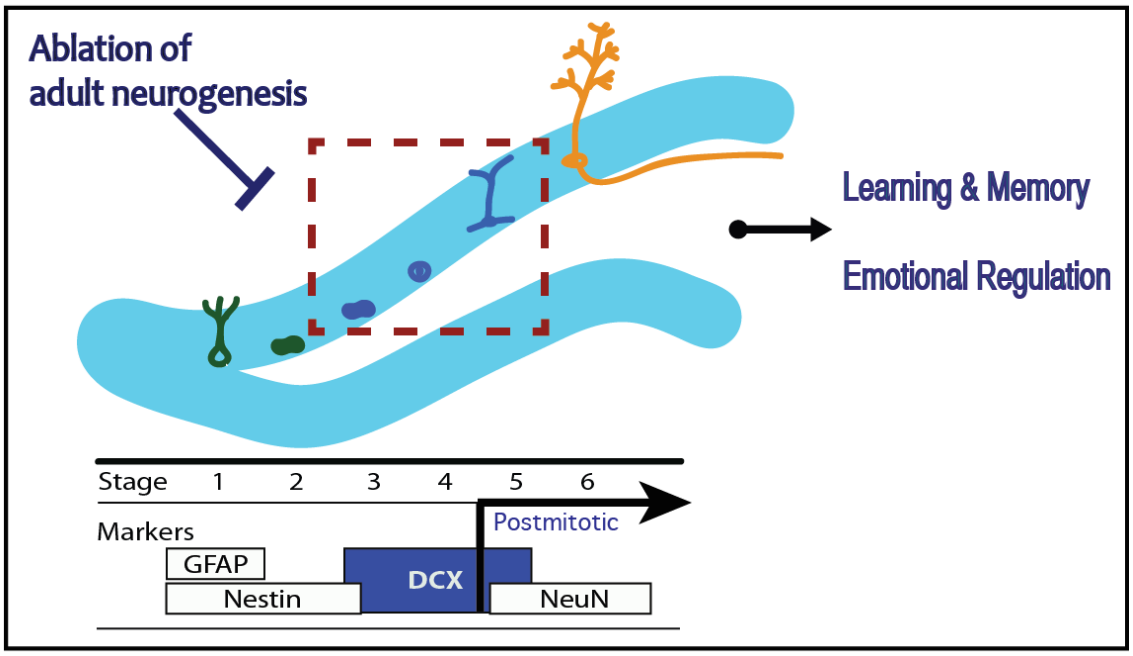


Figure 1.1. Hippocampal adult neurogenesis.

The diagram shows the developmental stages of adult-born neurons in the DG. Neurogenesis in the SGZ (granule cell layer is illustrated with a thick sky-blue line) is generally thought to include 6 stages based on morphology and immunochemical markers. The first stage is the division of multipotent stem-like cells. Some progeny differentiate into neuronal-committed transient amplifying progenitor cells (Stage 2,3,4), which produce immature neurons (Stage 5). Newborn neurons that survive for the long-term become mature dentate granule cells (Stage 6). DCX expressing cells are shown in blue in the illustration and the table below.

In *Chapter 4* we use this DCX-TK/GCV system to investigate the role of adult hippocampal neurogenesis in trace fear conditioning. The DCX-TK mice treated with GCV showed enhanced contextual fear memory compared to wild type (WT) controls. In the trace cue memory test, both DCX-TK and WT mice displayed similar levels of freezing behavior to the tone. This behavioral outcome was similarly observed in a

separate experiment using hippocampus-targeted low-dose x-irradiation to ablate adult-born neurons. Irradiated and sham-irradiated mice were conditioned 6-8 weeks following irradiation using delay or trace fear conditioning procedures. Irradiated and control mice displayed similar levels of tone fear in both delay and trace procedures. However, consistent with the DCX-TK experiment, irradiated mice displayed significantly higher context fear than control mice after trace conditioning, whereas, there were no significant differences between the groups after delay conditioning. The enhanced contextual fear after trace conditioning in mice lacking neurogenesis was unexpected considering that studies generally showed contextual fear impairment or no effect after fear conditioning in mice lacking adult neurogenesis (Drew, Denny, & Hen, 2010; Castilla-Ortega et al., 2011). However, the behavioral pattern was only observed after trace conditioning, not after delay conditioning, which suggests that adult-born neurons may modulate contextual fear through the perturbation of trace conditioning, rather than through direct modulation of contextual learning.

In *Chapter 5*, we demonstrate that the fear conditioning protocol we used produced both associative and nonassociative fear. Nonassociative fear was evidenced by significant freezing in response to the tone even in mice that did not receive tone-shock pairings. Additional experiments using the open field and elevated plus maze confirmed that fear conditioning causes generalized changes in emotionality. We demonstrated that the fear conditioning procedure is anxiogenic. Moreover, the anxiogenic effect was stronger in mice lacking neurogenesis than WT mice. The exaggerated emotional behavioral changes in mice lacking adult neurogenesis were evident in both open field and elevated plus maze tests.

In *Chapter 6* we present an alternate trace fear conditioning procedure that produces less nonassociative learning. With the new trace fear conditioning procedure, we revealed that there was an impairment of the trace CS-US association after DCX-TK-mediated arrest of adult neurogenesis. This result supports the hypothesis that the enhanced contextual fear after trace conditioning in DCX-TK mice in *Chapter 4* was due to the increased prediction value of the context for an aversive event trading off the impairment of the trace CS-US association.

In summary, accumulating evidence suggests that hippocampal adult neurogenesis contributes to memory and emotional regulation. However, there is no consensus on the nature of this contribution because of inconsistencies among published studies. We show that arrest of adult hippocampal neurogenesis can enhance or impair aspects of trace fear conditioning depending on the nature of the conditioning protocol. When the procedure engenders robust nonassociative changes in fear expression, neurogenesis-arrested mice displayed elevated fear behavior; in a trace conditioning procedure that minimizes nonassociative plasticity, neurogenesis-arrested mice displayed impaired associative fear. The data suggest that arrest of adult neurogenesis affects fear conditioning through opposing associative and nonassociative mechanisms. Contradictory results might be due to differences in emotional state in the studies, confounding interpretation of results. The current study provides valuable new insights into the interacting cognitive and emotional functions of adult neurogenesis.

# Chapter 2: Literature review

## 2.1 ADULT NEUROGENESIS AND HIPPOCAMPUS

In 1962, Joseph Altman first reported the proliferation of neurons in adult rats in auto-radiographic investigations (Altman, 1962; Altman & Das, 1965). Although the discovery ultimately overturned the dogma that the adult brain does not retain the capacity for the formation of new neurons, Altman's discovery did not take center stage for many years because of influential –but incorrect– reports that that neurogenesis is limited to prenatal development in primates (Rakic, 1974; Rakic, 1985). In the 1990's a series of studies using more precise techniques confirmed that new neurons are generated in the brain throughout adult life in various species including rodents, primates and humans, although each species showed different characteristics of adult neurogenesis in terms of the number and the location of neurogenic regions and the turnover rates of neurogenesis (Barker et al., 2011; Eriksson et al., 1998; Lois & Alvarez-Buylla, 1994; Nanry, Mundy, & Tilson, 1989; Kaplan & Hinds, 1977; Altman & Das, 1965; Gould et al., 1999b).

In the brains of rodents, there are two major discrete regions of adult neurogenesis: the subventricular zone (SVZ) around the lateral ventricles and the subgranular zone (SGZ) in the DG of the hippocampus (Ming & Song, 2005). There are reports that adult neurogenesis occurs in other mammalian brain areas beside SVG and SGZ (e.g. hypothalamus, piriform cortex, striatum), but this is controversial (Bonfanti & Peretto, 2011; Shapiro et al., 2007; Dayer, Cleaver, Abouantoun, & Cameron, 2005). SVZ is frequently referred to as the subependymal zone (SEZ) because neural stem cells (NSC) are situated beneath the thin ependymal cell layer of the lateral ventricles. In SVZ,

the NSCs proliferate beneath the ependymal cell layer and produce neuroblasts, which migrate a long distance (around 5 mm in mice) through the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into GABAergic interneurons as they are integrated into existing circuits either in the granule cell layer or periglomerular layer (Lois & Alvarez-Buylla, 1994). Eventually, when they become new mature neurons, they replace older neurons and integrate into the existing circuit in the olfactory bulb (Calof et al., 1996; Lois & Alvarez-Buylla, 1994; Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999; Imayoshi et al., 2008).

The SGZ is a small region located between the granule cell layer and hilus of the DG. Newborn neurons in SGZ differentiate and migrate only a short distance to the granule cell layer (GCL) and integrate into the tri-synaptic pathway of the hippocampus, similar to developmentally-born granule cells (Zhao, Teng, Summers, Ming, & Gage, 2006; Esposito et al., 2005; Tashiro, Sandler, Toni, Zhao, & Gage, 2006; Ge et al., 2006). After a short migration from SGZ to the GCL, the neuronal-committed progenitors begin to extend cellular processes. In the second week after birth, they become more neuron-like in shape with polarized processes extending into the molecular layer and towards CA3, but they are yet to be fully integrated in the existing neural circuits. At this age, immature neurons have different physiological characteristics compared to mature neurons. For example, GABAergic inputs are excitatory to immature neurons (Toni et al., 2008; Toni et al., 2007; Ge, Yang, Hsu, Ming, & Song, 2007; Zhao et al., 2006; Esposito et al., 2005).

By around three weeks after birth, the immature granule cells form synapses with the afferent axon fibers from EC, and the mossy fiber boutons of the cell form synapses

with neurons in CA3. During the third week after birth, dendritic spines begin to appear in dentate granule cells. In particular, dendritic filopodia, which are very motile and regulated by synaptic activity, are more abundant around this period, and they are found near to preexisting multiple-synapse boutons, indicating that hippocampal adult-born neurons are integrated into the preexisting network (Toni et al., 2008; Toni et al., 2007). The timing of the synaptic integration coincides with the excitatory-to-inhibitory transition of GABAergic inputs, which occurs as the resting membrane potential decreases (Ge et al., 2006). Dendritic synaptic integration leads to the survival of the new neurons through NMDA-type glutamate receptors, implying that new neurons selectively survive in a competitive, activity-dependent manner (Tashiro et al., 2006).

At 4-6 weeks of age, adult-born neurons have stronger synaptic plasticity characterized by a low threshold for long-term potentiation (LTP) induction and higher amplitude of LTP than older mature granule cells. The NMDA receptor subunit NR2B contributes to this enhanced synaptic plasticity (Ge et al., 2007; Snyder, Kee, & Wojtowicz, 2001). Also, recent studies show that young neurons (4-6 weeks) gradually develop local inhibitory inputs from GABAergic interneurons until they become mature neuron (8 weeks). Because the inhibitory input to the young neuron is still immature at 4-6 weeks, neurons of this age display increased excitation/inhibition ratio and a low activation threshold (Marin-Burgin, Mongiat, Pardi, & Schinder, 2012; Li, Aimone, Xu, Callaway, & Gage, 2012).

After 8 weeks of age the unique physiological characteristics of adult-born neurons, such as reduced threshold for LTP induction and increased amplitude of LTP, disappear, and the physiological properties of adult-born neurons become similar to

mature neurons (Ge et al., 2007). The unique physiological characteristics of adult-born neurons younger than 6 week olds imply that young adult-born neurons may contribute in a unique way to learning and memory. Indeed, recent studies showed mice lacking adult-born neurons specifically at the immature stage were deficient in the some cognitive functions (Denny et al., 2012; Deng et al., 2009).

To understand the role of hippocampal adult neurogenesis, it is important to take the neuronal circuitry of the hippocampus into account. Newborn neurons are integrated into the existing neuronal circuit and work as a part of a neural network (Figure 2). The hippocampus is comprised of three main subregions: DG, CA3, and CA1 (O'Reilly & McClelland, 1994). Each subregion receives direct input from the entorhinal cortex (EC) (Nakashiba, Young, McHugh, Buhl, & Tonegawa, 2008). It has been suggested that each subregion has its own function. For example, CA1 may work as a novelty detector comparing the processed information from the tri-synaptic (EC-DG-CA3-CA1) with the novel information from the monosynaptic pathways (EC-CA1) during new experience (Kumaran & Maguire, 2007). The Schaffer-collateral pathway of CA3 includes a recurrent loop, which might allow it to function as an auto-associator (Nakashiba et al., 2008; Leutgeb, Leutgeb, Moser, & Moser, 2007; McHugh & Tonegawa, 2009). The function of the DG is less well understood compared to the other two subregions, but the DG has been thought of as the first gate of the tri-synaptic pathway of the hippocampus in information processing.

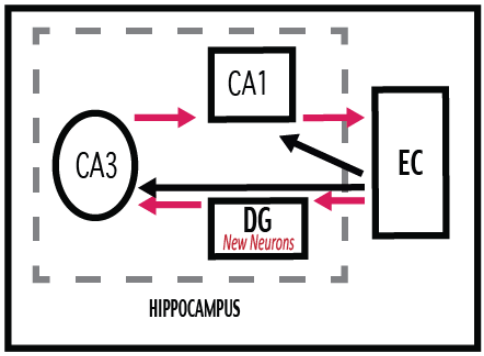


Figure 2.1. Diagram of the hippocampal neural network.

Each subregion of hippocampus (DG, CA3, CA1) receives direct input from the EC depicted by black arrows. The traditional excitatory trisynaptic pathway (EC-DG-CA3-CA1) is depicted by red arrows. The neurons in the EC send projections to the DG through the perforant pathway. The DG sends projections to the pyramidal cells in CA3 through mossy fibers. The pyramidal neurons in the CA3 send projections to CA1, and CA1 sends projections to the EC.

In the classical circuit of the tri-synaptic pathway, the information from layer II of EC is relayed to DG, CA3 and CA1 in order (Treves, Tashiro, Witter, & Moser, 2008). However, this concept is over-simplified. In reality signals do not always propagate in one direction via the tri-synaptic pathways, and more complex circuits exist both between and within subregions (Piatti, Ewell, & Leutgeb, 2013; Vivar & van Praag, 2013). For example, EC is not the only extrinsic input source into DG; there is back-projection from CA3 to DG through the axons of CA3 pyramidal neurons (Myers & Scharfman, 2011; Scharfman, 2007). Likewise, there is a direct input from layer II of EC to CA3, and a direct monosynaptic input from EC to CA1 is originated from layer III of EC (Witter & Amaral, 1991; Steward & Scoville, 1976). Furthermore, within each subregion, there are multiple different types of neurons producing complex microcircuits. In the DG there are interneurons and mossy cells, and each has unique functions and projections. For

instance, parvalbumin-expressing interneurons, but not somatostantin- or vasoactive intestinal polypeptide-expressing interneuron, in the DG regulate mitotic activity of local adult neural stem cells (Song et al., 2012; Wojtowicz, 2012; Klausberger & Somogyi, 2008). Mossy cells in the hilar region receive excitatory inputs from granule cells in the DG and pyramidal cells in the CA3 and have excitatory synapses onto granule cells and interneurons. The dendrites of mossy cells extend long distances in both medio-lateral and dorso-caudal directions. This unique anatomical characteristic suggests they regulate local and distal granule cell activity (Jinde, Zsiros, & Nakazawa, 2013; Scharfman, 1995).

The anatomical and physiological characteristics of the DG may help us understand how the DG processes information. One of the unique anatomical characteristics of the DG is that the number of DG granule neurons is larger than that of upstream neurons in the EC. This anatomical characteristic implies that signals from the EC can be encoded by a sparse set of neurons in the DG, which could help to avoid overlapped encoding between different stimuli. That is, the DG engages in sparse coding of incoming information from the EC (Treves et al., 2008). The granule cells in the DG receive strong tonic inhibition from local interneurons, and relatively few granule cells are active during any individual experience. Granule cells send signals to CA3 where pattern completion occurs (Li et al., 2012; Wojtowicz, 2012). The unique anatomical characteristics of the DG have led to the idea that it is a pattern separator. Pattern separation, as originally defined, is a computational process whereby similar information is separated into orthogonal neural representations (O'Reilly & McClelland, 1994). In behavioral studies, pattern separation is sometimes defined as the ability to discriminate

similar stimuli. The process can be understood through the example of animal distinguishing a similar new event from a previous experience, such as contextual fear discrimination task. For example, normal mice that received a shock in an environment show more freezing behavior in the same environment than in a different environment. Several studies showed mice with lesions to the DG or NMDA receptors knocked-out in the DG were impaired in discriminating between different contexts (McHugh et al., 2007), in that freezing behavior occurred in both the shock context and in a no-shock context. Because adult-born neurons are integrated into the DG circuit, it has been suggested that adult-born neurons might play a role in such pattern separation (Sahay et al., 2011; Drew et al., 2010; Clelland et al., 2009).

### 2.2 HIPPOCAMPAL ADULT NEUROGENESIS AND TRACE CONDITIONING

Trace conditioning is a hippocampus-dependent learning task produced by pairing a neutral cue (CS, e.g. a tone) with an US (e.g. footshock) involving a stimulus-free gap between the two stimuli (Pavlov, 1927; Kryukov, 2012). Trace conditioning has been hypothesized to constitute a form of declarative memory (Clark, Manns, & Squire, 2001). In the task, animals acquire information about temporal relations or the accurate timing of learned responses (Balsam, 1984), which distinguishes trace conditioning from other hippocampus-dependent tasks, which are usually spatial (Bannerman et al., 2014).

Some studies have supported that trace conditioning requires the DG to process the CS-US association. For example, rats trained in trace fear conditioning showed higher immediate early gene expression (e.g. *zif268*) in the DG than rats trained in the delay procedure after animals were re-exposed to the CS (Weitemier & Ryabinin, 2004). Another electrophysiological study showed that, while animals were trained in trace fear

conditioning procedure, the DG neural activity during the CS period gradually increased with training (Gilmartin & McEchron, 2005).

Despite the consensus that adult-born neurons in the DG are functionally significant with respect to learning and memory, the literature on trace conditioning has not reached a consensus regarding the contribution of adult-born neurons. Early studies showed that the formation of trace memory enhances the survival of adult-born neurons (Shors, 2004; Leuner et al., 2004; Gould et al., 1999a). However, it is still debated if those newborn neurons are necessary for the formation of the trace memory. Some studies showed that arresting adult neurogenesis impairs trace conditioning (Shors et al., 2001). Shors and colleagues (2001) treated rats with antimitotic drug (methylazoxymethanol acetate, MAM; saline for control group) to rats to arrest adult neurogenesis, and followed by training the rats in either delay or trace eye-blink conditioning. Rats with arrested adult neurogenesis showed impairment of learning in trace conditioning, but not in delay conditioning. The same groups obtained similar results in a separate study with trace fear conditioning (Shors et al., 2002). However, subsequent studies showed no impairment or even an enhancement of trace memory after reduction of adult neurogenesis (Cuppini et al., 2006; Jaholkowski et al., 2009). The neurogenesis ablation techniques used in the original studies (Jaholkowski et al., 2009; Achanta et al., 2009; Shors et al., 2002; Shors et al., 2001) affected non-neurogenic cell lineages, which make it difficult to determine whether the impairment (or enhancement) of performance was the effect of ablation of adult-born neurons. Finally, even if arresting adult neurogenesis impairs trace conditioning, it is still unknown how adult-born neurons are involved in the task and how the role of adult-born neurons in trace conditioning can

be unified with existing results showing that hippocampal adult neurogenesis is important for rapid processing of contextual memory or contextual discrimination (*Please see Section 2.1*). Therefore, as a first step toward reaching to a comprehensive theory of neurogenesis function, we will revisit the question whether or not adult-born neurons contribute to trace fear conditioning.

# Chapter 3: DCX-TK Transgenic mouse model

There have long been debates about whether or not adult neurogenesis is functionally crucial in cognition or emotional regulation (Castilla-Ortega et al., 2011; Sahay et al., 2007). One obstacle to reaching a conclusion is that most existing ablation techniques do not specifically target neurogenesis (Bush et al., 1999). Several techniques have been popularly used to arrest adult neurogenesis in the field, such as x-irradiation, antimitotic drugs, and conditional HSV-TK expressing transgenic mice targeting stem-cell promoters, which include glial fibrillary acidic protein (GFAP) or nestin (GFAP-TK or Nestin-TK) (Deng et al., 2009; Snyder et al., 2011; Saxe et al., 2006; Shors et al., 2001). However, these techniques prevent DNA replication or cell proliferation in a non-specific manner. These techniques cause the reduction of adult neurogenesis, but it is important to note that these may also affect the non-neuronal cell lineages. For example, GFAP-TK and Nestin-TK mice have been developed to control adult neurogenesis through inducible ablation of proliferating cells (Singer et al., 2009; Garcia, Doan, Imura, Bush, & Sofroniew, 2004). GFAP and Nestin are intermediate filament proteins expressed in a variety of cell types. GFAP is expressed in astrocytes, ependymal cells and oligodendrocytes in CNS, liver, kidney, and gut (Tennakoon et al., 2013; Apte et al., 1998; Bush et al., 1998; Bush et al., 1999). Therefore, the GCV administration will affect not only neurogenesis but also gliogenesis, and it may cause side effects. For example, a GFAP-targeted ablation system (GFAP-TK/GCV) has been associated with jejuno-ileitis (Bush et al., 1998). Nestin is associated with cells of neuroepithelial origin and might be a more specific marker for neuronal cell lineage than GFAP, but it is still expressed in the astroglial lineages. Singer and colleagues (2009) demonstrated that treating Nestin-TK

mice with GCV for 4 weeks results in near complete suppression of adult neurogenesis (Singer et al., 2009). However, it is also likely that the system suppressed gliogenesis because nestin is also expressed in the radial glial cells. This possibility was not evaluated in the study.

Therefore, to precisely assess the role of adult neurogenesis in behavioral processes, it is desirable to have a method in which proliferation of neurons, but not other cell types, can be reversibly turned off. Toward this end, we developed a transgenic mouse model that expresses HSV-TK driven by the DCX promoter. Thymidine kinase (TK) is a phosphotransferase, which is important in DNA synthesis during cell division. It exists in most living cells. However, TK derived from herpes simplex virus has a unique genetic sequence, such that the GCV is activated by the HSV-TK but not by endogenous mammalian TK. GCV is a synthetic analogue of 2′-deoxy-guanosine. HSV-TK catalyzes the formation of GCV triphosphate, which competes with deoxyguanosine triphosphate, causing chain termination during DNA synethesis (Boucher, Ostruszka, & Shewach, 2000). When DCX-TK mice are treated with GCV, the HSV-TK expressed by DCX positive cells phosphorylates the GCV, preventing DNA replication and causing the death of dividing cells (Figure 3.1).

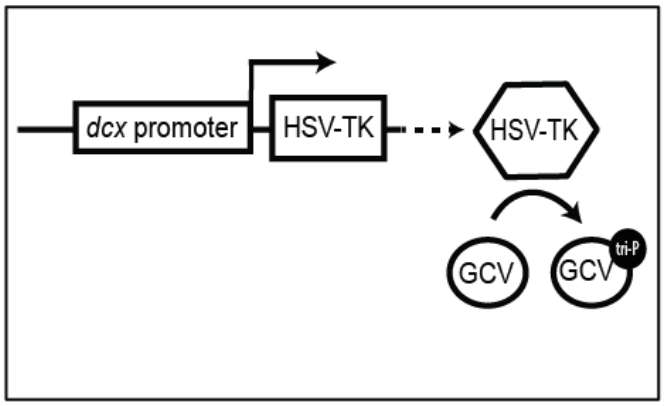


Figure 3.1. DCX-TK transgenic mouse model.

Transgenic mice express HSV-TK under control of the *dcx* gene promoter. HSV-TK catalyzes the formation of GCV-triphosphate, which prevents DNA replication and kills dividing cells.

DCX is highly expressed in the developing rodent brain and associated with the migration of neuronal progenitor cells during cortical development (des Portes et al., 1998; Gleeson, Lin, Flanagan, & Walsh, 1999; Francis et al., 1999). In adult rodent brains, DCX expression is very low, but is still retained in several brain regions including the major neurogenic niches (Couillard-Despres et al., 2005; Brown et al., 2003; Nacher, Crespo, & McEwen, 2001). In hippocampal adult neurogenesis, neural committed progenitor cells (type 2b/3 cells) during the mitotic period and immature neurons in the early post-mitotic period express DCX (Figure 1). However, DCX is not expressed in radial-glia-like stem cells (type-1) and multipotent progenitor cells (type-2a) (Brown et al., 2003). A recent fate mapping study with transgenic mice demonstrated that the DCX positive cells become mostly neurons, although the function of DCX in the regulation of adult neurogenesis is not clear (Spampanato, Sullivan, Turpin, Bartlett, & Sah, 2012; Cheng et al., 2011). Thus, DCX is a specific marker for adult neurogenesis that is not

expressed in multipotent stem-like cells. Therefore, GCV administration to DCX-TK mice should specifically arrest the proliferation of neurons, but not other non-neuronal committed dividing cells, such as glia.

In this chapter, we examine how efficiently and specifically the DCX-TK/GCV system ablates adult-born neurons. The results obtained in this chapter will help establish parameters for behavioral studies of adult neurogenesis function.

## 3.1 MATERIALS AND METHODS

### 3.1.1 Animals

A Bacterial Artificial Chromosome (BAC) clone (RP23-462G16) that contained the entire mouse *dcx* gene, plus at least 50kb of upstream and downstream flanking sequence, was obtained from the BACPAC Resources Center (BPRC, http://bacpac.chori.org). The DCX promoter was selected because DCX is expressed in lineage-restricted neural progenitor cells and immature adult-born neurons (Brown et al., 2003). The pBADTcTypeG plasmid (Dr. Manabu Nakayama, Kazusa Institute, Japan) was transferred into DH10B cells containing the BAC clone, enabling Red/ET homologous recombination (Nakayama & Ohara, 2005). The HSV-TK-polyA cassette was PCR amplified from the GFAP-HSV-TK plasmid (Addgene pTGB008, and described in Bush et al., 1998) and inserted at the translation initiation site of the *dcx* gene via homologous recombination. Correct clones were verified with PCR. The BAC clone contained a second gene, *Calp6*. To avoid transgene-mediated overexpression of *Calp6*, the *Calp6* coding sequence was replaced with Neo cassette in reverse orientation using homologous recombination. The recombinant BAC was purified, and the circular

BAC was injected into C57Bl/6 x DBA (F2) embryos. Ten transgenic DCX-TK founders were identified by PCR and confirmed by Southern blot. One transgenic line (line A) was used for further analysis. Copy number for line A was estimated by Southern blot to be 10.

DCX-TK mice were backcrossed onto the C57BL/6J background. Mice used for histology studies were from N2–N4 generations. Mice used for behavioral tests were from N3 to N6 generations. Approximately equal numbers of male and female DCX-TK and WT littermates were used in each experiment. Mice were housed maximum 5 per cage under a standard 12 h light/dark cycle with *ad libitum* access to food and water. All procedures involving animals were approved by the University Committee on the Use and Care of Animals of the University of Texas at Austin.

### 3.1.2 GCV administration

GCV was administered continuously for two weeks via an intracerebralventricular (ICV) cannula. Osmotic mini pumps (Alzet, model 1002) were filled with GCV (12.5 mg/ml in phosphate-buffered saline [PBS]) or PBS and connected to the cannula via a vinyl catheter (Brain Infusion Kit 3, Alzet). The cannula was implanted into the lateral ventricle and delivered drug at a rate of 0.25 $\mu$l/hour. The devices were primed in saline at 37°C overnight before the cannula implantation.

Cannula implantation surgery was performed on 8-week-old DCX-TK transgenic mice and their wild-type (WT) littermates. Mice were anesthetized with isoflurane and fixed to a stereotaxic frame. An incision was made on the scalp and a small subcutaneous pocket was opened on the back of the body using a hemostat. The pump was inserted into the pocket, and the connected ICV cannula was implanted into the lateral ventricle (tip

targeted to 0.22 mm anterior, 1 mm lateral, and 2.5 mm ventral to bregma). The cannula and skull were covered with dental cement.

### 3.1.3 BrdU administration

Bromodeoxyuridine (BrdU) was dissolved in sterile saline (10 mg/ml) and injected to mice (300 mg/kg IP) twice per day for 2 days, 1 week before mice were euthanized.

### 3.1.4 Tissue collection and immunohistochemistry

The mice were overdosed with a mixture of ketamine (150 mg/kg) and xylazine (15 mg/kg) and perfused with 20 ml of PBS followed by 15-20 ml of 4% paraformaldehyde (PFA). Brains were post-fixed overnight in 4% PFA, immersed in 30% sucrose for 2-4d at 4°C, and then flash-frozen and sectioned coronally at 35 $\mu$m thickness on a cryostat.

For immunofluorescence labeling, sections were incubated in blocking solution (PBS with 5% normal donkey serum and 0.25% Triton X-100) for 1 hour with gentle agitation before incubation with primary antibodies: Rabbit anti-DCX (1:4000, Abcam), goat anti HSV-TK (1:1000, Santa Cruz), mouse anti-NeuN (1:250, Millipore), rabbit anti-GFAP (1:2500, Dako), rat anti-BrdU (1:100, Accurate Chemical & Scientific Corp.), and rabbit anti-Iba1 (1:2000, Wako). Sections were incubated with primary antibodies at room temperature overnight and rinsed for 5 min in 3 changes of PBS. Secondary antibodies (Alexa Fluor 488-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-goat, and Alexa-Fluor 647-conjugated donkey anti-mouse, Jackson ImmunoResearch) and DAPI (Invitrogen) were diluted in PBS (Secondary antibodies at 1:250, DAPI at 300nM) with 5% normal donkey serum and 0.25% Triton X-100. Sections were

incubated for 2 hours at room temperature with gentle agitation, then rinsed in PBS, mounted, and cover slipped.

For BrdU immunohistochemistry, DNA denaturation was performed prior to application of the primary antibody using a procedure modified from Leuner et al (Leuner et al., 2004). Floating sections were incubated in 2N hydrochloric acid for 30 min at room temperature, and then neutralized in 0.1 M boric acid (pH 8.5) for 10 minutes. Sections were rinsed in PBS twice for 5 minutes each prior to application of primary antibodies, as described above.

### 3.1.5 Cell quantification

Labeled cells were counted in every 12th section throughout the DG (6 sections in total) by an experimenter blind to experimental condition. BrdU+ cells counted exhaustively under fluorescent illumination (Zeiss Axio Imager M2) using a 20x objective (Plan-Neofluar 0.5 NA). All BrdU+ cells located in the GCL or the SGZ were counted. DCX cells in the SGZ were quantified using optical fractionator (Stereo Investigator, MBF Bioscience). Counting was performed using a 40x objective (Plan-Neofluar 0.75NA), counting frame of 60 x 60 $\mu$m, sampling grids of 360 x 100 $\mu$m, and 1 $\mu$m guard zones. At least 166 cells were counted per WT mouse. Cell counts are reported separately for the anterior and posterior DG. Anterior was defined as approximately -0.94 to -2.54 mm relative to bregma and comprised sections 1-4 of the 6-section series (Paxinos, 2001). Posterior DG was defined as -2.70 to -3.40 mm, and comprised sections 5-6 of the 6-section series.

To assess co-localization of BrdU/NeuN and BrdU/GFAP, 20 BrdU+ cells were imaged per mouse. Z-stacks were collected for each cell using a 63x oil-immersion

objective (Plan-Aprochromat 1.4 NA) and a Zeiss ApoTome structured illumination module. The total number of double-labeled cells was estimated for each mouse by multiplying the proportion of double-labeled BrdU+ cells (in the 20-cell analysis) by the total number of BrdU+ cells (counted as described above).

### 3.1.6 Statistical analysis

Cell counts and body weights were analyzed with Prism6 (GraphPad software, La Jolla, CA) using student's *t* test for two-condition analyses and one-way ANOVA for analyses including more than two conditions. *Tukey's test* was used for *post-hoc* pairwise comparisons. Body weight data were analyzed with repeated measures ANOVA (RM ANOVA).

## 3.2 RESULTS

### 3.2.1 Generation of DCX-TK mice

We generated transgenic mice that express herpes simplex virus thymidine kinase (HSV-TK) under the doublecortin (DCX) promoter. HSV-TK catalyzes the conversion of a prodrug, GCV, into a toxic intermediate that terminates DNA synthesis, killing diving cells (Figure 3.1)(Beltinger et al., 1999). Thus, administration of GCV to DCX-TK transgenic mice suppresses hippocampal neurogenesis, while leaving the production of other cell types intact.

Ten transgenic DCX-TK founders were obtained and confirmed using Southern blot. Of these four were fertile. One line, line A, was maintained and used for further analysis. The mice appeared healthy and exhibited normal growth. In DCX-TK mice, TK+ cells were abundant in the SGZ (Figure 3.2*A*, *B*) and subventricular zones (SVZ)

(Figure 3.2*C*). In both regions, HSV-TK-expression was largely confined to DCX+ cells. The percentage of DCX+ cells expressing HSV-TK was greater than 80% (Figure 3.2*D*), and percentage of HSV-TK cells expressing DCX was greater than 90% (Figure 3.2*E*). Consistent with evidence that DCX is expressed at low levels outside of these canonical neurogenic niches, sparse HSV-TK expression was also observed in other cortical regions (see below).

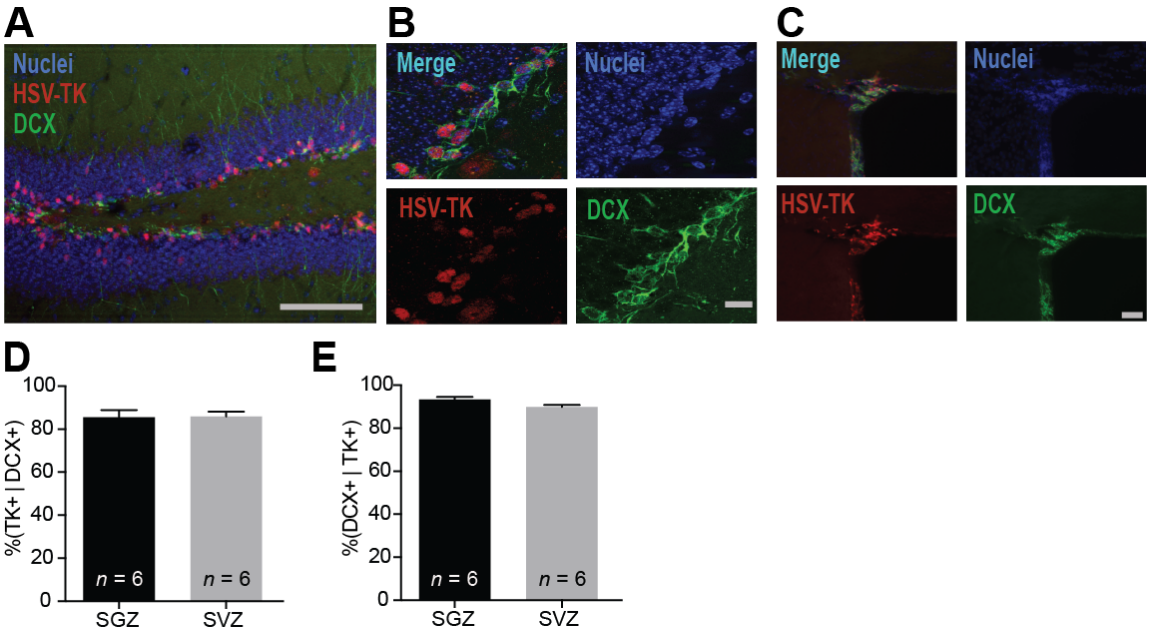


Figure 3.2. HSV-TK expression in DCX-TK transgenic mice.

***A*** - ***C***, DCX-TK mice expressed HSV-TK in DCX+ cells in the dentate gyrus (DG; ***A***, ***B***) and lateral ventricle (LV; ***C***). ***D***, Percentage of DCX+ cells expressing HSV-TK in the SGZ and SVZ. *E*, Percentage of HSV-TK+ cells expressing DCX. Scale bars represent 100 μm (***A*** and ***C***) or 10 μm (***B***).

### 3.2.2 Efficiency and specificity of ablation in the DG

To characterize the ablation efficiency and specificity, we treated the DCX-TK and WT mice with GCV or PBS for 2 weeks via ICV infusion. The mice were injected with BrdU one week before being euthanized (Figure 3.3*A*). Body mass increased over time during GCV administration and was not affected by genotype or drug (Figure 3.3*B*; RM-ANOVA: Group, $F_{(2, 11)} = 0.69$, $p = 0.52$; Time, $F_{(2, 22)} = 14.59$, $p < 0.001$; Group × Time, $F_{(4, 22)} = 0.70$, $p = 0.60$). To assess the effect of the treatments on neurogenesis, we quantified DCX+ cells in the SGZ. The number of DCX+ cells was greatly reduced (80 - 85%) in the anterior DG of DCX-TK/GCV mice as compared to the DCX-TK/PBS and WT/GCV groups (Figure 3.3*C*, *G*; $F_{(2, 11)} = 76.29$, $p < 0.001$; *Tukey*: DCX-TK/GCV Vs. WT/GCV or DCX-TK/PBS, $p < 0.001$). The control groups (WT/GCV or DCX-TK/PBS) did not differ from each other ($p = 0.26$). In posterior DG, the reduction of DCX+ cells in DCX-TK/GCV mice was somewhat smaller (60 - 65%) than that seen in the anterior GCL. Nevertheless, the reduction was significant (Figure 3.3*D*, *H*; $F_{(2, 11)} = 9.07$, $p = 0.005$; *Tukey*: DCX-TK/GCV Vs. WT/GCV or DCX-TK/PBS, $p < 0.001$).

Next, we sought to determine whether HSV-TK-expressing cells in non-neurogenic regions had been ablated by GCV administration. Consistent with evidence for DCX expression in neurons of the piriform cortex, DCX+/HSV-TK+ double-labeled cells were found in layer II of piriform cortex (Figure 3.3*E*, *F*). In contrast to DCX+ cells in the SGZ, these cells are not proliferative (Klempin, Kronenberg, Cheung, Kettenmann, & Kempermann, 2011) and, thus, should not be ablated by GCV treatment. Consistent with this hypothesis, the density of TK+ cells in piriform cortex did not differ between DCX-TK/GCV and DCX-TK/PBS mice (Figure 3.3*I*; $t_{(7)} = 0.12$, p = 0.430).

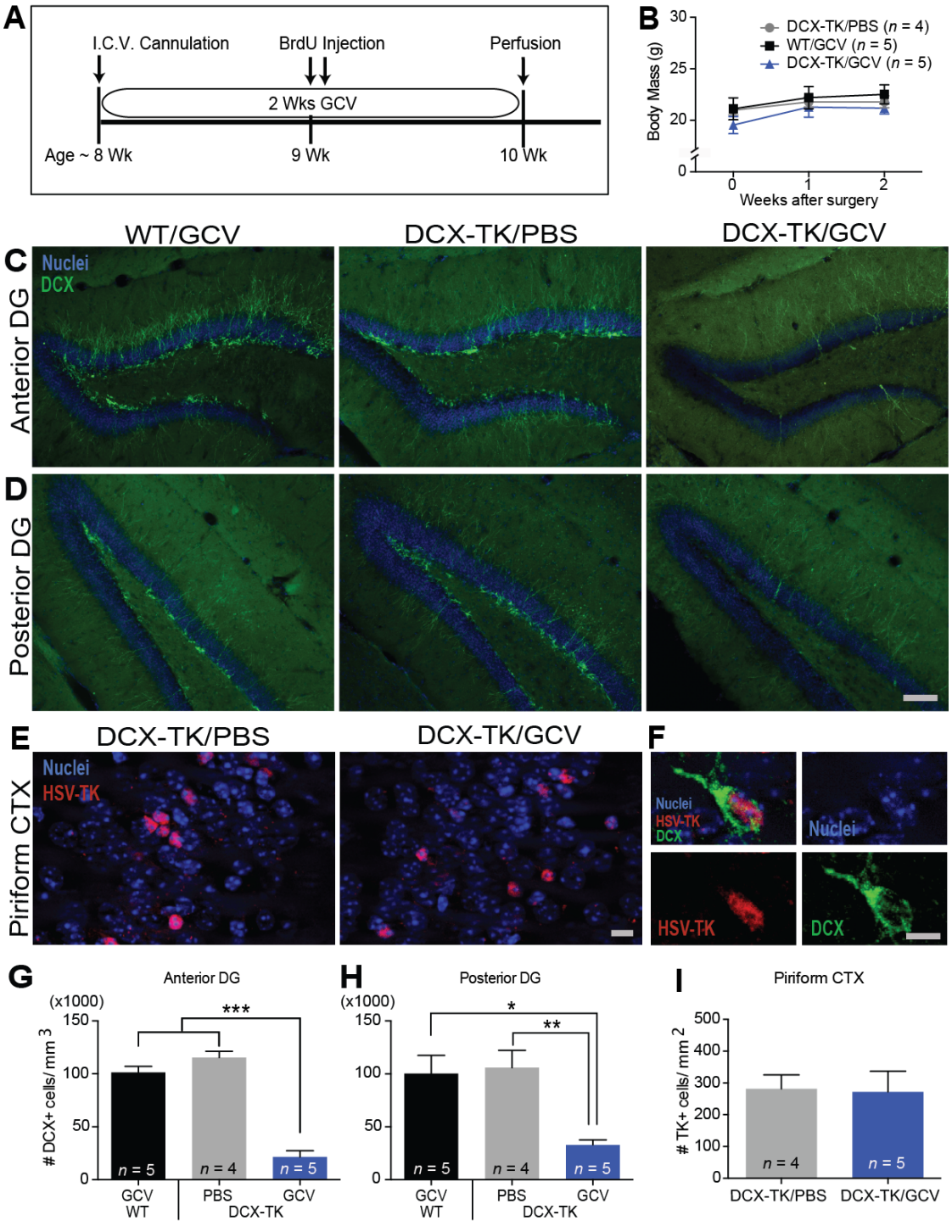


Figure 3.3. GCV treatment to the DCX-TK mice.

Figure 3.3

GCV administration to DCX-TK transgenic mice depletes DCX+ immature neurons in the DG but does not deplete putative quiescent DCX+ cells in the piriform cortex. A, DCX-TK and WT mice were treated with GCV or vehicle (PBS) for 2 weeks. Mice were injected with BrdU one week being euthanized. B, Body mass during the 2 weeks of GCV administration did not differ among DCX-TK/GCV, DCX-TK/PBS and WT/GCV mice. ***C***, ***D***, Representative images of DCX immunohistochemistry in the DG. ***E***, Immunohistochemistry against HSV-TK in the piriform cortex of DCX-TK transgenic mice. ***F,*** Representative image of a HSV-TK+/DCX+ double-labeled cell in the piriform cortex. Most HSV-TK+ cells in the piriform cortex also expressed DCX. ***G,H,*** The number of DCX+ cells in anterior and posterior DG was greatly reduced in DCX-TK/GCV mice relative to controls. ***E***, ***I***, In contrast, HSV-TK+ cells in the piriform cortex were not ablated by GCV treatment in DCX-TK mice. Scale bars represent 100 μm (***C*** and ***D)*** or 10 μm (***E*** and ***F)***. ***$p<0.001$.

We confirmed that the reduction in DCX+ cells in DCX-TK/GCV mice reflected a suppression of neurogenesis by quantifying BrdU+ cells. The number of BrdU+ cells was greatly reduced in the GCL of DCX-TK/GCV mice as compared to controls (Figure 3.4*A*). In the anterior DG, the number of BrdU+ cells was reduced by ~80% in DCX-TK mice as compared to controls (Figure 3.4*C*: anterior; One-way ANOVA: $F_{(2, 11)} = 11.82$, $p = 0.002$; *Tukey*: DCX-TK/GCV Vs. WT/GCV or DCX-TK/PBS, $p < 0.01$), and in posterior DG, there was ~70% reduction (Figure 3.4*C*: posterior; One-way ANOVA: $F_{(2, 11)} = 9.50$, $p = 0.004$; *Tukey*: DCX-TK/GCV Vs. WT/GCV, $p < 0.01$; DCX-TK/GCV Vs. DCX-TK/PBS, $p < 0.05$). The number of BrdU+ cells was also greatly reduced in the SVZ of DCX-TK/GCV mice as compared to controls (data not shown).

Next, we examined ablation specificity in the DG by assessing co-localization of BrdU with markers of neuronal (NeuN) and glial (GFAP) identity (Figure 3.4*B*). Because DCX is expressed in neuronal-committed progenitor cells, but not in multipotent stem-like cells (Brown et al., 2003; Wang, Kempermann, & Kettenmann, 2005; Kempermann et al., 2004), we predicted that GCV administration to DCX-TK mice would arrest neurogenesis but not gliogenesis. Consistent with this prediction, the number of BrdU+/NeuN+ adult-born neurons was greatly reduced in DCX-TK/GCV mice as compared to controls (Figure 3.4*D*; $t_{(8)} = 4.23$, $p = 0.004$), but the number of BrdU+/GFAP+ newborn glia did not differ among groups (Figure 3.4*E*; $t_{(8)} = 0.19$, $p = 0.596$). This result suggests that DCX-TK/GCV system specifically arrests adult neurogenesis.

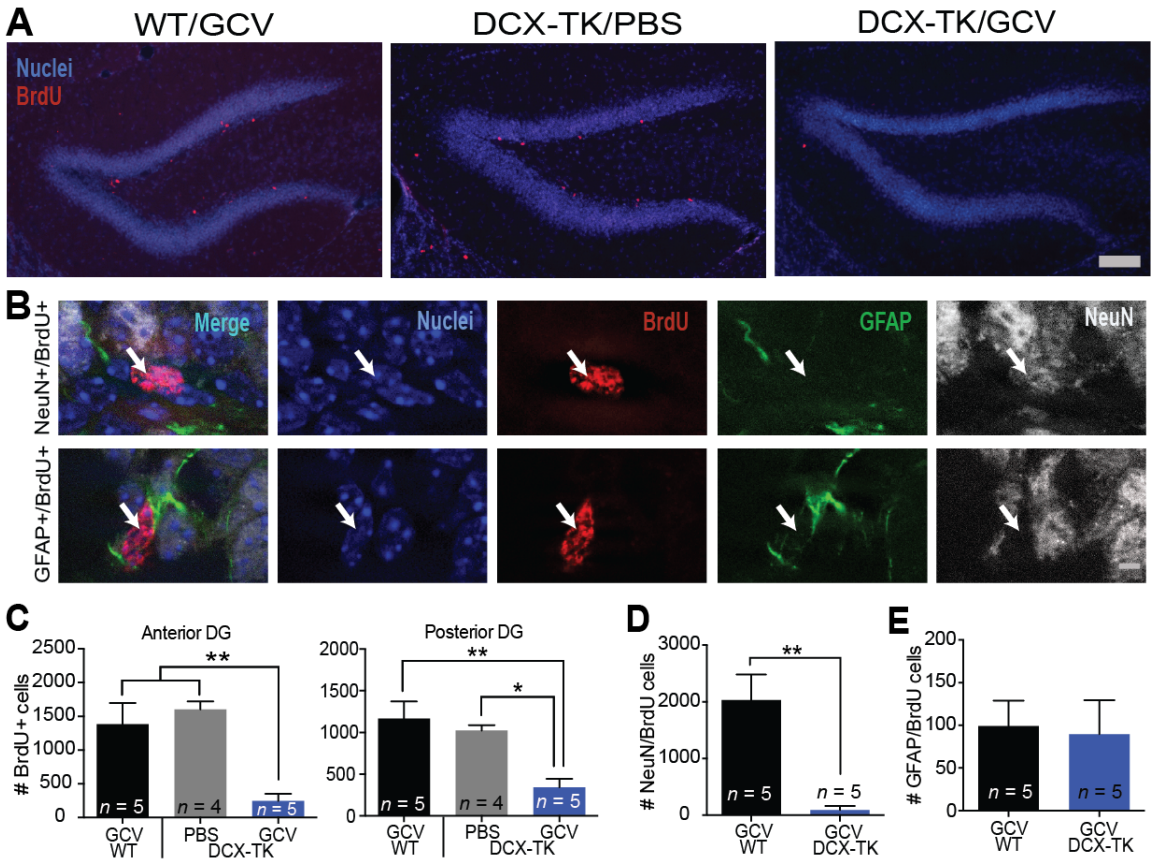


Figure 3.4. Specificity of ablation in two-week DCX-TK/GCV system.

GCV administration to DCX-TK mice suppresses DG neurogenesis but not gliogenesis. ***A***, Representative images of BrdU immunohistochemistry in the DG. ***B***, Examples of BrdU+ cells co-labeled with NeuN or GFAP. ***C***, In both anterior and poterior DG, the number of BrdU+ cells was greatly reduced in DCX-TK/GCV mice relative to controls ***D, E***, Quantification of BrdU/GFAP and BrdU/NeuN double-labeled cells. The proportion of BrdU cells expressing NeuN was reduced in DCX-TK/GCV mice relative to WT/GCV controls (***D***). However, the proportion of BrdU cells expressing GFAP did not differ between DCX-TK/GCV and WT/GCV mice (***E***). Scale bars represent 100μm (***A***) or 5 μm (***B***). *$p$<0.05, **$p$<0.01.

### 3.2.3 Inflammatory reaction in DCX-TK/GCV system

To determine whether GCV treatment to DCX-TK mice evoked an inflammatory response, we performed immunohistochemistry against Iba-1, a marker of microglia that is upregulated upon microglial activation associated with inflammation (Ito, Tanaka, Suzuki, Dembo, & Fukuuchi, 2001). Animals were treated with either GCV or PBS for 2 weeks (Group: WT/GCV, DCX-TK/PBS, or DCX-TK/GCV) and then euthanized (WT/GCV, $n = 5$; DCX-TK/PBS, $n = 4$; DCX-TK/GCV, $n = 5$) or two weeks ($n = 3$ in each group) after the end of GCV treatment. Iba-1 expression intensity was measured in the DG. No significant effects of Group or interaction were detected (Figure 3.5*B*; Two-way ANOVA: Group, $F_{(2, 17)} = 2.05$, $p = 0.160$; Group × Recovery Interval, $F_{(2, 17)} = 0.14$, $p = 0.870$). However, the main effect of Recovery Interval was significant (Figure 3.5*B*; $F_{(1, 17)} = 82.24$, $p < 0.001$) indicating that the microglial activation declines over time after surgery. These results suggest microglial activation was induced by surgery, not by GCV treatment or genotype *per se*.

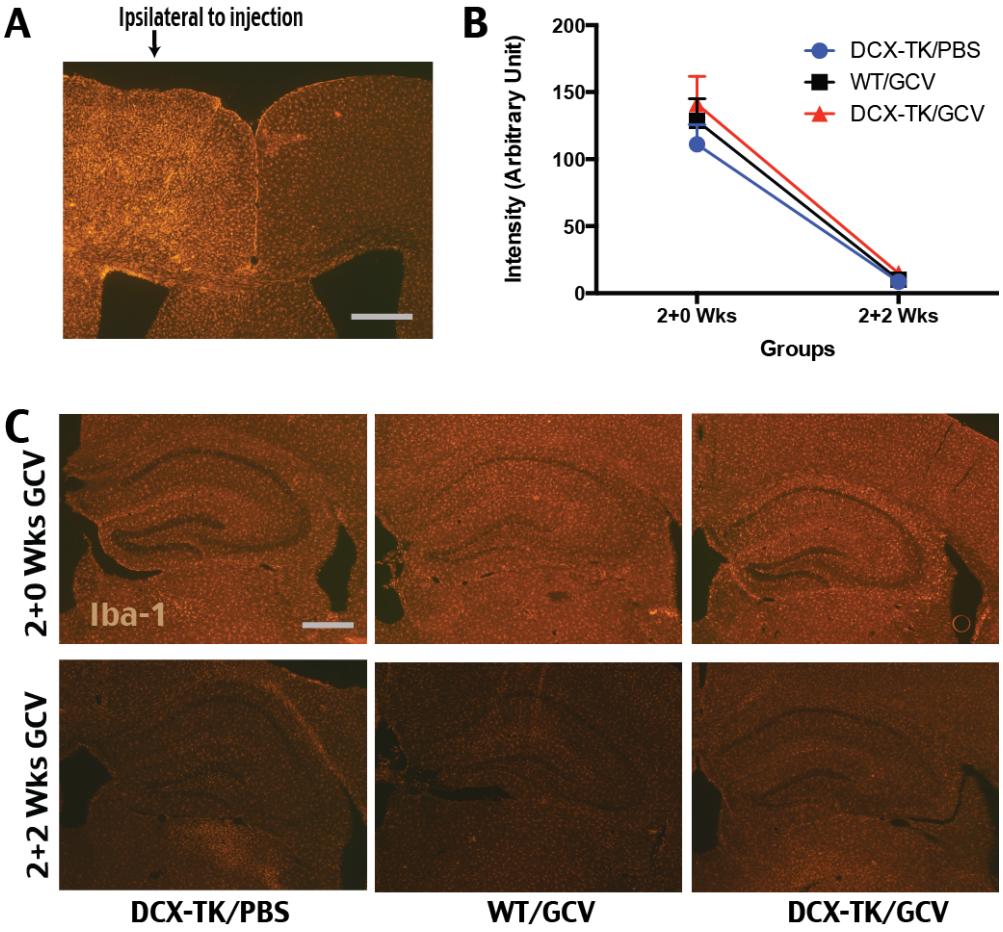


Figure 3.5. Microglia activity was measured using Iba-1.

Animals were treated with either GCV or PBS for two weeks (2+0 Wks) through ICV. injection or given a two-week drug free period after the end of two-week drug treatment (2+2 Wks). ***A***, The cannulated hemisphere two weeks after the surgery showed high intensity of Iba-1 expression. ***B*** and ***C***, In the region of the DG, the Iba-1 expression intensity was similar among groups two weeks after surgery (2+0 Wks). However, the Iba-1 expression dramatically reduced through the two-week recovery period (2+2 Wks). The scale bars (***C***), 500μm

## 3.3 DISCUSSION

We developed a new transgenic mouse model (DCX-TK) to achieve specific ablation of adult neurogenesis. The transgenic mice expressed HSV-TK in DCX-positive cells. The treatment, consisting of two weeks of GCV, dramatically reduced DCX- and BrdU-positive cells in the DCX-TK mice, but not in the WT mice, indicating that the DCX-TK/GCV system is an efficient method of arresting adult neurogenesis.

The two-week GCV treatment of the DCX-TK mice ablated adult-born neurons more efficiently in the anterior part of the DG than the posterior part (*Please see section 3.2.2*). There might be two explanations for the differences in the GCV-mediated ablation efficiency across the anterior-posterior axis. One is the low penetrance of the drug in the ventral area. In one-week GCV treatment experiment, we observed a partial ablation of adult neurogenesis, with the ablation efficiency decreasing with increasing distance from the cannula (data are not shown). The observation suggests that the drug did not reach high concentrations in the ventral area because it was farther from the cannulation site. However, this hypothesis does not explain the observation that cell proliferation as measured by BrdU did not differ across the anterior-posterior axis. The other possible explanation is the slow speed of cell maturation in the ventral area. Recent studies showed that newborn cells in the posterior DG mature more slowly than those in the anterior DG (Snyder, Ferrante, & Cameron, 2012; Piatti et al., 2011). We speculate that because the time window of development of adult neurogenesis is longer in posterior DG than anterior DG, turnover of the immature neuron population is slower in the posterior than anterior DG. Therefore, to completely ablate newborn neurons that are in the post-mitotic periods, it may be necessary to wait longer after the two-week GCV treatment

before assessing the role of adult neurogenesis in behavioral tasks. Our behavioral experiments were designed with this concern in mind.

In the current study, to specifically ablate neuronal precursors in the major neurogenic zones, we used the *dcx* gene to drive expression of HSV-TK. DCX has been reported to be expressed exclusively in the neuronal lineage. Therefore, we hypothesized that the GCV treatment in the DCX-TK mice would suppresses neurogenesis but not gliogenesis. As we predicted there were no changes in gliogenesis indicated by GFAP+/BrdU+ co-labeling while there were dramatic reductions in neurogenesis, indicated by NeuN+/BrdU+. These results suggest that the DCX-TK/GCV system will allow us to suppress adult neurogenesis with more cellular specificity than existing methods. This new method will enable us to more precisely characterize the role of adult hippocampal neurogenesis in cognition and emotional regulation.

## Chapter 4: The role of adult neurogenesis in trace fear conditioning

Different temporal relationships between stimuli produce different levels of association strength but also engage different neural systems. For example, in Pavlovian conditioning, two different experimental procedures are widely used with different temporal contiguity. In the delay fear conditioning procedure, a CS, such as a neutral tone is presented and remains present until a US, such as a footshock, is presented. In the trace conditioning procedure, there is a temporal gap between the offset of the CS and the onset of the US. Many studies have demonstrated that normal hippocampal function is necessary for acquiring a CS-US association in trace conditioning but not in delay conditioning (Raybuck & Lattal, 2013; Raybuck & Lattal, 2011; Quinn et al., 2005; Burman & Gewirtz, 2004; Knight et al., 2004; Quinn, Oommen, Morrison, & Fanselow, 2002; Tseng et al., 2004). Furthermore, some studies have suggested that the DG actively contributes to trace conditioning (Weitemier & Ryabinin, 2004; Gilmartin & McEchron, 2005).

The findings that the DG is a region where adult neurogenesis takes place have led efforts to investigate a link between hippocampus-dependent associative learning, such as trace conditioning, and hippocampal adult neurogenesis. Similar to studies showing that hippocampus-dependent learning (e.g. water-maze) enhances neurogenesis and the survival of the adult-born neurons (Dobrossy et al., 2003; Sisti et al., 2007), some studies have shown that trace conditioning, but not some forms of delay conditioning, also enhance the survival of adult born-neurons in the DG. The surviving new-born neurons induced by learning remain in the hippocampus for at least two months after learning, implying that newborn neurons may support the retention of trace memories

later (Gould et al., 1999a; Leuner et al., 2004). Further studies have suggested that the survival of newborn neurons was more effectively enhanced when learning tasks demand more effort. For example, CS pre-exposure before trace eyeblink conditioning (latent inhibition) or longer interstimulus interval (e.g. 250ms Vs. 500ms) trace eyeblink conditioning increase the survival of adult-born neurons compared to regular trace eyeblink conditioning procedure (Waddell & Shors, 2008; Waddell, Anderson, & Shors, 2011).

Nevertheless, it is still questionable whether the adult-born neurons are required for trace conditioning. Some studies have shown that arresting adult neurogenesis impairs trace conditioning (Shors et al., 2002; Shors et al., 2001). For example, Shors and colleagues (2001) showed that arresting adult neurogenesis using an anti-mitotic drug, MAM, results in the impairment of trace eyeblink conditioning in rats. The same group also demonstrated a similar effect in trace fear conditioning procedure in rats (Shors et al., 2002). However, the role of adult neurogenesis in trace conditioning remains debated because the ablation methods used in the aforementioned studies were not neurogenesis-specific. In addition, other studies have shown little or no effect of neurogenesis ablation in trace conditioning (Achanta et al., 2009; Cuppini et al., 2006). Cuppini and colleagues (2006) compared trace fear conditioning performance between different age groups of rats (2, 5, and 12 months). Neural proliferation declined with age. However, trace conditioning was not affected by age, even though the magnitude of neurogenesis decline with age (about 95% between 2 and 12 months of age) exceeded that caused by the antimitotic drug used by Shors' group (about 75%). The trace fear conditioning protocol was similar in the two studies. There is even a study showing enhanced trace cue memory

after the ablation of adult-born neurons. Jaholkowsky and colleagues (2009) investigated the role of adult neurogenesis using a mutant mouse model in which cyclin D2 protein is absent (D2 KO mice). In this mutant mouse model, major brain structures appear normally formed during development, but there was specific suppression of adult neurogenesis. Authors trained the D2 KO mice in trace fear conditioning protocol to test if adult born neurons contribute to trace learning. Mutant mice displayed normal sensory motor behavior. In the trace fear conditioning experiment, however, the D2 KO mice showed higher freezing response to the trace cue compared to the WT mice, which contradicts Shors and colleages' early findings (2001 and 2002). It is unclear what variables led to the inconsistency of the results because there has not been a systematic study. The inconsistencies could relate to differences in species, training protocol, ablation method, or other unidentified variables.

In this chapter, we revisit the role of adult neurogenesis in trace fear conditioning using methods that may provide a more specific arrest of adult hippocampal neurogenesis. First, we used the DCX-TK transgenic mouse model for the investigation, which may provide a more specific arrest of adult hippocampal neurogenesis than existing techniques (*please see chapter 3*). DCX-TK and WT mice were treated with GCV and trained in either delay or trace fear conditioning procedure. Next, we used hippocampus-targeted x-irradiation to ablate neurogenesis, specifically in the hippocampus area. Both the mice lacking neurogenesis and the intact mice displayed similar levels of tone fear in both delay and trace procedures. However, an unexpected difference in context-elicited fear emerged. The mice lacking neurogenesis displayed significantly higher context fear than the control mice after trace conditioning whereas

there were no significant differences between the groups after delay conditioning. The data suggest that adult neurogenesis is involved in the modulation of context memory in trace fear conditioning.

## 4.1 MATERIALS AND METHODS

### 4.1.1 Animals

For DCX-TK mice experiments (*Section 4.2.1*, *4.2.2*, *4.2.3*), the same transgenic mice described in *Chapter 3* were used. Approximately equal numbers of 8-week-old male and female DCX-TK and WT littermates were used in each experiment. For the hippocampus targeted x-irradiation experiment (*Section 4.2.4*), 8-week-old C57BL/6J male mice were used.

### 4.1.2 X-ray irradiation

X-irradiation was performed as described previously (David et al., 2009; Santarelli et al., 2003; Denny et al., 2012). Mice were anesthetized with sodium pentobarbital (6 mg/kg), placed in a stereotaxic frame and exposed to cranial irradiation using a Siemens Stabilopan X-ray system operated at 300 kVp and 20 mA. Mice were protected with a lead shield that covered the entire body, but left unshielded a 3.22 X 11-mm treatment field above the hippocampus (interaural 3.00 to 0.00). The dose rate was approximately 1.8 Gy per min at a source to skin distance of 30 cm. Three 5-Gy doses were administered over 8d.

### 4.1.3 Fear conditioning

Fear conditioning took place in Med-Associates conditioning chambers (30 cm wide x 24 cm deep x 22 cm high). The chambers could be configured as two distinct contexts, A and B. Context A was rectangular, had floors made of stainless steel rods (2 mm diameter, spaced 5 mm apart), had walls of aluminum and acrylic, was scented with acetic acid, and was cleaned with 70% ethanol between runs. Context B had a white acrylic floor, had an acrylic A-frame roof, and was cleaned with antiseptic wipes (Chlorox Fresh Scent). The training session and context test occurred in context A. The tone test occurred in context B. All sessions were recorded from the side using a digital camera. Freezing behavior was scored using a pixel-change algorithm (VideoFreeze, Med Associates Inc., St. Albans, VT).

#### *4.1.3.1 Protocol 1A for DCX-TK experiments (Figure 4.1.-4.3.)*

Training consisted of 4 conditioning trials during a 440s session in context A. The CS was a tone (85 dB, 5000 Hz). The US was footshock (2s, 0.8mA). The tone was presented at 120, 200, 270, and 360s into the session. In delay conditioning, the footshock occurred at tone offset. In trace conditioning, the footshock commenced 20s after tone offset.

On the day following training, the mouse was returned to context A for 5 min for a test of context-elicited fear. Neither tones nor shocks were presented. Freezing behavior was scored throughout the session. On the third (trace and shock-alone conditioning) or fourth (delay conditioning) day, the mouse was placed into context B for a test of tone-elicited fear. The tone was presented 4 times for 20s each at 120, 200, 270, and 360s into

the session. Delay fear-conditioned mice received a test session in context B to a novel auditory stimulus, a white noise, on the third day.

#### *4.1.3.2 Protocol 1B for x-irradiation experiments (Figure 4.4.)*

Context A was as described above. Context B was created by covering the walls with curved plastic inserts and covering the floor with bedding material. Context B was scented with lemon extract and cleaned with nonalcohol disinfectant wipes. On the day before training, mice received 3-min pre-exposures to context A and B. Training consisted of 5 pairings between a 20-s tone (85dB, 2000 Hz) and a footshock (2s, 0.65mA) in context A. The tones were presented at 190, 465, 690, 990, and 1190s into the session. The shocks occurred at tone offset (delay conditioning) or 20 s after tone offset (trace conditioning). The test of tone-elicited fear occurred in context B on the day after training. The tone was presented for 20s each at 190 and 330s into the session. The context test occurred on the following day. Mice were placed in context A for 5 min with no tones or shocks presented.

### 4.1.4 Shock reactivity

The unconditioned response to footshock was assessed by estimating horizontal distance traveled during the shock and by quantifying the frequency of four behaviors commonly exhibited during footshock: running, jumping, backward shuffling, and forward shuffling. Shuffling was defined as locomotion without full limb extension. Horizontal distance was estimated by quantifying crossings of a 4-cell grid superimposed over the conditioning chamber.

### 4.1.5 Statistical analysis

Behavioral data were analyzed with JMP11 Pro (SAS institute, Cary, NC). *Student's t*-test, *one-way* or *two-way ANOVA*s were used to analyze between-subjects designs. Repeated-measures designs were analyzed using mixed-effects restricted maximum likelihood (REML) model. The null hypothesis was rejected at the $p < 0.05$ level.

## 4.2 RESULTS

### 4.2.1 Effects of DCX-TK-mediated arrest of adult neurogenesis on trace and delay fear conditioning

To determine whether adult-born neurons are involved in trace fear conditioning, DCX-TK or WT mice were subjected to either the delay or trace fear conditioning procedure (*please see section 4.1.3.1 protocol 1A*).

We predicted that arrest of adult neurogenesis would impair hippocampus-dependent trace fear conditioning but have no effect on delay fear conditioning, which typically does not require hippocampal integrity. Delay and trace fear conditioning were assessed in separate groups of WT and DCX-TK mice treated with GCV. Fear conditioning occurred one week after the end of 2-week GCV treatment (Figure 4.1*A*). Mice received 4 pairings between a tone and shock using a delay (shock occurred at tone offset) or trace (shock occurred 20s after tone offset) protocol.

On the training day (Day1), freezing levels were measured in 10s bins across the entire session. All groups reached about 80% freezing after the last training trial. The freezing levels in time bins were analyzed using REML. During delay fear training, freezing levels increased more rapidly in WT/GCV than in DCX-TK/GCV mice (Figure

4.1*B*:Delay; Genotype × Time, $F_{(43,1075)} = 1.82$, $p = 0.001$*)*. The opposite pattern was observed in trace fear training (Figure 4.1*B*:Trace; Genotype × Time, $F_{(43,1161)} = 2.09$, $p < 0.001$). However, freezing levels after the final shock were equivalent between the two genotypes in both trace ($t_{(27)} = -1.89$, $p = 0.07$) and delay ($t_{(25)} = -0.56$, $p = 0.58$) conditioning.

On the next day, mice were placed into the training context for a test of context-elicited fear. Consistent with previous data (Clark et al., 2008; Drew et al., 2010), in delay fear conditioning, there was no effect of genotype on the level of context-elicited freezing (Figure 4.1*C*; Genotype, $F_{(1,25)} = 0.01$). After trace fear conditioning, however, DCX-TK/GCV mice froze significantly more than WT/GCV mice throughout the context test (Figure 4.1*C*; $F_{(1,27)} = 7.10$, $p = 0.013$).

On the third day, mice were placed into a novel context for a test of tone-elicited fear. Freezing was compared during the baseline period (20s before the first tone) to the mean freezing during the CS and the post-CS periods (20s after tone-off). The data were analyzed using Period (Baseline, CS, and Post-CS) × Genotype REML. Based on evidence that the hippocampus controls fear generalization in auditory fear conditioning (Cushman et al., 2012; Quinn, Wied, Liu, & Fanselow, 2009), delay-trained mice were tested for freezing in response to a novel auditory stimulus, a white noise, in addition to the original tone stimulus. Both genotypes displayed freezing to the tone and white noise (Figure 4.1*D*, *E*), and there was no effect of genotype on freezing to either the white noise (Genotype, $F_{(1,25)} = 0.68$, $p = 0.417$; Period, $F_{(2,50)} = 65.90$, $p < 0.001$; *Tukey*: Baseline Vs. WN or Post-WN, $p < 0.001$, WN Vs. Post-WN, $p = 0.949$) or the tone

(Genotype, $F_{(1,25)} = 0.46$, $p = 0.504$; Period, $F_{(2,50)} = 38.75$, $p < 0.001$;*Tukey*: Baseline Vs. CS or Post-CS, $p < 0.001$, CS Vs. Post-CS, $p = 0.014$).

Trace conditioned mice also exhibited freezing in response to the tone (Figure 4.1*D*, *E*), and there was no effect of genotype during the baseline, CS or post-CS periods (Genotype, $F_{(1,27)} = 2.36$, $p = 0.137$; Period, $F_{(2,54)} = 40.94$, $p < 0.001$; *Tukey*: Baseline Vs. CS or Post-CS, $p < 0.001$, CS Vs. Post-CS, $p = 0.851$). However, when the entire baseline period was analyzed (2 min before the first CS presentation), DCX-TK/GCV mice froze significantly more than WT/GCV mice ($t_{(27)} = -3.59$, $p < 0.01$).

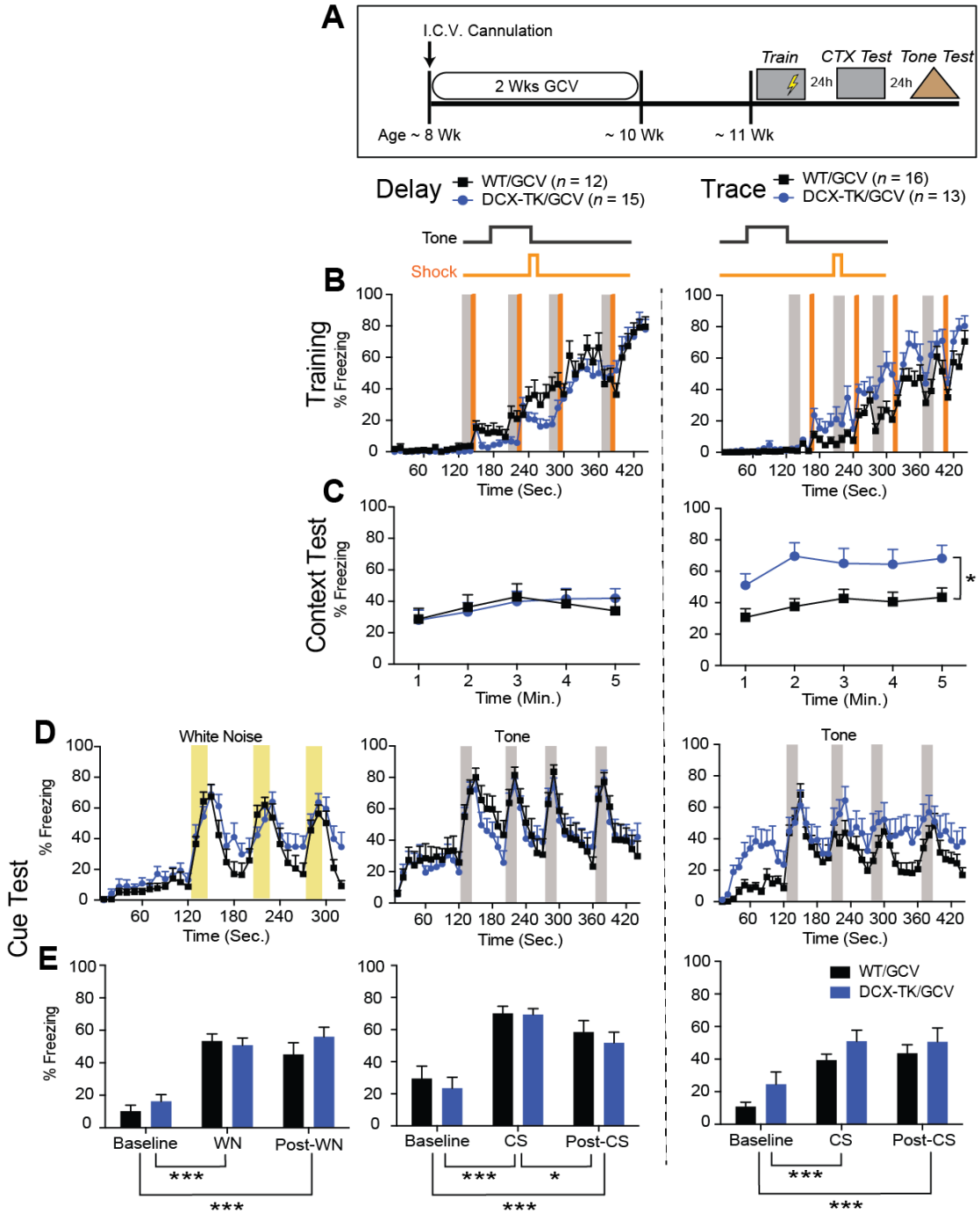


Figure 4.1. Effects of DCX-TK-mediated arrest of adult neurogenesis on trace and delay fear conditioning.

Figure 4.1

***A***. DCX-TK and WT mice were trained in delay or trace fear conditioning one week after a two-week GCV treatment. ***B***, During delay training, WT mice displayed a more rapid acquisition of freezing behavior than DCX-TK mice. In contrast, during trace training DCX-TK mice displayed a more rapid acquisition of freezing behavior. However, freezing levels in the final minute of the training session (after the final shock) were equivalent between the two genotypes in both Delay and Trace conditioning. ***C***, Freezing during the context test. In trace but not delay conditioning, DCX-TK/GCV mice displayed significantly higher freezing than WT/GCV mice. ***D***, Freezing in response to the tone (trained CS) or white noise (novel stimulus) in a novel context. ***E***, Mean freezing during the baseline (20s before presentation of auditory stimuli), during presentation of the auditory stimuli, and during the 20s after presentation of auditory stimuli. In delay conditioning, WT/GCV and DCX-TK/GCV displayed freezing in response to both the tone and white noise, and there was no effect of genotype on freezing to either stimulus. Similarly, in trace conditioning, mice displayed freezing in response to the tone, and there were no genotype effect. *$p<0.05$, ***$p<0.001$.

### 4.2.2 Shock reactivity during trace fear conditioning

To determine whether the increased context-elicited freezing in trace-trained DCX-TK/GCV mice related to a change in shock sensitivity, we assessed the behavioral response to shock during trace training. Figure 4.2*A* shows general activity (number of grid crossings) during each of the four shock presentations during the training. The number of grid crossings did not differ by genotype (Two-way ANOVA: Genotype, $F_{(1,27)} = 0.96$, $p = 0.943$; Trial, $F_{(3,81)} = 5.25$, $p = 0.002$; Genotype × Trial interaction, $F_{(3,81)} = 0.39$, $p = 0.760$). We also quantified the frequency of four behaviors commonly displayed during shock across the 4-trial session (running, jumping, shuffling, and backward shuffling). There was no effect of genotype on the relative frequency of each behavior (Figure 4.2*B*; Two-way ANOVA: Genotype, $F_{(1,108)} = 0.49$, $p = 0.488$; Genotype × Trial interaction, $F_{(3,108)} = 0.33$, $p = 0.807$).

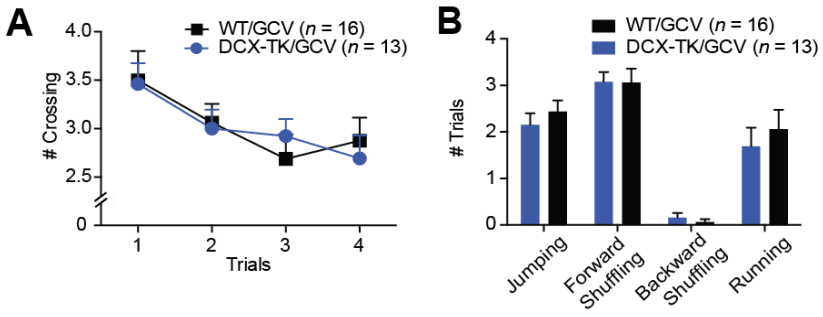


Figure 4.2. Shock reactivity during trace fear conditioning.

***A***, The number of crossings of the conditioning chamber did not differ between genotypes. ***B***, The two genotypes showed similar behaviors during the shock-induced activity burst.

### 4.2.3 Trace fear conditioning in vehicle-treated DCX-TK and WT mice

To confirm that the increased fear in trace conditioned DCX-TK mice was caused by neurogenesis ablation rather than an extraneous effect of the transgene (e.g., a site-of-integration effect), PBS-treated DCX-TK and WT mice were subjected to trace fear conditioning using the procedure described above.

In the training session there was no effect of Genotype or of the Genotype × Time interaction (Figure 4.3*A*; REML: Genotype, $F_{(1,30)} = 0.85$, $p = 0.365$; Genotype × Time, $F_{(43,1290)} = 0.82$, $p = 0.796$). On the day following training, contextual fear was assessed in the training context. There were no effects of Genotype or the Genotype × Time interaction (Figure 4.3*B*; REML: Genotype, $F_{(1,30)} = 0.12$, $p = 0.73$; Genotype × Time, $F_{(3,90)} = 1.10$, $p = 0.355$). On the third day, tone fear was assessed in a novel context. Both groups displayed freezing in response to the tone, and there was no effect of Genotype or the Genotype × Period (Baseline, CS, Post-CS) interaction (Figure 4.3*C*, *D*; REML: Genotype, $F_{(1,30)} = 1.27$, $p = 0.269$; Period, $F_{(2,60)} = 61.21$, $p < 0.001$; Genotype × Period, $F_{(2,60)} = 0.06$, $p = 0.94$; *Tukey*: Baseline Vs. CS, $p < 0.001$, Baseline Vs. Post-CS, $p < 0.001$, CS Vs. Post-CS, $p < 0.001$).

In summary, arrest of adult neurogenesis was associated with enhanced context fear in trace but not delay conditioning. The enhancement was not caused by increased shock sensitivity or by a nonspecific effect of the DCX-TK transgene.

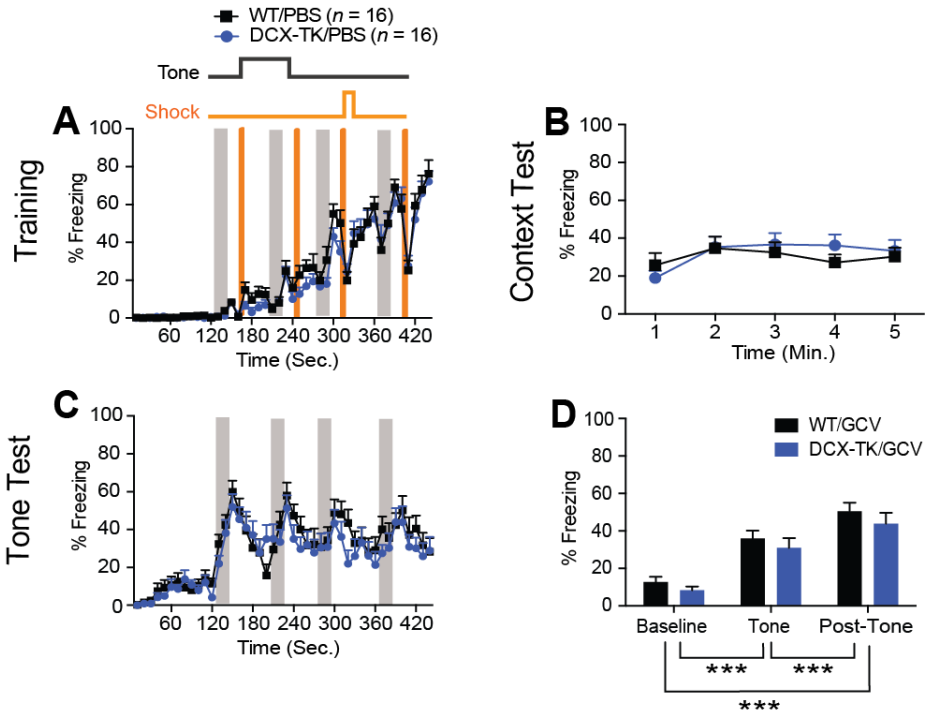


Figure 4.3. Trace fear conditioning in vehicle-treated DCX-TK and WT mice.

DCX-TK or WT mice were given trace conditioning one week after the two-week PBS treatment. ***A***, In the training session there was no effect of Genotype on freezing. ***B***, Freezing during the test of context-elicited fear. There was no effect of genotype on freezing. ***C***, Freezing during the test for tone-elicted fear in a novel context. ***D***, Mean freezing during the tone test. Both genotypes displayed freezing in response to the tone, and there was no effect of Genotype on freezing. *$p<0.05$, ***$p<0.001$.

### 4.2.4 Effect of targeted cranial irradiation on delay and trace fear conditioning

To confirm that the unexpected phenotype in trace fear conditioning was caused by the arrest of adult hippocampal neurogenesis rather than another effect of the ablation system, we assessed trace and delay fear conditioning using an alternate neurogenesis ablation method. Fear conditioning was conducted 6 weeks after targeted x-irradiation (Figure 4.4*A*, *B*). Mice received 5 pairings between a tone and shock using a delay or 20s trace protocol.

In the tone test session (Figure 4.4*C*, *D*), we compared freezing during the baseline period to the mean freezing during the CS and the post-CS periods. The data were analyzed using a Period (Baseline, CS, and Post-CS) × Treatment ANOVA. In each protocol there was a significant effect of Period ($F$'s$_{(2,36)} > 12$, $p$'s $< .001$), confirming that freezing during the CS and Post-CS periods exceeded that during the Baseline period. The effects of Treatment ($F$'s$_{(1,18)} < 1$) and the interaction ($F$'s$_{(2,36)} < 2.25$, $p$'s $> .12$) were not significant. Arrest of neurogenesis via x-irradiation failed to impair trace fear conditioning.

Consistent with the DCX-TK experiments, in the context test session, there was an effect of neurogenesis ablation in the trace protocol but not the delay protocol (Figure 4.4*E*). In delay conditioning there was no effect of x-irradiation on context-elicited freezing. A Time × Treatment ANOVA confirmed no effect of Treatment ($F_{(1,18)} < 1$) or of the interaction ($F_{(3,54)} = 1.4$, $p = .26$). In trace conditioning, however, x-irradiated mice displayed significantly stronger context-elicited freezing (Main effect of Treatment: $F_{(1,18)} = 5.2$, $p = .035$). Thus, ablation of adult neurogenesis by either DCX-TK/GCV or x-irradiation caused enhanced context fear in trace but not delay fear conditioning.

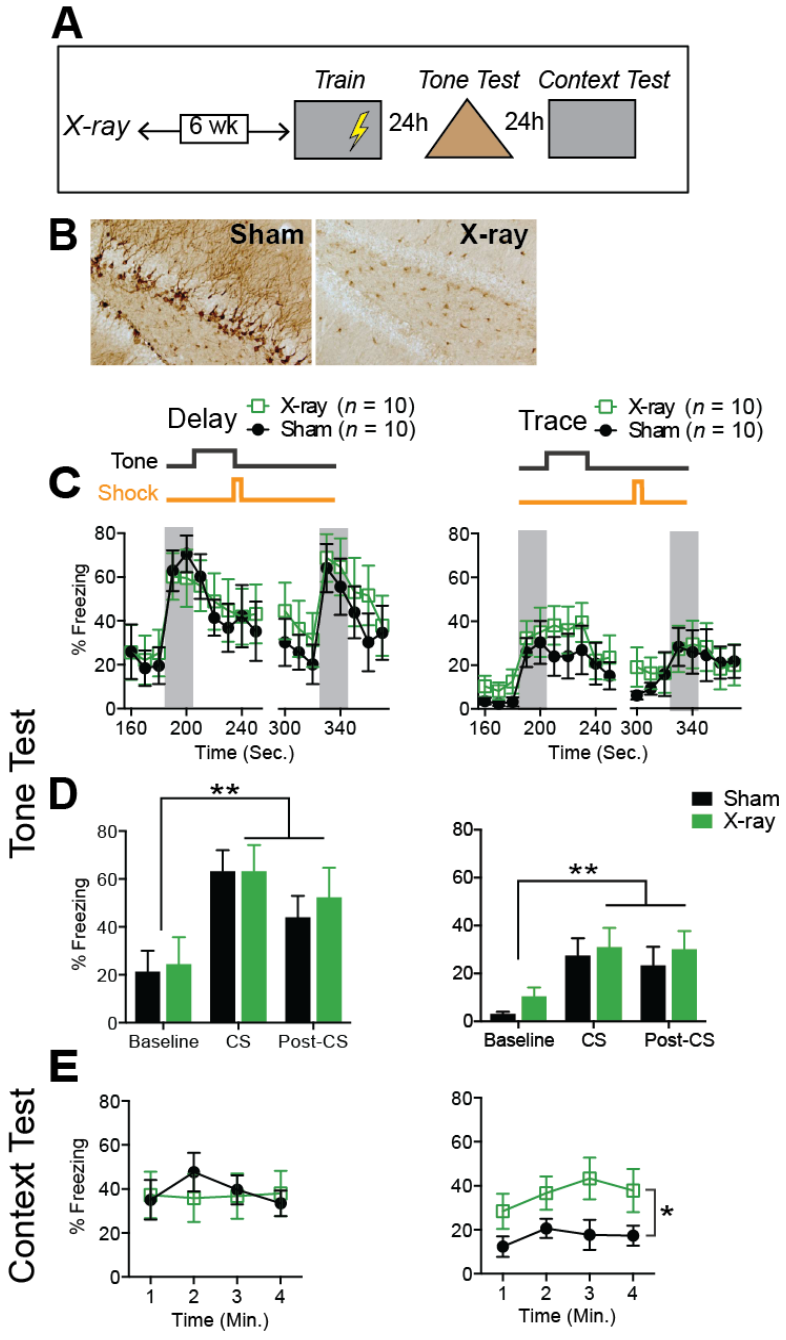


Figure 4.4. Effect of x-irradiation-induced ablation of adult neurogenesis on trace and delay fear conditioning.

Figure 4.4

***A***, Fear conditioning was conducted 6 weeks after the first of 3 doses of x-irradiation. ***B***, DCX immunhistochemistry confirmed that DCX+ immature neurons were greatly reduced in x-irradiated mice. ***C***, Freezing as a function of time during the tone test session. ***D***, Mean freezing during the baseline period (20s before presentation of the first tone), the tone presentations, and the 20s following each tone presentation. In both delay and trace training, there was a significant effect of Period but no effect of x-irradiation treatment and the interaction. ***E***, Consistent with the DCX-TK experiments, in the context test, there was an effect of neurogenesis ablation in the trace conditioning but not delay conditioning. In trace conditioning, x-irradiated mice displayed more context-elicited freezing than sham controls, but in delay conditioning the treatment groups did not differ. $*p<0.05$, $**p<0.01$.

## 4.3 DISCUSSION

Taking advantage of the specific ablation of adult neurogenesis using the DCX-TK/GCV system, we tested if adult-born neurons are involved in trace fear conditioning. DCX-TK or WT mice were treated with GCV for two weeks. One week after the cessation of the drug infusion, the animals were trained in either delay or trace conditioning. In both delay and trace training, DCX-TK and WT mice treated with GCV finished each task with similar levels of freezing. However, in the context memory test, the DCX-TK mice showed enhanced contextual fear after trace fear conditioning, but not delay fear conditioning. A similar pattern of results was observed when x-irradiation was used to ablate adult-born neurons.

The enhanced contextual fear memory in trace fear conditioning was an unexpected result, but we have observed this behavioral pattern consistently through two different hippocampal neurogenesis ablation systems: DCX-TK/GCV system and x-irradiation. Some hypotheses are considered to explain the underlying mechanism of this behavioral pattern. One hypothesis is that the enhanced contextual memory is due to the failure of neurogenesis-arrested mice to acquired the trace CS-US association during trace conditioning. Many learning theories include the idea that when multiple cues are presented together, each cue competes with the others for a limited amount of associative strength (Rescorla & Wagner, 1972; Urushihara & Miller, 2009). When animals are trained in tone-shock paring, the tone competes with the context (Marilyn, 1981). If the mice lacking neurogenesis failed to associate trace CS and US, this could enhance conditioning to the context. However, this cue competition hypothesis cannot be accepted at present, because, in the current experiment, we did not detect an impairment of trace tone fear in the DCX-TK mice. Nevertheless there are two reasons why our procedure

may have been insensitive to a real impairment in the tone-shock association in the DCX-TK mice.

First, there is the possibility that the different basal emotional state in the cue memory test masked the impairment of tone-shock association. In the tone tests, the different freezing level between the two genotypes in pre-CS periods complicates interpretation of the tone freezing. For example, the different initial emotional state may modulate tone response differently. In the current trace fear conditioning experiment, the DCX-TK mice froze more than the WT mice during the pre-CS period. It is possible that the higher fear level in the DCX-TK mice amplified weak cue memory, and eventually resulted in a similar freezing level to the cue with the WT mice. Thus, the baseline issue makes it difficult to conclude that animals have the same levels of the trace CS-US association between the two genotypes.

A second, but related, possibility is that nonassociative learning was influential in the current fear conditioning procedure. In the cue generalization task, the delay-conditioned animals were tested to a novel cue, white noise. Freezing to the novel cue differed from the freezing response to the original cue by only a small amount (5~10%). In another study (Cushman et al., 2012), normal mice showed about 30% difference between the two different stimuli (they also used tone and white noise as cues). The poor performance in our cue discrimination may be due to nonassociative learning, which can mask the response to a novel cue. Several studies have suggested that fear conditioning can produce nonassociative learning in mice. For example, mice exposed to footshocks freeze to a tone that was never paired with shock (Kamprath & Wotjak, 2004). Therefore,

further investigation is needed to determine if the current fear conditioning procedure produces nonassociative learning.

Another potential explanation for the enhanced contextual fear memory in mice lacking adult neurogenesis is that these mice were unable to discriminate between the trace interval and the ITI. One of the functional models of DG posits that this is involved in distinguishing similar information, a process known as pattern separation (Schmidt, Marrone, & Markus, 2012; McHugh et al., 2007; O'Reilly & McClelland, 1994). For example, mice lacking NMDA receptors specifically in the DG showed normal memory performance in regular contextual fear conditioning. However, they showed more generalization to an alternate context in a contextual discrimination procedure (McHugh et al., 2007). Recent studies have shown that ablating adult neurogenesis results in impairment in pattern separation-type tasks such as context discrimination (Kheirbek, Tannenholz, & Hen, 2012; Tronel et al., 2012), while stimulation of adult neurogenesis improves the contextual discrimination performance (Sahay et al., 2011). These results suggest that hippocampal adult neurogenesis contributes to the process of disambiguating information.

One theoretical account of trace conditioning is that the task requires discrimination between the trace interval and ITI. This hypothesis was tested using the trace–conditioning deficit phenomenon. Trace conditioning produces a smaller magnitude of conditioned response than delay conditioning when all other experimental conditions are the same. Bolles and colleagues (1978) showed that the trace-conditioning deficit was ameliorated by increasing the ITI or inserting an extra light signal to help discriminate better between the trace interval and the ITI. It has been speculated that the

hippocampus helps distinguish the trace interval from the ITI during conditioning (Bolles et al., 1978; Kryukov, 2012). Considering these theories, a possible explanation for the enhanced contextual fear is that, although the CS-US association is normally formed during trace conditioning, animals generalize between the trace interval and the ITI, which could enhance conditioning to contextual cues. If this hypothesis is correct, the enhanced contextual fear memory will disappear if the trace conditioning protocol is modified to increase the discriminability of the trace interval and ITI (e.g. increasing ITI).

Lastly, the stress level induced by trace fear conditioning deserves further discussion. Stress is known as a potent modulator of both neurogenesis and memory, and it is likely that the elevated levels of glucocorticoids produced during fear conditioning modulate memory. Recent evidence shows that hippocampal adult neurogenesis is involved in the negative feedback regulation of the HPA axis and moderates stress sensitivity (Hayashi et al., 2008; Schloesser et al., 2010; Snyder et al., 2011). Snyder and colleagues (2011) measured corticosteroid levels after exposing GFAP-TK mice to acute stress. In neurogenesis-ablated GFAP-TK mice, elevated corticosteroid levels lasted longer than those of control animals after acute stress, indicating an interrupted negative feedback of the HPA axis. This finding provides a new potential function of adult neurogenesis as a modulator of the interaction between stress and memory. This finding lends support to our earlier hypothesis that nonassociative fear masked a deficit in trace conditioning in neurogenesis-arrested mice. It is possible that the impairment of the negative feedback of the HPA axis in mice lacking adult neurogenesis leads to increase stress hormone level such as corticotropin-releasing factor and glucocorticoid. The increased stress hormone may stimulate neural substrates such as amygdala that are

involved in fear sensitization or anxiety behavior (Davis, 1989b; Davis, 1989a; Bale, Lee, & Vale, 2002). Eventually, the sensitized emotional state may cause an exaggerated freezing response to the neutral tone stimulus, which masks associative learning deficits in fear conditioning. For example, in the trace cue memory test, the tone response in the DCX-TK mice might be the product of sensitized emotional responses to the tone instead of the CR learned in training. This possibility will be explored further in *Chapter 5*.

In summary, we tested the contribution of hippocampal adult neurogenesis to trace fear conditioning using two neurogenesis ablation techniques. Unexpectedly, but consistently through the two experiments, mice lacking adult neurogenesis showed enhanced contextual fear after trace conditioning but not after delay conditioning. We did not detect an impairment of trace cue memory in the experiments. However, further investigation is needed to test if the behavioral responses to the tone in the tone test truly reflected an association between trace CS and US.

## Chapter 5: Nonassociative fear effects in fear conditioning

As shown in *Chapter 4*, DCX-TK mice displayed normal fear to the trace CS but increased contextual fear, which led us to speculate that the arrest of neurogenesis impaired formation of the tone-shock association across the trace interval. Why, then, did DCX-TK mice freeze in response to the tone? One possibility is that tone-elicited freezing was nonassociative.

Fear conditioning can cause nonassociative emotional changes, such as generalized fear and anxiety-like behavior that is expressed even in the absence of conditioned stimuli. For example, exposure to footshock can cause mice to display freezing responses to a tone that was never paired with shock (Kamprath & Wotjak, 2004). This issue becomes important for a correct measurement of the CR because the nonassociative response can mask the CR in memory tests. This nonassociative masking may be particularly problematic in trace conditioning, because trace conditioning is considered the more difficult task and it usually generates a smaller CR than delay conditioning. That is, nonassociative effects in trace conditioning can easily produce false positives in behavioral outcomes (Smith, Gallagher, & Stanton, 2007; Burman, Simmons, Hughes, & Lei, 2014). In this chapter, we sought to determine whether our fear conditioning procedure induced nonassociative behavioral phenotypes.

Kampath and colleagues (2004) showed that high levels of tone and footshock intensity increase the prevalence of nonassociative fear. In their study, tone was not paired with the footshock (0.7mA). However, when a tone was presented to the animals, they showed significant freezing response compared to naïve mice. Our current fear conditioning protocol consists of higher levels of tone (85 dB) and footshock intensity

(0.8 mA) than they used in the study. Therefore, we predict that animals will exhibit robust freezing to the tone after fear conditioning even though the tone was never paired with footshock before.

The response to the neutral tone could be due to the general changes in emotionality. In the Kampath and colleagues' same study (2004), a single footshock (0.7 mA) also led mice to more strongly avoid the light compartment in the light-dark avoidance task compared to naïve control mice, implying that fear conditioning increases anxiety-like behavior. In addition, a series of their studies confirmed that fear conditioning cause a long-lasting anxiogenic effect and the underlying neural mechanism might be independent of associative fear learning (Golub, Mauch, Dahlhoff, & Wotjak, 2009; Siegmund & Wotjak, 2007a; Siegmund & Wotjak, 2007b; Kamprath & Wotjak, 2004). We tested if the current fear conditioning procedure generally affected basal emotional state using other assays of emotionality.

Accumulating data support that hippocampal adult neurogenesis is involved in emotional regulation. In particular, recent studies showed that hippocampal adult neurogenesis moderates stress sensitivity through HPA axis negative feedback (Hayashi et al., 2008; Schloesser et al., 2010; Snyder et al., 2011). Considering the fear conditioning procedure has emotionally aversive properties, this raises a question if mice lacking adult neurogenesis are more susceptible to the stress associated with fear conditioning, and how this stress affects their future behavior. It is possible that mice lacking neurogenesis are more susceptible to the current fear conditioning procedure and display exaggerated emotional alteration compared to WT mice. To test this hypothesis, DCX-TK and WT mice were subjected to the open field test before or after fear

conditioning. In the experiment, although activity in the open field was not different between DCX-TK and WT mice without fear conditioning, DCX-TK mice showed lower center activity than WT mice after fear conditioning. The exaggerated emotional changes in mice lacking adult neurogenesis was also confirmed with another anxiety behavioral test, the elevated plus maze.

## 5.1 MATERIALS AND METHODS

### 5.1.1 Animals

Separated cohorts of 8-week-old transgenic mice described in *Chapter 3* were used for foreground contextual fear conditioning (*Section 5.2.1*), open field testing (*Section 5.2.2*), and elevated plus maze testing (*Section 5.2.3*). WT littermates were used as a control group. Approximately equal numbers of male and female mice were used in foreground contextual fear conditioning experiment. For the open field test, two separated male subgroups were subjected to the open field either without fear conditioning or after fear conditioning (*please see Section 5.1.3 for more details*). For the elevated plus maze test, the same cohort of male mice were first tested on the elevated plus maze, were then fear conditioned, and then tested on the elevated plus maze again (*please see Section 5.1.4 for more details*).

### 5.1.2 Foreground contextual fear conditioning (shock alone)

One week after two week GCV treatment, foreground contextual fear conditioning took place in the same apparatus described in *Chapter 4*. The training procedure was also similar to the trace fear conditioning procedure in *Chapter 4* except

that there was no tone presentation during the training. The US was presented at the equivalent time point as in the trace fear conditioning procedure. Contextual and cue memory tests were performed in the same way as in *Chapter 4*.

### 5.1.3 Open field test

Mice were treated with GCV for two weeks. One week after the last infusion of GCV, one subgroup of mice was subjected to the open field test without fear conditioning (before fear conditioning group). The other group was subjected to delay or foreground contextual fear conditioning, followed by a context fear test (day 2) and a cue fear test (day 3). On the 4$^{th}$ day, animals were subjected to the open field test (after fear conditioning group).

For the open field test, mice were placed in a 40 × 40 cm arena for 30 minutes. The walls were 35 cm high and made of opaque plastic. A light bulb mounted above provided 85 lux illumination, measured in the center of the arena. Sessions were recorded via a digital camera, and videos were analyzed offline using video tracking software (ANY-Maze, Stoelting Co.). The center was defined as an 18.5 × 18.5 cm zone in the center of the arena.

### 5.1.4 Elevated plus maze

Mice were treated with GCV for two weeks. One week after the last infusion of GCV, the animals were subjected to the elevated plus maze (day 1), followed by foreground contextual fear conditioning a day later (day 2), and tested for context fear (day 3) and cue memory (day 4) in succession. The same foreground fear conditioning procedure and parameters described in *Chapter 5* were used. On the 5$^{th}$ day, the animals were subjected to the elevated plus maze test again.

For the elevated plus maze test, an individual mouse was taken from the home cage and directly placed in the center of the plus-shaped maze for 5 minutes. Each arm was 30 cm in length and 5 cm in width. Two were "closed" arms with walls 13 cm in height while the other two were "open" arms. Light bulbs mounted above the apparatus provided a 55 lux illumination on the open arms. The maze was on a stand, approximately 93 cm above the floor. Traveled time and distance in each arm and center zone were analyzed offline using video tracking software (ANY-Maze, Stoelting Co.). The maze was cleaned with 70% ethanol after each run.

## 5.2 RESULTS

### 5.2.1 Results: freezing to the tone after fear conditioning

Experimentally-naïve groups of DCX-TK and WT mice were treated with GCV for two weeks. One week after the conclusion of GCV, mice received "foreground" contextual fear conditioning, in which the footshocks were presented alone without being preceded by a tone. The conditioning parameters (e.g., number, intensity, and timing of the shocks) were otherwise identical to those in the DCX-TK trace fear conditioning procedure described above.

For the training session, we assessed freezing behavior in 10-s bins across the entire session. There were no effects of Genotype or of the Genotype × Time interaction (Figure 5.1*A*, REML: Genotype, $F_{(1,17)} = 0.06$, $p = 0.809$; Genotype × Time $F_{(43,731)} = 0.69$, $p = 0.939$). On the day following training, mice were placed into the training context to test for contextual fear. There were no significant effects of Genotype or the Genotype × Time interaction (Figure 5.1*B*; REML: Genotype, $F_{(1,17)} = 0.04$, $p = 0.835$; Genotype ×

Time, $F_{(4,68)} = 1.56$, $p = 0.196$). On the third day, mice were placed into the novel context to measure nonassociative freezing to the tone. Although mice had not received tone-shock pairings, both WT/GCV and DCX-TK/GCV mice exhibited freezing in response to the tone (Figure 5.1*C*). As in the previous experiments, we analyzed freezing during three periods (Figure 5.1*D*): baseline (20s before tone-on), tone, and post-tone (20s after tone-off). There was a significant main effect of Period indicating that freezing during the tone and post-tone periods exceeded that during the baseline ($F_{(2,34)} = 45.78$, $p < 0.001$). *Tukey's test* confirmed that freezing during the tone and post-tone period was significantly higher than during the baseline (baseline vs. tone, p=0.001; baseline or tone vs. post-tone, $p < 0.001$). The main effects of Genotype and the interaction of Genotype × Period were not significant (Genotype, $F_{(1,17)} = 0.73$, $p = 0.404$; Genotype × Period $F_{(2,34)} = 0.71$, $p = 0.498$). As shown in Figures 4.1*B* and 4.3*A*, mice exhibited little or no freezing in response to the first tone presentation during delay and trace fear conditioning. This indicates that tone-elicited freezing was caused by prior shock exposure and does not occur in unshocked mice.

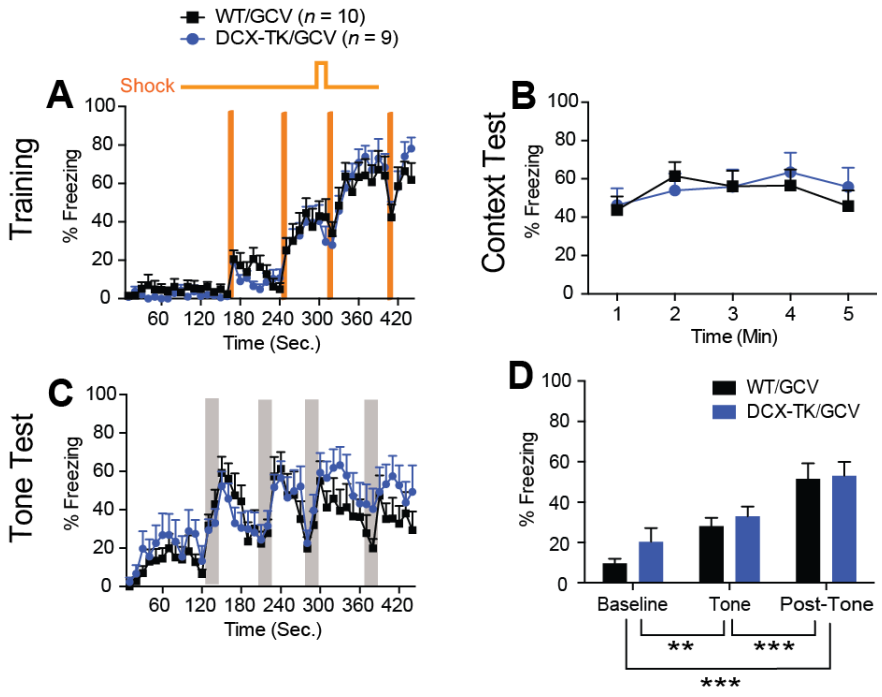


Figure 5.1. Evidence for nonassociative tone-elicited freezing.

DCX-TK/GCV and WT/GCV mice received shock-alone ("foreground") contextual fear conditioning. ***A***, ***B***, There was no effect of genotype on freezing during the training session (***A***) or the context test (***B***). ***C***, During the tone test in a novel context, both DCX-TK/GCV and WT/GCV mice displayed freezing in response to the tone. ***D***, Mean freezing during the tone and post-tone periods exceeded that during the baseline period. There was no effect of genotype. $^{**}p < 0.01$; $^{***}p < 0.001$.

### 5.2.2 Results: activity level in open field test after fear conditioning

These results suggest that fear conditioning induces a nonassociative change in emotional responsiveness that causes mice to exhibit fear in response to a tone stimulus that was never paired with shock. If fear conditioning, in fact, induces generalized changes in emotionality, these changes should be evident in another assay of emotional function. To test this hypothesis, we assessed open field behavior in WT and DCX-TK

treated with GCV as described above. One subgroup of mice was subjected to fear conditioning (using the delay or shock-alone protocols described above) prior to open field testing. The other subgroup was tested in the open field without prior exposure to fear conditioning.

Consistent with earlier studies (Jaholkowski et al., 2009; David et al., 2009), in mice that were not fear conditioned, there was no significant effect of neurogenesis ablation on open field activity. (Figure 5.2*A-D*; REML: Center Time: Genotype, $F_{(1,25)} = 0.30$, $p = 0.588$, Genotype × Time $F_{(5,125)} = 0.84$, $p = 0.527$; Center Distance: Genotype, $F_{(1,25)} = 2.97$, $p = 0.097$, Genotype × Time, $F_{(5,125)} = 1.14$, $p = 0.341$; Marginal Distance: Genotype, $F_{(1,25)} = 0.03$, $p = 0.873$, Genotype × Time, $F_{(5,125)} = 1.33$, $p = 0.256$). However, among mice that were previously fear conditioned, DCX-TK/GCV mice displayed reduced time and distance in the center zone, as well as a small reduction in marginal distance (Figure 5.2*E-H*; REML: Center Time: Genotype × Time, $F_{(5,125)} = 4.86$, $p < 0.001$; Center Distance: Group, $F_{(1,25)} = 6.30$, $p = 0.019$; Marginal Distance: Genotype × Time, $F_{(5,125)} = 3.25$, $p = 0.009$). These results suggest that, after fear conditioning, neurogenesis-arrested DCX-TK/GCV mice displayed elevated anxiety-like behavior in the open field compared to WT mice.

In the comparison of open field activity between mice that were not fear conditioned and fear conditioned mice, the fear conditioned mice showed the reduction of traveled center distance (WT: Genotype × Time $F_{(3,71)} = 9.23$, $p < 0.001$; DCX-TK: Genotype × Time, $F_{(3,83)} = 82.61$, $p = 0.014$).

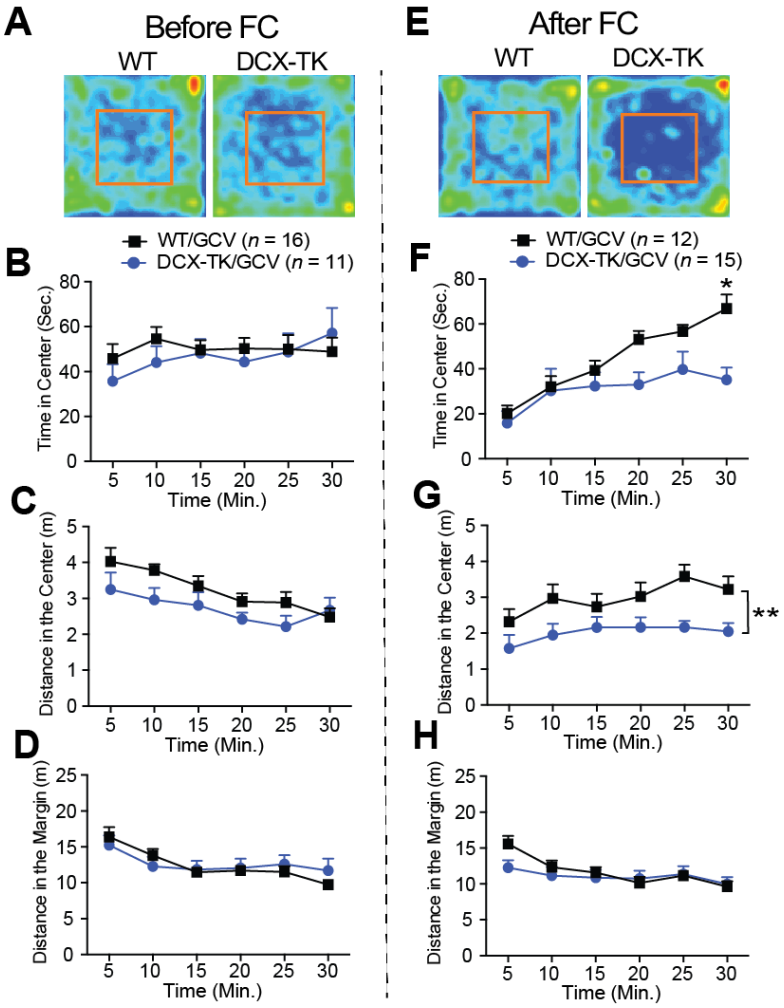


Figure 5.2. Effect of fear conditioning on behavior in the open field.

Separate groups of DCX-TK/GCV and WT/GCV mice were tested in the open field without prior fear conditioning or 3d after fear conditioning. ***A***, ***E***, Occupancy plots for representative individual mice. ***A-D***, Among mice without prior fear conditioning, there was no effect of genotype on open field behavior. ***E-H***, In contrast, after fear conditioning, DCX-TK/GCV mice displayed reduced center time (***F***), center distance (***G***), and marginal distance (***H***) as compared to WT/GCV mice. $*p < 0.05$, $**p < 0.01$.

### 5.2.3 Results: anxiety-like behavior in elevated plus maze test after fear conditioning

In the open field assay, we found that mice lacking neurogenesis display an exaggerated emotional change after footshocks. However, animal studies related to anxiety behavior using the same/different type of emotional behavior tests have shown highly variable and sometimes contradictory behavioral results as consequence of experimental treatments such as anxiolytic drugs (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002). Therefore, we tested if fear conditioning induces generalized changes in emotionality in a different anxiety test besides the open field test.

The mice were treated with GCV for two weeks. One week after the last infusion of GCV, the mice were subjected to the elevated plus maze and the same groups of animals were subjected to the elevated plus maze again after they received footshocks using the "foreground" fear conditioning procedure. Considering the direction of effect in this experiment is that the DCX-TK/GCV mice display higher anxiety behavior compared to WT mice after fear conditioning, we used a *one-tailed t-test* to confirm the hypothesis.

Consistent with the open field results, there was no significant effect of neurogenesis ablation on elevated plus maze activity in either open or closed arms (Figure 5.3*A*-*D*:Before FC; Open_time, $t_{(17)} = -0.03$, $p = 0.510$; Open_distance, $t_{(17)} = 0.30$, $p = 0.382$; Closed_time, $t_{(17)} = -0.15$, $p = 0.558$; Closed_distance, $t_{(17)} = 0.01$, $p = 0.504$, *one-tailed t-test*). However, after fear conditioning, the DCX-TK/GCV mice displayed reduced time and distance in the open arms (Figure 5.3*A* and *C*: After FC; Open_time, $t_{(17)} = 1.78$, $p = 0.047$; Open_distance, $t_{(17)} = 1.87$, $p = 0.040$, *one-tailed t-test*), but not in the closed arms (Figure 5.3*B* and *D*: After FC; Closed_time, $t_{(17)} = -1.32$, $p = 0.898$; Closed_distance, $t_{(17)} = 0.49$, $p = 0.317$, *one-tailed t-test*). These results

confirm that, after fear conditioning, neurogenesis-arrested DCX-TK/GCV mice displayed elevated anxiety-like behavior compared to WT mice.

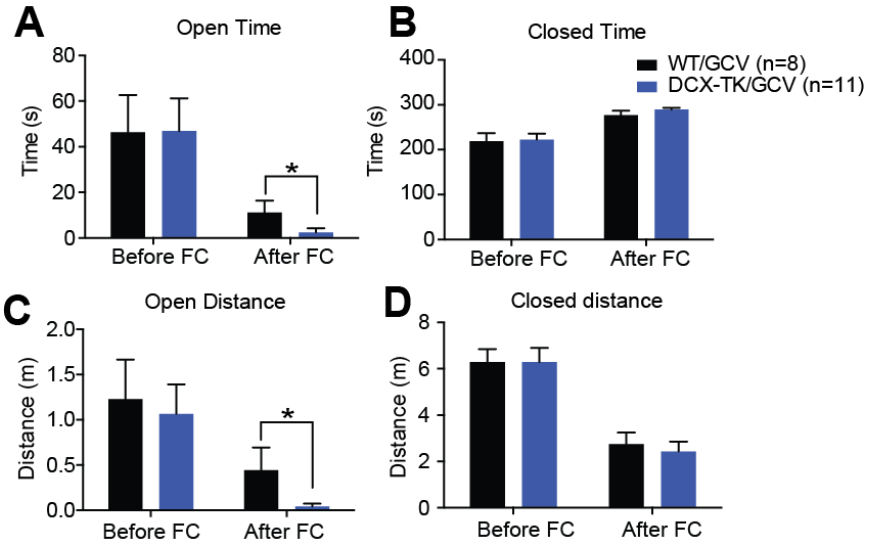


Figure 5.3. Anxiety-like behavior in elevated plus maze after fear conditioning

DCX-TK/GCV and WT/GCV mice were tested in the elevated plus maze before fear conditioning (before FC) and after fear conditioning (after FC). ***A-D***, Before FC, there was no effect of genotype in the activity level on either open arms or closed arms. ***A*** and ***C***, However, after fear conditioning, mice lacking neurogenesis showed lower time (A) and traveled distance on the open arms (*C*). *$p < 0.05$.

## 5.3 DISCUSSION

We tested if the current fear conditioning procedure produces nonassociative freezing. Animals were exposed to footshocks using a foreground contextual fear conditioning procedure, but they were never exposed to the tone during training. Nevertheless, in the tone test, animals displayed freezing response to the neutral tone, which confirmed that fear conditioning in mice can produce considerable nonassociative

fear of the conditioned stimulus, as previously reported (Kamprath & Wotjak, 2004). Importantly, the magnitude of the tone response was similar to the tone response in the trace fear conditioning (*please see Chapter 4*; during cue, ~40%; during post-CS, ~60% in WT), which implies that, in the previous trace fear conditioning experiment, shock-induced nonassociative behavioral response may have masked the trace conditioned response. Also, this nonassociative behavioral response may explain why animals showed poor cue discrimination performance in the cue generalization experiment in the previous chapter. Animals might sensitively freeze to the novel cue because of the shock-induced nonassociative behavioral response.

In the nonassociative fear experiment using foreground contextual fear conditioning procedure, there were no genotype effects in the context test. The result is consistent with studies showing that multiple-trial contextual fear conditioning is not adult neurogenesis dependent (Drew et al., 2010). It is important to note that, in the trace conditioning (*Chapter 4*), the freezing response to the contextual fear in the WT mice was around 40%. However, in the current 'foreground' contextual fear conditioning where there was no paring tone with shock during the training, the WT mice showed around 60% freezing behavior in the contextual fear test implying the lack of competition with tone cue increases the strength of the context-shock association. In the trace conditioning experiment, the DCX-TK mice showed around 60% context freezing, similar level to the WT in 'foreground' context fear conditioning. This behavioral pattern supports the possibility that the enhanced contextual fear in DCX-TK mice is explained by the failure for form a trace CS-US association.

In the open field and elevated plus maze tests, neurogenesis ablation did not cause general activity changes or axiogenic behavior in mice. These results are consistent with previous reports that neurogenesis does not affect general locomotor behaviors or anxiety (Sahay et al., 2011; Saxe et al., 2006; Jaholkowski et al., 2009; Garrett, Lie, Hrabe de Angelis, Wurst, & Holter, 2012) although there are some exceptions (Revest et al., 2009; Bergami et al., 2008). After fear conditioning, both DCX-TK and WT mice treated with GCV showed decreased general activity levels. The data are consistent with some studies showing acute stress (e.g. restraint stress) results in decreased activity in the center zone in the open field indicating anxiogenic effect of the fear conditioning (Kubota, Amemiya, Yanagita, Nishijima, & Kita, 2014; Zimprich et al., 2014). In particular, the DCX-TK mice treated with GCV showed more anxiety-like behavior after fear conditioning compared to WT mice, suggesting that mice lacking neurogenesis are more susceptible to fear conditioning-induced stress. The result is also consistent with recent data suggesting that neurogenesis-arrested mice are more susceptible to effects of restraint or psychosocial stress on anxiety-like behaviors (Schloesser et al., 2010; Snyder et al., 2011).

In summary, we demonstrated that the fear conditioning procedure used in *Chapter 4* produce a mixture of associative and nonassociative fear. In addition, the nonassociative emotional alteration was more severe in mice lacking adult neurogenesis compared to naïve mice. For a proper interpretation of the behavioral results in the fear conditioning studies, it is important to consider the relation between the fear conditioning procedure and two learning mechanisms: associative and nonassociative.

# Chapter 6: Trace fear conditioning procedure that minimized nonassociative tone freezing

We investigated the role of adult neurogenesis using trace fear conditioning as a behavioral model system. Our previous experiments, using DCX-TK/GCV transgenic system and x-irradiation to ablate adult-born neurons, consistently showed that the arresting of adult neurogenesis causes the enhancement of contextual fear in trace fear conditioning, but not in delay conditioning. We did not detect any significant impairment in the trace cue memory in the mice lacking adult neurogenesis different from some other studies (Achanta et al., 2009; Shors et al., 2002; Shors et al., 2001).

One explanation for the enhanced contextual fear in mice lacking adult neurogenesis is that the failure to acquire a trace CS-US association strengthened the context-US association. One caveat of this hypothesis is that we did not detect the behavioral impairment of trace CS-US association in *Chapter 4*. However, the involvement of nonassociative fear in the current fear conditioning protocol complicates the interpretation of the role of adult neurogenesis in the trace CS-US association. In *Chapter 5*, we showed that the mice exposed to the shock alone displayed freezing in response to the tone, and the freezing levels were similar to the trace fear conditioned animals. This indicates that fear conditioning in mice can produce considerable nonassociative fear of the CS, and it implies that the nonassociative fear masked the impairment of the trace CS-US association. Therefore, we hypothesized that an alternate trace fear conditioning protocol minimizing nonassociative fear would unmask the impairment of the trace CS-US association in neurogenesis-arresed mice.

In this chapter, first, we developed a new trace fear conditioning protocol where nonassociative factors are minimized. The literature on nonassociative learning processes

in mouse fear conditioning show that shock intensity, tone intensity, and ITI length are active variable in the involvement of nonassociative fear (Kamprath & Wotjak, 2004; Burman et al., 2014; Smith et al., 2007). Because those studies did not investigate the factors causing nonassociative effects in the trace fear conditioning in parametric manner, it is difficult to know which of these factors is the most influential in producing nonassociative learning. However, a characteristic common to all studies was that low-intensity sensory stimuli (e.g. shock and tone) and longer ITI length reduced the nonassociative effects in fear conditioning (Kamprath & Wotjak, 2004; Burman et al., 2014; Smith et al., 2007). In the Kamprath and Wotjak (2004) study, the mice received 0.7 mA footshocks displayed significant freezing response to a neutral tone indicating nonassociative fear learning occurred in the procedure. However, when the shock intensity was reduced to 0.4 mA, the nonassociative fear response significantly reduced. Also, they showed that high amplitude tones (more than 90 dB) provoked freezing responses in naïve mice. Similar to these results, other studies showed that, when the protocol consisted of high tone intensity and short ITI, trace and unpaired training produced similar tone-elicited freezing. However, when the protocol was revised to with lower intensity and longer ITI, the trace-trained animals displayed significantly higher freezing to the tone compared to the unpaired control group (Smith et al., 2007; Burman et al., 2014). Therefore, we established a new fear conditioning protocol by reducing the footshock intensity and tone intensity and increasing ITI length. Afterwards, the neurogenesis-ablated mice using DCX-TK/GCV system were trained in the alternate trace fear conditioning.

## 6.1 MATERIALS AND METHODS

### 6.1.1 Animals

Separated 12-week-old naïve C57BL/6 male mice were used to develop an alternate trace fear conditioning procedure (*please see Section 6.1.2 for procedure*). To investigate the effect of arresting of adult neurogenesis on the alternate trace fear conditioning experiment, approximately equal numbers of 8-week-old male and female DCX-TK and WT littermates were used (*please see Section 6.1.3 for procedure*).

### 6.1.2 Developing alternate trace fear conditioining

The apparatus for fear conditioning was the same that was described in *Chapter 4*. The procedure for fear conditioning was similar to the previous fear conditioning procedure described in *Chapter 4*, but with some parameters changed. First, naïve WT mice were handled for 5 days (~2 min/day, compared to minimum handling in the previous fear conditioning procedure). In the training day (day 1), the tone intensity was reduced from 85 dB (5000 Hz) to 75 dB (5000 Hz) for the CS. The US intensity was also reduced from 0.8 mA (2s footshock) to 0.5 mA (2s). In the trace conditioning procedure, the CS was presented at 180, 370, 620, 900, 1060s. Therefore, the ITI was increased from 40s to 180s, and the number of trials also increased from 4 trials to 5 trials to increase the CS-US association strength. The CS was followed by a 20s trace interval followed by the US.

To test if any nonassociative learning processes mask the trace CS-US association learning response, the foreground contextual fear conditioning procedure (shock alone) and unpaired fear conditioning procedure (unpaired) were used as control groups. In foreground contextual fear conditioning, the shock occurred at 220, 410, 660, 940, and

1100s into the session, and the tone was not presented. For unpaired training, the tone (20s, 75 dB) was presented at 180, 370, 620, 900, and 1060s into the session, and the shock occurred at 120, 300, 500, 820, and 1160s into the session.

In the context memory test session (day 2), the mice were placed back into the original conditioning chamber for 5 minutes without footshock delivery (same to the previous procedure in *Chapter 4*). For the tone test (day 3), the mice were placed into an alternate chamber (a triangular plastic chamber comparable in size to the original chamber) and the tone was presented at 180, 280, 390, 510s into the session.

70% ethanol was used to clean the grids between runs for the conditioning and contextual memory sessions, but on the cue memory test session, Clorox Fresh Scent disinfecting wipes were used to clean the grids and chamber for context shift.

### 6.1.3 Alternate trace fear conditioining

Either WT mice or DCX-TK mice underwent the same ICV surgeries for GCV administration as in *Chapter 3*. After three weeks, the animals were trained in the alternate trace fear conditioning procedure, as described in the previous section (*Section 6.1.2*) for the alternate trace fear conditioning.

Briefly, mice were handled for 2 min per day for 5 days prior to conditioning. Training occurred in Context A and consisted of five tone-shock pairings during a 1280s session. In trace conditioning, the tone (20s, 75 dB) was presented at 180, 370, 620, 900, and 1060s into the session. The shock (0.5 mA, 2s) occurred 20s after tone offset.

The test for context-elicited fear occurred on the following day and consisted of a 5 min exposure to context A. Neither the tone nor shock was presented. The test for tone-

elicited fear occurred in Context B on the following day. The tone was presented for 20s at 180, 280, 390, 510s into the session.

### 6.2 RESULTS

Separate groups of WT mice were trained with trace, unpaired, or shock-alone protocols. In the tone-test session, REML model revealed significant main effects of Protocol and Period (Figure 6*B*; Protocol, $F_{(2,15)} = 10.32$, $p = 0.002$; Period, $F_{(2,30)} = 21.14$, $p < 0.001$). There was no effect of conditioning protocol on freezing during the baseline (pre-tone) period (*Oneway ANOVA*: $F_{(2,17)} = 2.64$, $p = 0.104$). However, the trace-trained animals froze significantly more than the unpaired or shock-alone groups during the CS ($F_{(2,17)} = 5.37$, $p = 0.017$; *Tukey*: Trace Vs. Un-paired or Shock alone, $p < 0.05$) and the Post-CS periods ($F_{(2,17)} = 10.80$, $p = 0.001$; *Tukey*: Trace Vs. Un-paired or Shock alone, $p < 0.01$). The data confirm that this protocol produces tone-elicited fear that is largely associative.

Next, we trained WT/GCV and DCX-TK/GCV mice in the alternate trace conditioning protocol. On the training day (Day 1), both DCX-TK/GCV and WT/GCV group reached about 80% freezing after the last trial of training. The freezing levels in time bins were analyzed using REML. There was no effect of Genotype or the Genotype x Time interaction (Figure 6*C*; Genotype, $F_{(1,19)} = 1.14$, $p = 0.299$; Genotype × Time, $F_{(119,2261)} = 1.13$, $p = 0.172$). On the next day, mice were placed into the original training context for a test of context-elicited fear. The genotypes did not differ significantly (Figure 6*D*; Genotype, $F_{(1,18)} = 0.22$, $p = 0.644$; Genotype × Time $F_{(4,72)} = 0.55$, $p = 0.670$). On the third day, mice were placed into the novel context to test tone-elicited

fear. As in the previous experiments, we analyzed Genotype × Period (Baseline, CS, and Post-CS) using REML model. There was a significant Genotype × Period effect (Figure 6*F*; Genotype × Period, $F_{(2,38)} = 3.48$, $p = 0.04$). A subsequent *t-test* between genotype groups in each period revealed that there was no effect of genotype on baseline freezing ($t_{(19)} = 0.82, p = 0.375$). However, during the CS and post-CS period, the freezing levels of DCX-TK/GCV mice were significantly lower than those of WT/GCV mice (Figure 6*F*: CS, $t_{(19)} = 2.66$, $p = 0.015$; Post-CS, $t_{(19)} = 3.12$, $p = 0.006$). Arrest of adult neurogenesis impaired associative trace fear conditioning.

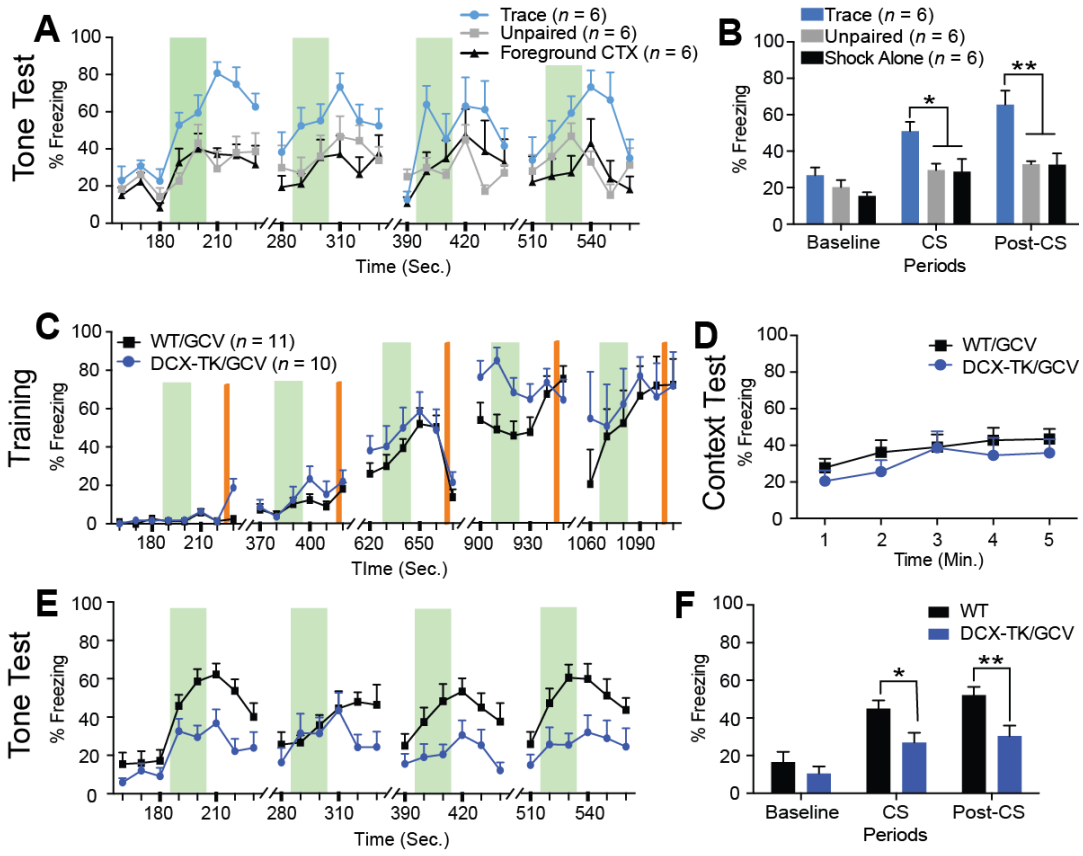


Figure 6.1. Effect of neurogenesis ablation in an alternate trace fear conditioning.

Figure 6.1

Effect of DCX-TK-mediated ablation of adult neurogenesis in an alternate trace fear conditioning procedure that minimized nonassociative tone freezing. ***A, B,*** Tone test for WT mice previously trained using trace, unpaired, or shock-alone protocols (training data not shown). ***B***, Mean freezing during the tone and post-tone periods was significantly greater in mice that received trace conditioning than in those that received unpaired or shock-alone training. There was no effect of conditioning protocol on baseline freezing ***C-F***, Performance DCX-TK/GCV and WT/GCV in the alternate trace conditioning protocol. ***C***, There was no effect of genotype on freezing during the training session. ***D***, Freezing during the test of context-elicited fear. There was no effect of genotype. ***E***, ***F,*** Freezing during the test for tone-elicited fear in a novel context. ***F***, There was no effect of genotype on baseline freezing, but DCX-TK/GCV mice displayed reduced freezing relative to WT/GCV mice during the tone and post-tone periods. $*p<0.05$, $**p<0.01$.

## 6.3 DISCUSSION

In the current chapter, we showed that the reducing the tone and shock intensity and increasing ITI reduced the amount of nonassociative freezing produced by fear conditioning. The WT mice trained in the alternate trace fear conditioning procedure showed clearly distinguishable freezing response to the tone compared to the mice received shock alone or tone-shock unpaired. Therefore, the response to the tone in the alternate trace trained animals can be interpreted as true conditioned responses uncontaminated by nonassociative factors.

Using this new trace training protocol, we showed that the neurogenesis ablation results in the impairment of the trace cue memory. This result is consistent with studies showing that the hippocampal adult neurogenesis is involved in the process of trace conditioning, and also it supports the hypothesis that the enhanced contextual fear in the DCX-TK mice resulted from the impairment of trace CS-US association (*Chapter 4*). However, in the current trace fear conditioning experiment, we did not observe the enhanced contextual fear in the DCX-TK mice. One possible explanation is that there was a formation of enhanced contextual fear due to the trace-conditioning deficit, but the enhanced contextual fear might be extinguished due to the long ITI (Barela, 1999).

In the field of functional adult neurogenesis, investigators have tried to find links between adult-born neurons and cognitive function using hippocampus-dependent learning paradigms. It is well established that trace conditioning is hippocampus-dependent learning paradigm, but it has been still unclear if adult-born neurons contribute to trace conditioning (*please see Chapter 1 and Chapter 2*). In the previous trace fear conditioning experiments using two different neurogenesis ablation systems in *Chapter 4*, we did not detect the impairment of trace cue memory. However, *in Chapter 5*, we found

that the fear conditioning procedure produces nonassociative fear, which may mask the trace-conditioning deficit. In the current chapter, we showed an impairment of trace cue conditioning in mice lacking adult neurogenesis when the conditioning procedure minimized nonassociative fear. These data imply that the inconsistency of the results in in published studies of adult neurogenesis might be due to the confounding of associative and nonassociative learning mechanisms.

# Chapter 7: General discussion

Since it was discovered that the adult mammalian brain generates new neurons, there has been great progress in understanding the cellular/molecular mechanism of adult neurogenesis regulation. However, the understanding of the functional role of adult neurogenesis is still immature (Castilla-Ortega et al., 2011; Deng et al., 2010; Zhao, Deng, & Gage, 2008; Sahay et al., 2007).

We characterized trace and delay fear conditioning and anxiety-like behavior using a novel chemogenetic approach for selectively ablating neural progenitor cells in adult mice. There were three main results. First, selective ablation of proliferating DCX+ cells caused a robust and specific arrest of adult neurogenesis, consistent with evidence that DCX+ progenitors are a lineage-restricted population (Brown et al., 2003). Second, the effects of arresting neurogenesis on trace fear conditioning were dependent on the conditioning protocol. When the protocol produced significant nonassociative fear, arrest of adult neurogenesis potentiated fear behavior; with a procedure that minimized nonassociative plasticity, arrest of neurogenesis impaired associative fear. Finally, the effects of neurogenesis ablation on anxiety-like behavior were modulated by the fear conditioning protocol. Consistent with previous studies (Jaholkowski et al., 2009; David et al., 2009), arrest of neurogenesis had no effect on anxiety-like behavior in mice not subjected to fear conditioning. However, after fear conditioning, neurogenesis-arrested mice displayed increased anxiety-like behavior in the open field. The results suggest that adult neurogenesis modulates emotional learning via two distinct but opposing mechanisms: it supports associative trace conditioning while also buffering against the generalized, nonassociative fear and anxiety caused by fear conditioning.

Although fear conditioning is usually studied as a model of associative learning, it can induce changes in behavior that are not readily explained via associative mechanisms. For instance, mice previously exposed to footshock display increased anxiety-like behavior in the open field and display fear responses to a tone never paired with shock. There is debate about whether these behaviors are truly nonassociative (Davis, 1989a; Davis, 1989b; Richardson, 2000), but recent evidence indicates that such behaviors can be induced even when the conditioning procedure fails to produce associative fear (Sauerhofer et al., 2012). In the current study, exposure to footshocks caused generalized changes in behavior similar to those reported previously (Kamprath & Wotjak, 2004). Fear conditioned mice displayed reduced center exploration in the open field as compared to mice that were not fear conditioned. In addition, mice exposed to footshock displayed freezing responses to a tone that was never paired with shock. Arrest of neurogenesis appeared to potentiate the nonassociative effects of fear conditioning. In the absence of fear conditioning, arrest of neurogenesis had no effect on open field behavior. However, among mice that were fear conditioned, neurogenesis-arrested mice displayed reduced exploration in the open field. The results suggest that arrest of neurogenesis sensitized mice to the anxiogenic effects of fear conditioning.

This observation is consistent with recent data suggesting that adult born neurons buffer the behavioral and endocrine response to acute stressors. Acute restraint or novel environment stress causes a larger corticosterone surge and more severe induction of anxiety-like behavior in neurogenesis-arrested than control mice (Schloesser et al., 2010; Snyder et al., 2011). The circuit mechanisms for these effects are not well understood, but it is thought that suppression of adult neurogenesis impairs hippocampal feedback

regulation of HPA axis. Corticosterone and its upstream modulator corticotropin-releasing factor (CRF) appear to be necessary for acute stress to induce long-term increases in anxiety-like behavior (Adamec et al., 2010; (Jakovcevski, Schachner, & Morellini, 2011; Clay et al., 2011). Thus, fear conditioning may be more anxiogenic to neurogenesis-arrested mice because these mice experience a more pronounced induction of corticosterone and/or CRF by fear conditioning.

An unanswered question is whether the increased anxiety-like behavior in neurogenesis-arrested mice after fear conditioning itself represents a cognitive impairment. One possibility is that the increased anxiety-like behavior by neurogenesis-arrested mice in the open field reflects overgeneralization of associative context fear, similar to that reported in context fear discrimination experiments (Niibori et al., 2012; Sahay et al., 2011; Tronel et al., 2010). The data are not consistent with this hypothesis. Neurogenesis-arrested and control mice displayed similar levels of context-elicited fear in both the training and alternate contexts in delay and shock-alone conditioning, indicating that arrest of neurogenesis does not cause a general increase in context fear generalization. The only significant effect of neurogenesis ablation on context-elicited freezing emerged in the trace procedure, and in that case the effect was present in both the training and alternate contexts. Thus, we attribute the elevated alternate-context freezing of neurogenesis-arrested mice to increased context conditioning rather than to increased fear generalization *per se*.

With an intensive trace fear conditioning procedure neurogenesis-arrested mice displayed elevated context fear compared to WT controls. We propose a cue competition mechanism to explain this phenotype. Context and discrete cues can compete for

associative strength (Urcelay & Miller, 2014; Urushihara & Miller, 2009). As a consequence, manipulations that degrade the contingency between a discrete CS and US sometimes increase the strength of context conditioning (Urcelay & Miller, 2010; Detert, Kampa, & Moyer, 2008). We hypothesize that the increased context conditioning in neurogenesis-arrested mice reflects impaired associative conditioning to the trace CS. In the intensive conditioning protocol, this impairment was not observable because of the high level of nonassociative tone-elicited freezing. However, when a protocol of more moderate intensity was used, nonassociative tone fear was reduced, and neurogenesis-arrested mice displayed impaired conditioned fear to the trace CS.

While there is abundant evidence that participating in trace conditioning enhances survival of adult-born neurons, studies examining the requirement of adult neurogenesis in trace conditioning have reached contradictory conclusions. Arrest of neurogenesis via systemic administration of an antimitotic compound or x-irradiation in rats impaired trace fear and trace eyelid conditioning (Achanta et al., 2009; Shors et al., 2002; Shors et al., 2001). However, two studies examining the role of neurogenesis in mouse trace conditioning failed to detect impairments (Jaholkowski et al., 2009; Cuppini et al., 2006). Mice and rats show different cellular and behavioral responses to stress (Bain, Dwyer, & Rusak, 2004; Griebel, Perrault, & Sanger, 1997; Armario & Castellanos, 1984), which raises the possibility that the effects of neurogenesis ablation are more readily masked by nonassociative fear in mice than in rats.

Two mechanisms have been proposed to explain the hippocampus-dependence of trace conditioning. One idea is that hippocampus maintains the CS representation during the trace interval. Supporting this idea, neurons in entorhinal cortex and hippocampus

display sustained activity during the retention interval in working memory tasks (Axmacher et al., 2007; Young, Otto, Fox, & Eichenbaum, 1997). An alternate but not mutually exclusive idea is that hippocampus plays a discriminative role. Successful trace conditioning requires that subjects distinguish between the trace interval and the ITI (Bolles et al., 1978). Distinguishing between these two stimulus-free periods appears to require the hippocampus, because deficits in trace conditioning caused by hippocampus lesions can be rescued by providing a cue to help subjects differentiate between the trace and ITI (Bangasser, Waxler, Santollo, & Shors, 2006). Recent evidence that adult neurogenesis supports behavioral pattern separation –the ability to discriminate between complex stimuli with shared or similar features (Clelland et al., 2009; Sahay et al., 2011; Tronel et al., 2010)– suggests the possibility that adult neurogenesis contributes to trace conditioning by supporting the discrimination between the trace and ITI.

Ablation of adult neurogenesis by DCX-TK/GCV or x-irradiation caused increased context fear in trace conditioning. The consistency of the phenotype across two ablation methods suggests that the phenotype was caused by ablation of neurogenesis rather than a side effect of either procedure. Furthermore, because the targeted x-irradiation procedure arrests neurogenesis in hippocampus but not SVZ (Santarelli et al., 2003), the increased context fear can be attributed to the loss of hippocampal neurogenesis. Nevertheless, the phenotypes of DCX-TK/GCV and x-irradiated mice were not identical. DCX-TK mice displayed elevated pre-tone fear in the novel context as compared to controls, whereas in x-irradiated mice pre-tone fear was significantly elevated. The difference may relate to differences in the conditioning protocol. In the irradiation experiment, mice were pre-exposed to the conditioning and alternate contexts

prior to training, whereas in the DCX-TK experiments mice were not pre-exposed. Context pre-exposure can affect the strength of context conditioning and modulate context generalization (Urcelay & Miller, 2010; Iordanova & Honey, 2012). Perhaps more important, the training and novel contexts were more different in the x-irradiation experiment than DCX-TK experiments. In the DCX-TK experiments, both contexts had metal bar floors, whereas in x-irradiation experiments the novel context floor was covered with bedding. Across a variety of learning paradigms, the effects of neurogenesis manipulations on generalization/discrimination tend to become more pronounced as the discriminability of the stimuli decreases (Bekinschtein et al., 2014; Creer, Romberg, Saksida, van Praag, & Bussey, 2010; Nakashiba et al., 2012; Niibori et al., 2012; Sahay et al., 2011). Thus, the DCX-TK conditioning protocol may be been more sensitive to effects of neurogenesis ablation on context generalization, owing to the use of more similar contexts.

The role of adult neurogenesis in behavior continues to be a subject of debate, owing in part, to a literature that includes inconsistent and sometimes contradictory findings (Castilla-Ortega et al., 2011). The conflicts among studies raise the possibility that the contribution of adult neurogenesis to behavior is modulated by experiential factors. One implication of the current experiments is that the effects of neurogenesis ablation depend on the stress history of the animal. In particular, exposure to strong aversive stimuli can induce an anxiety-like phenotype in neurogenesis-arrested mice that can mask associative deficits in fear conditioning. More generally, the results suggest that adult neurogenesis modulates emotional learning via dual mechanisms: it supports the ability to learn predictive contingencies involving aversive events while buffering against

the generalized anxiogenic effects of those events.

# Appendix

| | |
|---|---|
| BAC | Bacterial Artificial Chromosome |
| BrdU | Bromodeoxyuridine |
| CA1 | Cornu Ammonis areas 1 |
| CA3 | Cornu Ammonis areas 3 |
| CS | Conditioned Stimulus |
| DCX | Doublecortin |
| DG | Dentate Gyrus |
| DNA | Deoxyribonucleic acid |
| EC | Entorhinal Cortex |
| EPM | Elevated Plus Maze |
| GCL | Granule Cell Layer |
| GCV | Ganciclovir |
| GFAP | Glial fibrillary acidic protein |
| HPA | Hypothalamic–pituitary–adrenal axis |
| HSV | herpes simplex virus |
| ICV | intracerebralventricular |
| ITI | Intertrial Interval |
| LTP | Long-Term Potentiation |
| NSC | Neural Stem Cell |
| OF | Open Field |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| RMS | Rostral migratory stream |
| SGZ | Subgranulla Zone |
| SVZ | Subventricular Zone |
| TK | Thymidine kinase |
| US | Unconditioned Stimulus |
| WT | Wild-type |

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