Modulation of Adult Neurogenesis in Opioid Addiction

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YUE ZHANG

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Dedication

This thesis is dedicated to my mom. I love you.

Abstract

One of the major problems in treatment of opioid addiction is the repeated reuse and long-term memory of the drug-experience even after prolonged periods of abstinence. During the past decades, there has been enormous expansion in our understanding of how opioid drugs act on the nervous system. A complex brain network including the mesolimbic dopamine system, ventral striatum, extended amygdala, prefrontal cortex and hippocampus is suggested to be associated with the addiction cycle, in particular, the adult neurogenesis taken place in the dentate gyrus (DG) of the hippocampus has a functional implication in opioid addiction. It is intriguing to study the convergence between the modulation of adult neurogenesis and opioid addiction, since the adult-born granule cells were shown to play a role in neuroplasticity of hippocampus function and in the development and retention of drug-contextual memory.

In the first part of my study, I attempted to define the temporal window of morphine's inhibitory effect on adult neurogenesis with a transgenic mouse model. Four days of conditioned place preference (CPP) training with morphine significantly reduced the number of late stage progenitors and immature neurons in the sub-granular zone (SGZ) of mouse hippocampus but did not affect the number of early progenitor cells. The results from colocalization of cell-type selective markers suggested that under the condition of CPP training, morphine affects the transition of neural progenitor/stem cells differentiate into immature neurons. When the transcription factor neural differentiation1 (NeuroD1) was over-expressed in DG by stereotaxic injection of lentivirus, it rescued the loss of immature neurons and prolonged the extinction of morphine-trained CPP.

Next, a synthetic small molecule KHS101 which was reported to increase NeuroD1 mRNA in cultured neural progenitors and induce neuronal differentiation in the DG of hippocampus, was utilized to mimic the effect of lentivirus-mediated NeuroD1 overexpression on morphine-primed CPP. The results indicated that subcutaneous injection of KHS101 before conditional training could enhance the retention of drug-related memory and prolong CPP extinction; while the same treatment after conditional training disrupted the drug-contextual associations and shortened CPP extinction. Such KHS101's effect paralleled that observed when the over-expression of NeuroD1 was temporally controlled with an inducible tetracycline system. Furthermore, the KHS101's effect could be abolished by the stereotaxic injected NeuroD1 shRNA lentivirus.

These studies suggest that morphine decrease the total numbers of newborn neurons in the SGZ by interfering with neural progenitors' differentiation via a mechanism involving NeuroD1. Since adult neurogenesis serves as an important form of neural plasticity, we assume that certain immature neurons contribute to the formation and consolidation of drug-contextual association memory, and NeuroD1 plays a key role during this process. Such assumption is supported by the observation that compounds such as KHS101 that could regulate NeuroD1 expression in the hippocampus possess the ability to manipulate the extinction of drug contextual memory. In conclusion, the regulation of NeuroD1 activity leads to modulation of adult neurogenesis, thus affecting the drug-association memory.

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CHAPTER 1
INTRODUCTION

1.1 Opioid receptor signaling

1.1.1 Opioid receptor

According to National Institutes of Health (NIH) Pathways to Prevention Workshop, there are an estimated 5 to 8 million Americans use opioids for long-term management of chronic pain, and there is a dramatic increase in opioid prescriptions and use over the past 20 years (Reuben et al. 2015). Opioids include opiate alkaloids, which are the natural opioids or their derivatives extracts of the opium poppy, such as morphine, heroin or etorphine; semi-synthetic opioids derived from morphine, such as hydrocodone, hydromorphone, oxycodone, and oxymorphone; synthetic opioids which are not derived from morphine, such as [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO), fentanyl, buprenorphine, and methadone; and endogenous opioids such as enkephalins, β-endorphine and dynorphine (Goldstein & Cox 1977; Basbaum & Fields 1978) . All of these different opioids bind to the opioid receptors and activate the G-protein coupled opioid receptor signaling pathways.

The first identification of opioid receptors was reported in 1973 by S. Snyder, E. Simon, and their coworkers (Pert & Snyder 1973; Simon et al. 1973). Since then, three types of opioid receptors have been well defined and cloned from central and peripheral nervous system: mu (μ)-opioid receptor (MOR) (Chen et al. 1993; Thompson et al. 1993), delta (δ)-opioid receptor (DOR) (Evans et al. 1992; Kieffer et al. 1992) and kappa (κ)-opioid receptor (KOR) (Yasuda et al. 1993; Fukuda et al. 1993; Knapp et al. 1995).

Opioid receptors belong to the superfamily of seven transmembrane (7TM) G protein-coupled receptors (GPCRs), and class A (Rhodopsin) Gi/Go-coupled receptor

subfamily (Pierce et al. 2002; Waldhoer et al. 2004). The three subtypes of opioid receptors all have a putative structure of seven transmembrane domains, extracellular N-terminus with multiple glycosylation sites, and intracellular carboxyl tail (Pogozheva et al. 1998). The MOR, DOR and KOR show about 60% homology between their genes, with the greatest identity found in the transmembrane domains (73– 76%) and intracellular loops (86–100%) (Law et al. 2000).

1.1.2 Opioid receptor signal transduction

After binding of a ligand, opioid receptor undergoes conformational changes which give rise to the dissociation of the heterotrimeric $G_{i/o}$ protein complex into G_{α} and $G_{\beta\gamma}$ subunits (Figure 1.1). The opioid receptor activation leads to multiple signaling transduction cascades, and can regulate a large spectrum of effectors, including adenylyl cyclase (Chen et al. 1993; Meng et al. 1993; Yasuda et al. 1993), N- and L-type voltage-gated Ca^{2+} channels (Tallent et al. 1994; Piros et al. 1996), phospholipase C (Johnson et al. 1994; Spencer et al. 1997), inward rectifying K+ channels (GRIK) (Henry et al. 1995), and mitogen-activated protein kinases ERK1 and ERK2 (Li & Chang 1996; Fukuda et al. 1996).

The inhibition of adenylyl cyclases (AC) by opioid receptor leads to decrease in cAMP production, while cAMP is an important second message involved in the activation of protein kinases and expression of specific genes (Sharma et al. 1977). The suppression of Ca^{2+} influx by opioid receptor is suggested to result in attenuate the excitability of neurons and reduce the release of neurotransmitters (Law et al. 2000;

Wang et al. 2010). Activation of GIRK induces postsynaptic hyper-polarization, thereby preventing neuronal excitation and/or propagation of action potentials (Luscher et al. 2010; Nockemann et al. 2013). All of these activities of opioid receptor signaling decrease the transmission of nociceptive stimuli at molecular, cellular and neuron circuit levels and profoundly reduce the perception of pain (Stein 2016).

The activity of opioid receptors can be regulated by phosphorylation like those of other GPCRs. Various kinases can phosphorylate intracellular regions of opioid receptor including the G protein–coupled receptor kinases (GRKs), which promotes binding of arrestin molecules (Pak et al. 1997; Hasbi et al. 1998). The formation of arrestin-receptor complexes not only uncouples the receptor from the respective G protein that transduces the signal thus blunt the receptor signaling (receptor desensitization), but also leads to agonist-induced, clathrin-coated vesicles–mediated receptor internalization (Law & Loh 1999; Zuo 2005; Williams et al. 2013). The internalized dephosphorylated opioid receptors go through either recycling and integration into the plasma membrane to reinstate signal transduction, or targeting to lysosomes leads to degradation (Ko et al. 1999; Pradhan et al. 2012). Many studies have shown a comprehensive correlation between opioid receptor phosphorylation, desensitization, trafficking and the development of opioid tolerance (Stafford et al. 2001; Nagi & Piñeyro 2011).

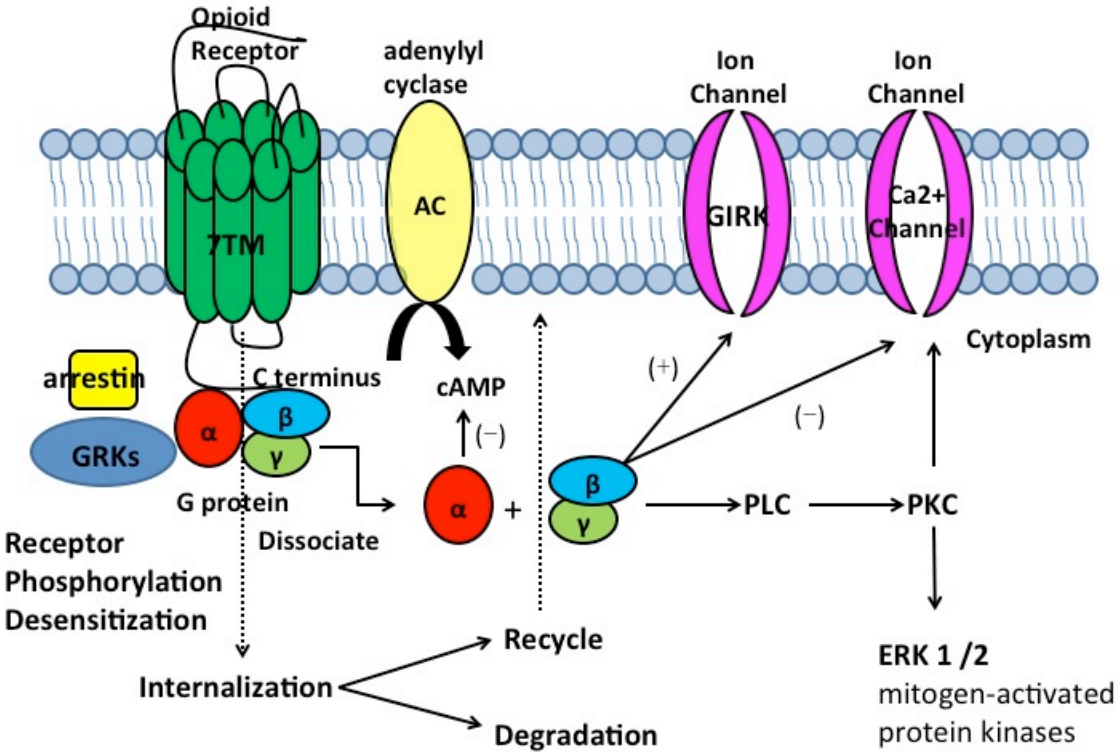


Figure 1.1 A simplified scheme of opioid receptor signaling pathways in cells.

Opioid receptor ligands induce a conformational change at the receptor that allows uncoupling of G proteins to the receptor. The heterotrimeric G protein dissociates into active Gα and Gβγ subunits, which can inhibit adenylyl cyclase and reduce cAMP, decrease the conductance of voltage-gated Ca^{2+} channels, or open rectifying K^{+} channels. In addition, the phospholipase C/phosphokinase C pathways can be activated to modulate Ca^{2+} channel activity in the plasma membrane. Opioid receptor signaling also activates ERK1/2 via PKC or arrestin-dependent pathways. The G protein–coupled receptor kinases (GRKs) phosphorylated the opioid receptors and recruit arrestin proteins, leading to receptor desensitization and internalization.

1.2 Addiction to opioid

1.2.1 Background

Opioids are not only among the most effective analgesics but also highly addictive drugs of abuse. According to the American Chronic Pain Association (ACPA), "Medication-related problems would rank 5th among the leading causes of death in the United States if they were considered a disease. In particular, the overuse, misuse and abuse of opioid (narcotic) pain medications have now become a national issue. The abuse of prescription opioid pain medications now ranks second - only behind marijuana - as the nation's most prevalent drug problem."(ACPA Resource Guide to Chronic Pain Treatment, 2016 Edition)

Opioid addiction has been conceptualized as a chronically relapsing disorder characterized by compulsion to take the drug, loss of control in limiting intake, and emergence of a negative emotional state reflecting a motivational withdrawal syndrome when access to the drug is prevented (Koob & Volkow 2010). Chronic opioid exposure causes long-term changes to the body and brain, which leads to the transition from drug taking to drug dependence. Classic symptoms associated with physical dependence include intoxication, tolerance, escalation in intake, profound dysphoria, physical discomfort, and somatic withdrawal signs (weight loss, marked diarrhea, piloerection, irritability, muscle rigidity and anorexia) during abstinence (Koob 2009). The studies of the neurobiology of opioid addiction focus on both the acute impact of opioid administration and the long-term neuroadaptive changes in the brain that result in relapse. To understand the genetic, cellular, and molecular mechanisms that mediate the transition

from occasional controlled drug use to the loss of behavioral control over drug craving and chronic relapse, animal models of addiction to opioids and human imaging studies of drug abuse patients have been used. The classical animal models used to study opioid addiction include the open-field test (Walsh & Cummins 1976; Veilleux et al. 2010), the condition place preference (CPP) (Iwamoto 1985; Shippenberg & Herz 1987; Cunningham et al. 2006), and the opioid self-administered paradigm (Young & Khazan 1987; Glick et al. 1995; Becker et al. 2000). Behavioral animal experiments help to explore the different stages of addiction cycle and effects of repeated opioid exposure on neuroplasticity in the brain circuits. The imaging studies reveal the discrete brain regions and changes in circuits associated with opioid administration and development of addiction (Younger et al. 2011; Lee et al. 2012).

1.2.2 Neurocircuitry of opioid addiction

A composite addiction cycle compose of three stages: 'binge/intoxication', 'withdrawal/negative affect', and 'preoccupation/anticipation' (Koob & Le Moal 1997). Evidences from animal experiments and human imaging studies suggest that different stages of the addiction involve neuroplasticity changes in a widely distributed network in the brain, like the mesolimbic dopamine system, ventral striatum to dorsal striatum, orbitofrontal cortex and prefrontal cortex, cingulate gyrus, and extended amygdala (Koob & Volkow 2010). The details of neurocircuitry in addiction cycle are summarized below.

The binge/intoxication stage. The acute administration of opioid activates the mesolimbic dopamine system (Di Chiara & Imperato 1988). Animal studies of

intracranial self-administration and intracranial place conditioning have shown that opioids were directly self-administered into the ventral tegmental area (VTA) and nucleus accumbens (NAc). Opioids also produce conditioned place preference when injected into the VTA and NAc (McBride et al. 1999). Lesions of the ventral pallidum, which is a major output from the NAc, are particularly effective in blocking the motivation for intravenous heroin self-administration (Hubner & Koob 1990). Human brain imaging studies have shown that drug-induced increases in dopamine in the striatum (including the ventral striatum where the NAc is located) are associated with subjective descriptors of reward (e.g., pleasure, high, euphoria) (Volkow et al. 1996). All of these evidences support the assumption that the initial action of opioid reward is dependent on dopamine release and opioid receptor activation in the VTA and NAc.

The withdrawal/negative affect stage. During acute morphine withdrawal, the activity of the mesolimbic dopamine system and serotonergic neurotransmission in the NAc are both decreased in animal brains (Pothos et al. 1991; Rossetti et al. 1992). Adrenocorticotropic hormone, corticosterone, and corticotropin-releasing factor (CRF) are elevated during acute withdrawal from chronic administration of addictive drugs such as opioid, producing an aversive or anxiety-like state (Koob & Kreek 2007). The place aversion paradigm was used to measure the aversive stimulus effects of opioid withdrawal (Hand et al. 1988). Suppression of CRF in the amygdala attenuated opioid withdrawal induced place aversions (Heinrichs et al. 1995). Microinjection of beta-noradrenergic-receptor antagonists, or an alpha2-receptor agonist into the bed nucleus of the striaterminalis (BNST) blocked opioid withdrawal-induced place aversion,

implicating the importance of noradrenergic stimulation in the stress responses that follow acute opioid withdrawal (Delfs et al. 2000). Vasopressin, substance P, neuropeptide Y (NPY), endocannabinoids, and other neurotransmitter systems are also reported to be involved in emotional dysregulation of the motivational effects of opioid withdrawal (Koob et al. 1992; Scavone et al. 2013). These studies indicate that the extended amygdala and NAc may have a key role in integrating brain arousal–stress systems with reward systems to produce the negative emotional states of the withdrawal/negative affect stage.

The preoccupation/anticipation stage. Animal studies suggest that opioid-induced drug reinstatement appears to dependent on glutamate release in the nucleus accumbens (Ribeiro Do Couto et al. 2005; LaLumiere & Kalivas 2008; Tahsili-Fahadan et al. 2010). The cue-induced reinstatement of opiates is mediated by the basolateral amygdala (Schulteis et al. 2000), and also involves the glutamate transmission from the prefrontal cortex (PFC) to the NAc core, (Bossert et al. 2005), and GABAergic interneurons in the PFC (Van den Oever et al. 2010). In contrast, stress-induced reinstatement of opioids seems to be associated with the activation of the corticotrophin releasing factor (CRF) receptor and adrenergic receptor in elements of the extended amygdala (Shaham et al. 1998; Highfield et al. 2001; Wang et al. 2006).

In addition, accumulating evidences suggest that one of the major neural substrates of memory and conditioned learning, the hippocampus, is implicated in the modulation of the reinforcing actions of opioids and the reinstatement of drug-seeking behavior (Floresco et al. 2001; Luo et al. 2011). It has been well established that

hippocampus plays a key role in the processing of contextual cues by which memories can be accessed and retrieved, linking the affective conditions or circumstances with drug-taking experiences (McDonald & White 1993; Nestler 2002). As one of the possible contributors to hippocampal neural plasticity, adult hippocampal neurogenesis has been proposed to be involved in modulating different addiction cycles (Mandyam & Koob 2012). It has been hypothesized that an alteration in the rate of adult neurogenesis in the hippocampus contributes to the regulation of drug seeking and relapse, and new born neurons in the dentate gyrus (DG) of hippocampus may block memories associated with the contextual reinstatement of drug seeking or enhance extinction learning (Canales 2007; Canales 2012). Evidences from *in vitro* and *in vivo* studies suggest that opioids can significantly disrupt neurogenesis in the adult hippocampus (Eisch et al. 2000; Mandyam et al. 2004; Kahn et al. 2005; Willner et al. 2014; Xu 2014), which is discussed in detail in the following sections.

1.3 Effect of opioids on adult hippocampal neurogenesis

1.3.1 Introduction to adult neurogenesis

It has been well established that new neurons were born continuously throughout life in the brains of many species, including human (Riksson et al. 1998; Gage 2000). In normal conditions, adult neurogenesis appears to be restricted in two discrete brain regions: the sub-ventricular zone (SVZ) of the lateral ventricle (Doetsch et al. 1997), and the sub- granular zone (SGZ) of the hippocampal dentate gyrus (DG) (Georg & Gage 1996). Since then, substantial research has been made to study the intrinsic and extrinsic factors that regulate adult hippocampal neurogenesis, for newborn neurons in the SGZ could contribute to specific hippocampal functions such as spatial learning, pattern discrimination, and mood regulation (Deng et al. 2010; Aimone et al. 2014).

Results from combined immunohistochemistry and autoradiography on rat brains show that most progeny of neural stem/progenitor cells (NSPCs) differentiate into dentate granule cells (GCs), whereas a small population becomes glia (Cameron et al. 1993). The proliferation, differentiation and maturation of adult-born granule cells are controlled by a series of genetically programmed fate choices (Hevner et al. 2006), and NSPCs in adult hippocampus could be divided into several types according to their different developmental stages. For instance, radial-glia-like stem cells which express glial fibrillary acidic protein (GFAP) and nestin, and have several other astrocytic features are defined as Type-1 cells (Fukuda et al. 2003). Type-2 cells are oval-shaped, highly proliferative cells with short processes which express nestin but not GFAP (Filippov et al.

2003). Type-3 cells are neuroblasts which express doublecortin (DCX) and polysialylated form of the neural cell adhesion molecule (PSA-NCAM) (Kempermann et al. 2004).

Besides the distinct morphological stages, many studies also work on the functional maturation process of these newborn granule cells (GCs) in the DG. During the first week after birth, after exiting the cell cycle and committing to the neuronal lineage, the adult-born GCs migrate a short distance into the inner granule cell layer (GCL) of the DG and start initial axon and dendrites growth (Zhao et al. 2006). At this stage, these GCs are excitatory activated by neurotransmitter γ-aminobutyric acid (GABA) (M. Soledad Esposito et al. 2005) (S Ge et al. 2006a). During the second week after birth, the dendrites of the adult-born GCs extend towards the molecular layer and axons grow through the hilus towards CA3; spines appear on the dendrites by approximately day 16, forming synapses with the local neuronal network (Zhao et al. 2006). These immature neurons have a higher membrane resistance and different firing properties comparing to the adjacent mature granule neurons (M. Soledad Esposito et al. 2005). They receive synaptic GABAergic input from local interneurons, and the resulting responses have slow rising and decay kinetics (Overstreet Wadiche et al. 2005; Markwardt et al. 2009). The GABA-mediated activity seems to be important for the survival and maturation of adult-born GCs. For instance, knock-down of Na+-K+-2Cl- co-transporter SLC12A2 reduces the number of adult-born GCs surviving the second week after birth and caused defects in the synapse formation (Jagasia et al. 2009), while administration of a GABA receptor agonist promotes dendrite growth of adult-born DGCs (S Ge et al. 2006b).

In contrast, glutamatergic afferents are detected by the fourth week in neurons displaying mature excitability and morphology (M. Soledad Esposito et al. 2005). During a short, critical period between the second and third weeks of age, the survival of adult-born GCs depends on N-methyl-d-aspartate (NMDA) glutamate receptor mediated cell-autonomous activity (Tashiro et al. 2006). During the 4th to 6th weeks after birth, the immature neurons show a lower threshold for the induction of long-term potentiation (LTP) and higher LTP amplitude, which is mediated by NMDA receptor subunit NR2B (Ge et al. 2007). It is not until 8 weeks after birth when the basic physiological properties and synaptic plasticity of the new-born GCs are indistinguishable from those of mature GCs (Toni et al. 2007; Deng et al. 2010). Thus, the maturation, survival and the development of functional synapses of the newly born GCs involve the excitatory and inhibitory inputs from the existing circuit in the DG of the hippocampus.

1.3.2 Regulation of adult neurogenesis

Adult neurogenesis can be regulated by numerous intrinsic and extrinsic factors associated with an animal's behavioral and cognitive states, such as genetic background, neurotransmitters, growth factors, physical activity, stress and aging (Zhao et al. 2008). Several class of addictive drugs have been shown to alter adult neurogenesis, including methamphetamine (Teuchert-Noodt et al. 2000), cocaine (Noonan et al. 2008), and opioid (Eisch et al. 2000).

Opioid drugs can cause long-lasting changes in the brain, which influence many different forms of neural plasticity, such as the stability of dendritic spines (Liao et al.

2005) and long-term potentiation (Nugent et al. 2007). Adult hippocampal neurogenesis is also among forms of neural plasticity mechanism that regulated by opioids. However, the effects of opioid on hippocampal neural progenitors are controversial in many cases, and are largely dependent on the manner in which the drug was administered (Fischer et al. 2008a). Also, since adult neurogenesis is a long and continuous process consisting of a series of developmental events, opioids can exert their action on multiple types and stages of the neural stem/progenitor cells (NSPCs). Here, we summarize the most recent work correlating opioid effects on regulating proliferation, differentiation or survival of adult-born hippocampal GCs (Table 1).

Table 1.1 Effects of drugs on different stages of adult neurogenesis

Drugs	Species	Administration Paradigm	Effects			References
			Proliferation	Neural Differentiation	Survival	
Morphine	Rat	Acute injection	-		-	(Eisch et al. 2000)
Morphine	Rat	Pellet implantation	↓		↓	(Eisch et al. 2000)
Heroin	Rat	Self-administration	↓		↓	(Eisch et al. 2000)
β-endorphin	Rat	in vitro, Chronic	↑			(Persson, Thorlin, Bull & Eriksson 2003)
#Naloxone	Rat	in vitro, Chronic	↓	↑		(Persson,

						Thorlin, Bull, Zarnegar, et al. 2003)
#Naltrindole	Rat	in vitro, Chronic	↓ ↓			(Persson, Thorlin, Bull, Zarnegar, et al. 2003)
#Naltrexone	Rat	Acute injection				(Holmes & Galea 2002)
Morphine	Mouse	Pellet implantation	↓			(Mandyam et al. 2004)
Morphine	Rat	Multiple injections	↓			(Kahn et al. 2005)
Morphine	Mouse	Pellet implantation	↓	↓		(Fischer et al. 2008a; Arguello et al. 2008; Brekken & Eisch 2010)
Morphine	Mouse	Multiple injections	-			(Fischer et al. 2008a)
Met-enkephalin	Zebra finche	in vitro, Chronic	↓			(Khurshid et al. 2010)
# Naloxone	Zebra finche	in vitro, Chronic in vivo, Chronic	↑			(Khurshid et al. 2010)
Heroin	Rat	Extinction of Self-administration		↑		(Hicks et al. 2012)
Buprenorphine	Mouse	Multiple injections	↓		↑	(Pettit et al. 2012)
Methadone	Rat	Multiple injections	-	-	-	(Sankararaman

						et al. 2012)
Morphine	Mouse	Multiple injections	-	↓		(Zheng et al. 2013)
Fentanyl	Mouse	Multiple injections	↑	-		(Zheng et al. 2013)
Morphine	Mouse	in vitro, Chronic	↓		↓	(Willner et al. 2014)
Morphine	Mouse	Multiple injections		↓		(Xu et al. 2014)

↑, Up-regulation; ↓, Down-regulation; - , no significant differences; #, opioid receptor antagonist

1.3.3 Opioids modulate adult neural progenitor proliferation

The most traditional and commonly used method to detect proliferating cells in adult brain is by using exogenous markers of DNA synthesis, such as the thymidine analog bromodeoxyuridine (BrdU), to label and track the birth of new born cells (Kriss & Revesz 1962; del Rio & Soriano 1989). The first report connecting opioid and adult neurogenesis was in 2000, A.J Eisch *et al* showed that chronic morphine, administered via subcutaneous pellet, decreased the number of proliferating cells labeled with BrdU in the SGZ in rodents; similar effect was also observed in rats after chronic self-administration of heroin (Eisch et al. 2000). Since then, evidences were accumulated from both sides to established opioid's negative impact on proliferation of adult born GCs (Table 1). For instance, proliferating cells in SGZ marked by two endogenous cell cycle markers, proliferating cell nuclear antigen (PCNA) and phosphorylated histone H3 (pHisH3), were largely reduced by chronic morphine, and triple labeling for BrdU,

PCNA, and pHisH3 revealed that morphine-treated mice have a shorter Gap2/mitosis (G(2)/M) phase (Mandyam et al. 2004). Rats injected with morphine sulfate (20 mg／kg) daily for 1 week were shown to have a strong reduction of cellular proliferation marked by fewer cells immunoreactive (IR) for PSA-NCAM, a cell surface protein that transiently expressed by newly generated neurons during development. Such reduction was followed by a rebound increase after 1 week withdrawal and a return to normal after 2 weeks withdrawal (Kahn et al. 2005). It was demonstrated that morphine pellet implantation for 24-96 hours decreased the proliferating cells labeled by BrdU and cycle marker Ki67 in DG (Arguello et al. 2008). Other opiate analgesics like buprenorphine, administered via subcutaneous injections (0.05 mg/kg) over a 3-day period in mice, also decreased the number of actively proliferating 5-iodo-2-deoxyuridine (IdU) labeled cells (Pettit et al. 2012), while no such effect was observed with synthetic opiate methadone (Sankararaman et al. 2012). On the contrary, knock-out of mu-opioid receptor was shown to enhance ischemia-induced generation of immature hippocampal neurons (Kolodziej et al. 2008). Following extinction from heroin-seeking behavior, the formation of immature neurons in the DG was increased, represented by DCX-IR cells (Hicks et al. 2012). In addition, there are also reports suggest that chronic morphine treatment influence neurogenic microenvironment in DG by regulating certain growth factors, such as increasing the pro-proliferative factor vascular endothelial growth factor (VEGF) (A. A. Arguelloa, Stephanie J. Fischera, Joshua R. Schonborna, Richard W. Markusa, Rolf A. Brekkenb 2012) .

Opiate effects on adult neurogenesis seems to be dependent on the paradigm of the experiment design, such as test in vitro or in vivo, and drug administration paradigm. In isolated rat hippocampal neural progenitor cells, incubation with β-endorphin for 48 h increased the total DNA content, and the number of cells expressed of PCNA and pHisH3. This proliferative effect was antagonized by naloxone (Persson, Thorlin, Bull & Eriksson 2003). The same group also reported that mu- and delta-opioid receptor (MOR and DOR) antagonists decrease proliferation of cultured neural progenitor cells (Persson, Thorlin, Bull, Zarnegar, et al. 2003). Similarly, a longer acting opioid antagonist naltrexone, was shown to decrease cellular proliferation in the adult rat hippocampus (Holmes & Galea 2002). These results are conflicting with more recent observations, which showed cultured mouse hippocampal neural progenitor cells treated with morphine for 24h demonstrated decreased BrdU expression in a dose dependent manner (Willner et al. 2014). This discrepancy in morphine's effect on neural proliferation remains within in vivo experiments, in which implantation of morphine pellets resulted in negative effect on adult neurogenesis (Eisch et al. 2000; Arguello et al. 2008), while intraperitoneally injection of escalating dose of morphine failed to show any significant influence(Fischer et al. 2008b). Such inconsistency of morphine's effect may due to difference in blood levels of morphine, when implant with morphine pellet, the drug level in blood is relevantly stable and caused decrease in number of proliferating cells, whereas the injection paradigms that produced transient spikes in drug blood levels fails to produce significant effect on hippocampal neural proliferation (Fischer et al. 2008b). Nevertheless, studies in our lab support the assumption that opiate negatively regulate

neural proliferation, for morphine daily injection in a condition place preferences (CPP) paradigm decreased the number of neural progenitors in DG labeled by DCX and other neurogenesis markers in mice (Zheng et al. 2013). Further research in this model reveals that such reduction may due to morphine's effect in modulating neural progenitors' differentiation, rather than regulating proliferation, which will be discussed in detail in the following section.

1.3.4 Opioid modulate adult neural progenitors differentiation and maturation

The radial-glia like neural stem cells in SGZ went through asymmetric cell division and gave rise to different types of progeny, including progenitors retaining self-renew capability, neuroblasts, astrocytes and oligodendrocytes (Gage et al. 1998; M S Esposito et al. 2005). A growing body of literature indicates that opiate drugs not only influence hippocampal GCs proliferation, but also interfere with differentiation and future developmental processes. In adult rat hippocampus, repeated morphine treatment altered the GABAergic phenotype of adult hippocampal GCs by significantly increasing the transcription of glutamate decarboxylase-67 mRNA, a GABA synthesizing enzyme (Kahn et al. 2005). By examining the co-staining of BrdU and cell cycle marker ki67 in mouse SGZ, it was found that morphine treatment increase the percent of BrdU-IR cells that were type 2b and decreased the percent that were immature neurons (Arguello et al. 2008). Analysis of the double-labeled cells in cultured mouse hippocampal progenitors treated with morphine showed a decrease in cells co-stained for BrdU with nestin and an increase in cells co-stained with BrdU and neuron-specific class III beta-tubuline (TUJ1) compared to cells treated with saline (Willner et al. 2014). Incubation of adult

hippocampal progenitors with endogenous opioid peptide beta-endorphin resulted in a threefold increase in oligodendrogenesis but no significant change in astrogliogenesis (Persson et al. 2006). Overall, these observations indicate that opioids could play a role in regulating adult hippocampal neural differentiation and maturation.

A recent study in our lab showed in detail that morphine exposure affected hippocampal neurogenesis by modulating cell-lineage in isolated hippocampal progenitor cells (Xu 2014). In cultured NSPCs, morphine treatment activates MOR and downstream signaling pathways, including extracellular signal-regulated kinase (ERK) activation (Belcheva et al. 2001). Phosphorylated ERK in cytosol is capable of phosphorylating the HIV-1 Trans-activation response element (TAR) RNA-binding protein (TRBP), a cofactor of Dicer, and the Dicer activity enhancement promotes the maturation of miR-181a. This drives down-regulation of Prospero homeobox protein 1 (Prox-1) and an up-regulation of Notch1 expression, while the Notch1 signaling plays an important role in regulating cell fate of the adult born hippocampal GCs (Breunig et al. 2007; Ables et al. 2010). Thus, morphine favoring the progenitor cells differentiation into glia instead of neuron by regulating Prox1/Notch1 activities via its control of miR181a level. Another opiate drug fentanyl did not show this effect, since fentanyl activated ERK via a β-arrestin-dependent pathway, and the activated ERK translocates to the nucleus (Zheng et al. 2008).

Furthermore, the activity of a transcriptional factor, neurogenic differentiation 1 (NeuroD1) was also shown to be regulated by morphine treatment (Zheng et al. 2010). NeuroD1 is a basic helix-loop-helix transcription factor that are expressed during

glutamatergic neurogenesis in the developing cerebellum and in the adult hippocampal DG (Hevner et al. 2006; Miyachi et al. 1999). It was shown to be involved in the differentiation of the progenitor cells and migration of immature neurons in the dentate gyrus (Liu et al. 2000). Several in vivo study support the fact that NeuroD1 has an important role in neuronal fate determination during both embryonic and adult neurogenesis (Lee 1997; Roybon et al. 2009), and is essential for the survival and maturation of adult-born neurons (Gao et al. 2009). Thus, by negatively regulating NeuroD1 activity, morphine impaired the differentiation of newborn GCs, leading to a reduction in neuroblasts and immature neurons expressing DCX and TUJ-1.

1.3.5 Opioids modulate adult neural progenitor survival and apoptosis

After being generated by neural stem cells in the DG of hippocampus, a large portion of these progenitors die within a few days following their birth (Dayer et al. 2003). It is reasonable to assume that neural precursors that fail to differentiate into functional immature neurons would go through apoptosis. The massive cell death of adult-born granule neurons may serve as a natural selective mechanism since it has been demonstrated that cell survival and death are both important during learning and memory (Dupret et al. 2007). Whether opiates interfere with this process remains to be demonstrated.

Chronic morphine and heroin treatment was shown to decrease GCs survival in vivo, by decreasing the number of 4 weeks old BrdU-labeled cells in the granule layer of the DG in drug-group rats compared to control rats (Eisch et al. 2000). However, direct evidence of opiate drugs inducing apoptosis of adult hippocampal progenitors is deficient

and inconsistent. Chronic morphine transiently increases cell death in the SGZ of mice, the activated caspase-3 cell count was increased after 24 but not 96 h (Arguello et al. 2008). Morphine exposure in cultured NSPCs led to a significant increase in caspase-3 activity in the nestin and GFAP positive cells, but not in TUJ1 positive neurons (Willner et al. 2014; Meneghini et al. 2014). Knock-out of mu-opioid receptor, in the contrary, was shown to enhance adult-born hippocampal GCs' survival, suggesting endogenous opioids have a negative effect on adult hippocampal neurogenesis (Harburg et al. 2007). However, minimal buprenorphine treatment was shown to increase the survival of newly born cells in mice DG of hippocampus (Pettit et al. 2012). In other cases, opioid drugs such as morphine were not associated with hippocampal neural apoptosis (Bajic et al. 2013).

So far, accumulating evidences have demonstrated that multiple opiate drugs interfered with proliferation, differentiation, maturation and survival of developing adult-born hippocampal neural precursors. These studies represent that most of the opiates have an adverse effect on adult hippocampal neurogenesis, by decreasing the total number of proliferating cells and cell survival in the SGZ of DG area; but there are also some exceptions. For neural differentiation, opiates such as morphine are likely to impede early progenitors differentiating into neuroblasts, but favor differentiation into glia.

The detail mechanism of the regulation of hippocampal neurogenesis by opiates remains to be clarified. Opiate may directly act on neural progenitors with MOR and DOR on the cell surface (Persson, Thorlin, Bull & Eriksson 2003) (Persson, Thorlin, Bull, Zarnegar, et al. 2003), or they may modulate the neurogenic microenvironment of

the DG, to indirectly influence the cell proliferation by growth factors in the hippocampus (Brekken & Eisch 2010). It has been reported that immature neurons (14–28 days postmitotic) are not inhibited, but excited by GABAergic activity (Shaoyu Ge et al. 2006). Also, exposure to novel environments increases GABAergic tone in the DG and facilitates the generation of LTP(Saxe et al. 2006). Thus, an alternative explanation for opioids' regulation of adult hippocampal neurogenesis is early neural progenitors need exciting signals from existing circuit for future differentiation and maturation, and opioid agonists interfere with this process by decreasing GABA release from interneurons (Zieglgansberger et al. 1979; Neumaier et al. 1988). It is intriguing to further investigate these possible mechanisms and to determine whether morphine exhibits its effect directly or indirectly on neural progenitors in the SGZ of hippocampus.

1.4 Adult neurogenesis and drug-associated memory

1.4.1 **Role of adult neurogenesis in learning and memory**

The hippocampus is a crucial structure for the formation of certain types of memory, such as declarative memory and spatial memory (Squire 1992). During development, newborn neurons in the adult SGZ migrate into the granule cell layer of the dentate gyrus and integrate into existing hippocampal circuit (Shaoyu Ge et al. 2006; Duan et al. 2007). Immature neurons have higher input resistance, more depolarized resting membrane potentials and small, broad action potentials compared to mature neurons (X. S. Liu et al. 2000), so they are more flexible in changes of neural plasticity, and may have a substantial roles in hippocampal function during learning and memory.

Correlations between the rate of adult hippocampal neurogenesis and learning and memory have been shown in several animal studies. For instance, by using the antimitotic agent methylazoxymethanol acetate (MAM), the ablation of adult neurogenesis in rats resulted in deficits in hippocampus-dependent trace conditioning tasks, but did not affect contextual fear conditioning or spatial navigation learning, or other hippocampus-independent delay conditioning tasks (Shors et al. 2001; Shors et al. 2002). Ablation of the neural progenitor cells by irradiation of the hippocampus or genetic ablation impaired contextual fear conditioning, but not cued conditioning (Saxe et al. 2006). In the spatial-navigation learning of Morris Water Maze (MWM), ablation of adult-born hippocampal neurons by transgenic strategy caused impairment of spatial relational memory (Dupret et al. 2008; Zhang et al. 2008). There are some animal studies show that reduced adult neurogenesis by either irradiation or lentivirus-mediated overexpression of dominant-

negative Wnt in mice impaired performance in two spatial discrimination tasks, also impaired the long-term but not the short-term retention of spatial memory (Snyder et al. 2005; Jessberger et al. 2009; Deng et al. 2009). In addition, when animals were infused with pan-caspase inhibitor z-Val-Ala-Asp-fluoromethyl-ketone (zVAD) in the lateral ventricles to block the learning-induced apoptosis, they showed an impaired spatial memory and impeded cell proliferation in the hippocampus (Dupret et al. 2007). In summary, the new neurons born in DG of hippocampus are involved in associating events that occur within a short time span, so the depletion of adult neurogenesis affect many behavior tasks such as trace conditioning, spatial learning and fear conditioning (Deng et al. 2010).

1.4.2 Role of adult neurogenesis in drug related memory

Over the past decades, there has been growing understanding that the process of drug addiction shares striking commonalities with molecular and cellular plasticity associated with natural reward learning and memory (Nestler 2002; Kelley 2004). Learning and memory and drug addiction not only have similarities in basic cellular mechanisms leading to adaptive changes in gene expression and synaptic plasticity, but also share common brain circuits involving a complex regions of dopaminergic and glutamatergic neuronal networks, including the hippocampus, cerebral cortex, ventral and dorsal striatum, and amygdala (Swanson 2000; Cardinal et al. 2002; Cardinal & Everitt 2004; Morris et al. 2003). As I summarized in the previous section, modulation of adult hippocampal neurogenesis impaired animal's performance in the spatial learning and

some memory task, so the similar regulation of adult neurogenesis may also affect animal's behavior in drug reward and relapse (Kelley 2004; Koob & Volkow 2010).

Recent studies suggest that hippocampal neurogenesis in DG may has substantial roles in opioid addiction cycle. For instance, suppression of adult neurogenesis by long-term stress, had significant positive relationships with ratings of craving for heroin (Preston & Epstein 2011). Some positive regulators of hippocampal neurogenesis like environment enrichment, voluntary exercise, on the contrary, prevented the development of morphine induced CPP (Lett, Virginia L Grant, et al. 2002; Rozeske et al. 2011), decreased the rewarding effect of heroin (El Rawas et al. 2009b) and the maintenance of heroin self-administration (Smith & Pitts 2012). These results suggest a negative correlation between the level of adult neurogenesis and opioid addiction.

Animal studies also indicate that opiates' rewarding effect and contextual memory is related by the manipulation of adult-hippocampal neurogenesis. When hippocampal neurogenesis is enhanced by physical excise or environment enrichment, the animals show a lower response to drug craving and reward, including opioids (Lett, Virginia L Grant, et al. 2002; Rozeske et al. 2011; El Rawas et al. 2009b; Smith & Pitts 2012). Following over-expression one of the transcription factor during neural development, neurogenic differentiation 1 (NeuroD1) in dentate gyrus to induce neural differentiation, animals showed much longer memory of their drug experience, represented by morphine-primed CPP extinction time; knocking down NeuroD1 with RNA interference method had an opposite effect (Zheng et al. 2013). These results indicate that the progenitors at

certain stage of development might serve as key players during drug contextual memory formation.

Current studies suggest that opioids are involved in the proliferation and fate determination of adult-born GCs in the SGZ of hippocampus, and the manipulation of adult hippocampal neurogenesis in return, influences rewarding effect and drug-contextual memory that associate with the opioid addition. So the aims of my thesis are (1) to investigate the detail mechanisms of the opioids' manipulation of adult neurogenesis and (2) to explore an efficient compound or method that regulate adult neurogenesis with a positive contribution to opioid addiction.

CHAPTER 2
OPIOID IMPEDES NERURAL PROGENITORS' DIFFERENTIATION

2.1 Introduction

As we discussed in Chapter One, opioid influences many different forms of neural plasticity (Liao et al. 2005; Nugent et al. 2007), including adult neurogenesis in the sub-granular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Adult neurogenesis has a substantial role in hippocampus function during learning and memory, and it has been implicated in drug reward and relapse (Shors et al. 2001; Saxe et al. 2006). Several addictive drugs have been shown to alter adult neurogenesis, for instance, the psychomotor stimulants methamphetamine and cocaine decreased proliferation or maturation of hippocampal neural stem cells (Teuchert-Noodt et al. 2000), and withdrawal from cocaine normalizes deficits in the proliferation of adult-born GCs (Noonan et al. 2008). In previous chapter, section1.3, we discussed the effect of opioids on the proliferation, differentiation and maturation of adult-born granule cells (GC) with different experiment paradigm (Eisch et al. 2000; Hicks et al. 2012; Harburg et al. 2007; Arguello et al. 2009; Willner et al. 2014). In section 1.4, we also mentioned some recent studies that suggested a negative correlation between drug addiction and level of adult hippocampal neurogenesis (Noonan et al. 2010; Mustroph et al. 2011; Recinto et al. 2012). Since neural stem/progenitor cells (NSPCs) in the adult hippocampus could be divided into several types according to their different developmental stages, it is important to determine the specific types and developmental stages of NSPCs on which addictive drugs such as morphine exert their actions, to further speculate about the potential mechanisms underlying the opioid's regulation.

Former studies suggested that morphine's negative effect on adult neurogenesis was largely dependent on the manner in which the drug was administered. Implantation of morphine pellets resulted in a negative effect on adult neurogenesis (Eisch et al. 2000), while intraperitoneal (i.p.) injections of morphine failed to show any significant influence (Fischer et al. 2008b). It was demonstrated that chronic morphine affected proliferation of type-2b and type-3 NSPCs but not others (Arguello et al. 2008). This discrepancy in morphine's negative effect remains in a conditioned place preference (CPP) paradigm, in which morphine injection decreased the number of neural progenitors in the DG labeled by Doublecortin (DCX) and other neurogenesis markers (Zheng et al. 2013). Furthermore, a transcriptional factor, NeuroD1, was shown to participate in morphine's regulation of adult neurogenesis. NeuroD1 is one of the conserved transcription factors that are expressed in the same order during glutamatergic neurogenesis in the developing cerebellum and in the adult dentate gyrus (Hevner et al. 2006). It has been shown to be involved in the differentiation of the progenitor cells and migration of immature neurons (Von Bohlen and Halbach 2007). Several in vivo studies support the fact that NeuroD1 has an important role in specifying the neuronal fate during hippocampal neurogenesis (Roybon et al. 2009), and it is essential for the survival and maturation of adult-born neurons (Gao et al. 2009). Thus, a probable mechanism for morphine's inhibition on adult neurogenesis is the drug regulation of progenitor cells' differentiation of immature neurons via its control of NeuroD1 activity. Our recent in vitro studies in isolated hippocampal progenitor cells suggest that by regulating Prox1/Notch1 activities via its control of the miR181a level, morphine alters the cell

lineage of the progenitor cells during differentiation, favoring the differentiation into glia instead of neurons (Xu et al. 2014). Whether the in vivo effects of morphine are similar to in vitro observations remains to be demonstrated.

To delineate the precise mechanism in which morphine modulates adult neurogenesis, we utilized a transgenic animal model in which a subpopulation of newborn granule neurons of the DG was labeled by EGFP under the control of a pro-opiomelanocortin (POMC) promoter. EGFP expression at the SGZ is transient, peaking at two weeks after the radial glial (neural progenitor) cells' mitosis and turning off by one month (Overstreet et al. 2004). The POMC-EGFP mice were trained under the 3-chambers CPP paradigm with either saline or morphine. We observed after 4 days of CPP training with saline, the number of EGFP-labeled newborn GCs in sub-granular zone (SGZ) hippocampus significantly increased compared to mice injected with saline in their homecage. CPP training with morphine significantly decreased the number of EGFP-labeled GCs, whereas no significant difference in the number of EGFP-labeled GCs was observed with the homecage mice injected with the same dose of morphine. By classifying neural progenitors in different developmental stages on the basis of the morphology of EGFP+ cells and co-expression with other immunohistochemical markers that reflect different stages of neurogenesis, we were able to demonstrate that morphine modulates adult neurogenesis by interfering with the transition of NeuroD1-expressing type-II and type-III neural precursors into DCX and Tuj-1 expressing immature neurons. Furthermore, a decrease in co-localization between BrdU+ and DCX+ cells and an increase in co-localization between BrdU+ and GFAP+ cells were observed.

Overexpression of NeuroD rescued the negative regulation of morphine on adult neurogenesis and strengthened the animal's memory of the drug experiences. Thus, our current in vivo studies support our in vitro reports that morphine's negative effect on adult neurogenesis is mainly due to the drug's action on the transition of progenitors into immature neurons. The over-expression of NeuroD1 facilitates the transition, negating morphine's effect on adult neurogenesis and the subsequent retention of drug experience.

2.2 Materials and Methods

2.2.1Animals

Eight-week-old C57BL/6J-Tg (POMC-EGFP) 1Low/J male mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) (http://jaxmice.jax.org/strain/009593.html). The transgene from which the mouse line was generated was described by Overstreet et al. (Overstreet et al. 2004). Mice were mated and all offspring were genotyped as described in a protocol from Jackson Laboratory Genotyping Database (Stock number: 009593). Three-month-old male transgenic carriers were used in the behavior study. Animal maintenance and procedures were conducted according to the Institutional Animal Care and Use Committee (IAUCUC) policies at the University of Minnesota.

2.2.2 Condition Place Preference (CPP)

Mice stereotaxically injected with lentivirus were allowed to habituate for at least one week in a 12/12 h reverse-light-cycle room prior to the CPP paradigm. The experiment was carried out in a three chamber apparatus supplied by Panlab (Harvard

Apparatus, Holliston, MA) according to the protocol (Cunningham, C LPatel & Milner 2006) with a slight modification, as described previously (Zheng et al. 2013). Briefly, in the habituation stage (day 1-2), mice were allowed free access to all three chambers for 15 min per day. On day 3, mice's place preference between two chambers (black and white) was tested and recorded as a pre-conditioning preference. Mice with strong bias to one of the chambers were excluded from the subsequent study. The rest of the mice were trained with saline or morphine for four days, received a saline injection subcutaneously in the morning, spent 30 min in one chamber (black or white, randomly chosen), received either 5 mg/kg morphine or an equal volume of saline injection (s.c.) in the afternoon, and then spent another 30 min in the complementary chamber. On day 8, mice were tested for post-conditioning preference. During the extinction stage, the place preference of each mouse was tested once a week until no significant preference was detected in two continuous tests (Figure 2.1). AnyMaze software (Stoelting, Wood Dale, IL) was used to track and record the position of mice in the apparatus during the testing period. The place preference of each individual mouse was calculated as follows:

CPP score = Time in drug-paired chamber (after training- before training) (second)

2.2.3 Lentivirus construction

A Lentivirus V5-DEST construct expressing NeuroD1-cDNA was prepared as described previously (Zheng et al. 2010). The TRC Lentiviral shRNA of NeuroD (5'-CCGGGCTCAGCATCAATGGCAACTTCTCGAGAAGTTGCCATTGATGCTGAGC TTTTTG-3') and a corresponding negative control construct were obtained from University of Minnesota Genomics Center. Virus particles were assembled in 293FT cells

by transfecting V5-DEST constructs or shRNA constructs together with pLP1, pLP2 and pLP-VSVG constructs from the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen, Grand Island, NY) following the manufacturer's instructions. Viral titers (~1.0 × 10^8 TU/mL) were determined by assessment of DNA sequences in transduced 293FT cells (Sastry et al. 2002). NeuroD1 gene expression and knock down were tested by western blot analysis and immunostaining.

2.2.4 Stereotaxic Injection

Stereotaxic injection of lentivirus was carried out as described (Zheng et al. 2013). Mice were anesthetized with isoflurane inhalation and a hole of 0.5 mm was drilled at 1.9-2.0 mm posterior to the bregma and ±1.0 mm lateral to the midline of each side. A needle of 28 gauges was connected with a flexible tube filled with virus and lowered 2.1 mm below the meniscus. Using a micro-syringe pump controller, 2 μl of virus (1x10^8 TU/ml) was injected at a constant rate over a 5 min period. The virus was allowed to diffuse for 5 min and the needle was then raised slowly at a constant rate over a 2-min period. The holes were closed with bone wax and the wound on scull was sealed with Vetbond™ Tissue Adhesive (3M™, St. Paul, MN). Mice were allowed to recover for one week before any behavior study. As we have reported earlier, expression of NeuroD1 was observed in all cell types within the DG immediately after virus injection, but only distinct cells showed NeuroD1 expression 1 week after injection (Zheng et al, 2013)(Figure 2.2). Such observation is in agreement with the literature report that only adult generated neural progenitors are target for lentivirus-mediated transgene delivery, 1 week after injection (van Hooijdonk et al. 2009).

2.2.5 Bromodeoxyuridine (BrdU) labeling, Immunohistochemistry and Quantification

Mice were injected with BrdU (200 mg/kg i.p.) for three continuous days prior to CPP training (Figure 2.3A) or 2 h prior to perfusion. A subset of mice was sacrificed after CPP training, and the rest were maintained and tested until their place preference was totally extinct (Figure 2.10A). Mice were deeply anesthetized with ketamine and xylazine and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were post-fixed with 4% paraformaldehyde, transferred into 10% sucrose and 30% sucrose sequentially for dehydration and then embedded in an O.C.T. compound. 30 μm coronal sections were cut through the dentate gyrus of the hippocampus (-0.82 to -3.70 mm post bregma) with a cryostat and every one in eight sections were mounted on the same Superfrost plus glass slides (Thermo Fisher Scientific, Waltham, MA) for future analysis. Thus, there were approximately 10 to 12 sections on the same slide, and any two of them were 0.24 mm apart in the stereotaxic Y-axis of hippocampus.

For immunohistochemistry, mouse coronal sections were rinsed with TBS three times, blocked with 5% goat serum or donkey serum (with 1.5% BSA in TBS) at room temperature for 1 h and then incubated with the primary antibody. An extra antigen retrieval step was needed for BrdU and NeuroD1 staining prior to serum blocking: sections were treated with 1 M HCl at 40 ˚C for 30 min, and then they were incubated with 10 mM of Boric Acid for 10 min and washed with TBS for 5 min. The primary antibodies used in our studies were listed in **Table 2.1.**

Table 2.1 Primary Antibodies used in immunohistochemistry studies

Antibody	Species	Dilution	Company
anti-GFP (Monoclonal)	Mouse	1:2000	Life technologies, Grand Island, NY
anti-BrdU	Rat	1:800	Abcam, Cambridge, MA
anti-Nestin	Mouse	1:500	Abcam
anti-Sox2	Rabbit	1:500	Abcam
anti-DCX	Rabbit	1:1000	Abcam
anti-NeuroD	Goat	1:400	Santa Cruz Biotechnology, Santa Cruz, CA
anti-Tuj1	Mouse	1:1000	Covance, Princeton, NJ
anti-GFAP	Rabbit	1:1000	Dako, Carpinteria, CA
Anti-Caspase-3	Rabbit	1:1000	Cell Signaling, Danvers, MA
Anti-MOR	Rabbit	1:1000	Genentech, *Customer ordered; San Francisco, CA

Secondary antibodies were used as follows: Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 594 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-rat IgG, Alexa Fluor 647 goat anti-rabbit IgM, Alexa Fluor 594 donkey anti-goat IgG, Alexa Fluor 647 donkey anti-goat IgG (Life technologies). The dilution of all secondary antibodies is 1:2000.

For the TUNEL Assay, we use Click-iT® Plus TUNEL Assay for *in situ* apoptosis detection with the Alexa Fluor® dyes kit (Life technologies, Grand Island, NY; Catalog number: C10618) following the manufacturer's instructions.

Sections were mounted with DAPI Fluormount G (Sigma-Aldrich, St. Louis, MO) and visualized using a Leica DFC365 FX 1.4 mp monochrome digital camera connected to a Leica DM5500 B upright microscope (Leica, Germany). Leica Application Suite (Leica, Germany), Metamorph (Sunnyvale, CA) and ImageJ (NIH, MA) software were

used to perform cell counting, fluorescent intensity, and co-localization analysis of images. To quantify the total number of fluorescent-positive cells in the SGZ, images of every section on the same slide throughout the dentate were taken by camera (10-12 sections in total). The number of marker positive cells from each section were counted by the cell-counting program ImageJ and added together to represent the number of marker positive cells of one animal, and then the average summation of every animal in one group was used to represent the expression level of one neurogenesis marker. The stereotaxic quantification from the front to the back through the hippocampus was also provided to represent the average distribution of these marker positive cells (6-8 animals).

Statistical analysis was performed with the GraphPad Prism 5.0 software. Statistical significance was determined by a student t-test, one-way or two-way ANOVA with a Bonferroni test for post hoc comparisons.

2.3 Results

2.3.1 CPP paradigm is essential for a morphine-induced decrease in POMC-EGFP-expressing neurons.

In POMC-EGFP transgenic mice, the EGFP-expressing cells in the sub-granular zone (SGZ) of the dentate gyrus represent adult-born neurons, which are 3-28 days post-mitotic (Overstreet et al. 2004). Four groups of male POMC-EGFP transgenic mice received saline (0.9% NaCl) or morphine (5 mg/kg) subcutaneous injections, a.m. and p.m., respectively. Two groups of mice only received injections in their home cages (Group 1/2), while the other two groups went through a CPP training paradigm (Group

3/4; Figure 2.4A). On day 8, these two groups of mice were tested for CPP preferences (Figure 2.1). Immunohistochemistry analysis showed that there was a significant difference in the number of green fluorescent cells in the dentate gyrus between the saline group (1319±69.1; N=10) and the morphine group (1010±54.0; N=10) after CPP training, but there was not a significant difference in the two groups without training (saline: 930±18.8; morphine: 758±94.2; N=10) (Figure 2.3B, D; two-way ANOVA, effect of training: $F_{1,36}=21.16, p<0.01$; effect of drug: $F_{1,36}=5.91, p<0.05$).

When the S-phase marker BrdU was used to label the proliferating cells right before mice were exposed to morphine or saline, similar results were obtained. A subset of animals from the four groups received three injections of BrdU (200mg /kg, i.p.) three days prior to the CPP training (day 1-day 3), while the rest of the animals received only one injection 2 h prior to perfusion (day 9) (Figure 2.3A). For mice injected with BrdU during day 1 to 3, the groups trained with morphine show a significant decrease in the number of BrdU labeled cells in their SGZ (saline: 272±10.3; morphine: 215±21.6; n=6), but no significant difference was found in groups without training (saline: 209±20.8; morphine: 199±15.3; n=6) (Figure2.3 C, E; two-way ANOVA, effect of training: $F_{1, 20}= 5.102, p<0.05$; effect of drug: $F_{1, 20}=3.463, p<0.05$). However, for mice injected with BrdU on day 9, 2 h prior to perfusion, no significant differences in the number of BrdU-labeled cells were detected between the four groups (Figure2.3 F-G). Comparing the fluorescent images of BrdU labeled cells under the microscope, we found that 50%-60% of the BrdU-labeled cells were also EGFP positive (Figure2.3F, left panel) from mice injected with BrdU on days 1-3, while there was no colocalization between BrdU and

EGFP positive cells (Figure 2.3F, right panel) from mice injected with BrdU on day 9. Our observation supported the former report that in this animal model, EGFP labeled adult-born neurons were 3-28 days post-mitotic (Overstreet et al. 2004), but they were not early proliferating neural stem cells. These results indicated that CPP training increases the number of neural progenitors labeled by EGFP and enhanced the survival of adult-born neurons, but this effect was blocked by morphine treatment.

2.3.2 Morphine decreased the number of late stage progenitors and immature neurons after CPP training, but not early type-I, type-II progenitors in SGZ.

Further immunohistochemistry analysis showed that neither morphine injection in the home cage nor that during CPP training changed the numbers of early stage of NSPCs. Mice from different groups have no significant differences in the number of proliferating cells, as shown by the S-phase marker BrdU (Figure 2.3) or cellular proliferating marker Ki-67 (Figure 2.4). Additionally, the four groups of transgenic mice showed similar levels of early neurogenesis marker expression, including nestin (Figure2.5 A, F), Sox2 (Figure 2.5 B, G) or NeuroD1 (Figure 2.5 C, H) labeled type I and type II cells.

On the other hand, mice trained with morphine in CPP showed a decreased number of both DCX (Figure2.5D, I; two-way ANOVA, effect of training: $F_{1,20}=28.15$, $p<0.05$; effect of drug: $F_{1,20}=22.69$, $p<0.05$) and Tuj-1 (Figure 2.5 E, J; two-way ANOVA, effect of training: $F_{1,20}=19.05$, $p<0.01$; effect of drug: $F_{1,20}=3.857$, $p<0.05$) labeled type III cells or immature neurons. No change in the DCX or Tuj-1 labeled cells

was found in groups without CPP training (DCX: saline-no training: 1604±54.8; morphine-no training: 1588±24.2; saline-CPP: 1836±38.5; morphine-CPP: 1596±18.2; N=6) (Tuj-1: saline-no training: 79±4.1; morphine-no training: 98±11.2; saline-CPP: 126±6.3; morphine-CPP: 97±8.5, N=6). In a previous study, we reported that morphine decreased NeuroD1 activity but not the NeuroD1 protein level in rat hippocampal primary neuron cultures (Zheng et al. 2010). A similar morphine effect was now observed *in vivo*. In the hippocampus from the transgenic mouse trained with morphine in the CPP paradigm, we failed to detect a significant difference in the numbers of NeuroD1-labeled neural progenitors (Figure 2.5 H); instead we observed a significant increase in the colocalization between NeuroD and EGFP-labeled neurons in CPP group trained with morphine (Saline-CPP: 22.03±0.89%; Morphine-CPP: 30.88±1.47%, N=6, $p<0.01$) (Figure 2.6). We also observed a decrease in the numbers of DCX-labeled neural progenitors in the group trained with morphine compared to saline (Figure 2.5 I). Since DCX is one of the transcriptional targets of NeuroD1, such results indicate NeuroD1 activity was affected. These data imply that, compared to its direct influence on early proliferation, morphine is more likely to interfere with certain late stages of development of adult-born neural progenitors.

Our conclusion is further supported by morphology analysis because morphine training increases the ratio of NSPCs in an oval shape and without noticeable neurite growth; while in the meantime, it decreases the ratio of NSPCs with a long or branching dendrite protruding inner molecular layer (Figure 2.7).

To confirm the change in cell numbers with quantification throughout the entire hippocampus, we added the stereotaxic quantification for each neurogenesis marker mentioned in Figure 2.3 and Figure 2.5 (Figure 2.8).

2.3.3 Morphine changed the lineage of neural progenitors' differentiation.

After analyzing the lineage of surviving progenitors labeled by BrdU after injection from day 1 to day 3 (Figure 2.3 A), we observed a decrease in the co-localization between DCX and BrdU-labeled neurons 9-12 days postmitosis (Figure2.9 A, C; two-way ANOVA, effect of training: $F_{1, 20}= 0.11, p=0.75$; effect of drug: $F_{1, 20}= 4.74, p<0.05;$ N=6). In the meantime, there was an increase in the co-localization between GFAP and BrdU-labeled newborn glia (Figure2.9 B, D; two-way ANOVA, effect of training: $F_{1, 20}= 2.95, p=0.10$; effect of drug: $F_{1, 20}= 29.10, p<0.01;$ N=6). These observations are consistent with our previous report showing that in cultured neuron stem cells, morphine decreases the percentage of immature neurons such as beta-tubulin 3(TUJ-1) positive cells in neural stem cell cultures, but it increases the percentage of GFAP-expressing glia (Xu et al. 2014).

So how did morphine decrease the number of immature neurons? Because a subset of the neural progenitors in the SGZ started to express the μ-opioid-receptor (MOR) a few days after birth (Figure 2.10 A-E), one probable mechanism could be that the activation of MOR in these progenitors resulted in apoptosis. If this hypothesis is correct, there should be less EGFP+ cells that are also MOR+ in the morphine-trained group than the saline-trained group. However, we observed that morphine did not alter

the percentage of EGFP+ cells colocalized with MOR in the total EGFP-labeled neurons (Figure 2.10M), which suggested that the activation of MOR on the surface of the immature neurons by morphine did not lead to the reduction of EGFP+ cells. This conclusion is confirmed by TUNEL-assay (Figure 2.10 F-I) and Caspase-3 staining (Figure 2.10 J-L) of SGZ, in which a significant difference in the number of apoptosis cells between the saline and morphine groups was not detected (Figure 2.10 N-O). These data indicated that morphine exerts its effect on hippocampal neural progenitors by increasing the immature NeuroD1-expressing neuron level and immature glia level, meanwhile decreasing the more mature DCX-expressing neuron level. Such effects of morphine on cell lineage did not involve a mechanism of inducing apoptosis.

2.3.4 NeuroD1 over-expression in the hippocampus by lenti-viral injection restored the population of immature neurons and prolonged the extinction phase of morphine-induced CPP.

Our previous studies have indicated that NeuroD1 is one of the essential targets during morphine's regulation of adult neurogenesis. To examine whether NeuroD1 activity is the basis for morphine's negative effect on the EGFP+ cells, we established lentiviral constructs that expressed cDNA of NeuroD1, a small-interfere RNA specifically targeting NeuroD1, and an empty viral vector. Lentivirus was stereotaxically injected into the mouse hippocampus before the CPP paradigm (Figure 2.11 A). We tested lentiviral gene expression by transducing three groups of viruses in the HEK293T cell line, which has no endogenous NeuroD1 expression (Figure 2.11 B). We also tested

NeuroD1 expression *in vivo* by delivering lentivirus in the mouse hippocampus (Figure 2.11 C, One way ANOVA, $F= 19.40, p<0.05;$ N=4). In the subsequent CPP study, we found that mice injected with NeuroD1 siRNA showed the shortest period of extinction phase, and their place preferences related to drug memory were gone (comparing pre- and post-conditioning preference) within 29 days after acquisition (Figure 2.11 D). The place preference of drug lasted for 43 days for mice that received the control virus and approximately 78 days for mice that received the NeuroD1 virus (Figure 2.11 D; student t-test comparison, $p<0.05$; N=6-8). The magnitude of place preference and the length of extinction days after training were almost identical between groups of mice that received the control virus injection and mice that did not have a stereotaxic injection (Figure 2.1), indicating that the surgery did not impair mice's ability to form and retain drug-memory.

When we examined the brain sections of mice that were injected with the control or NeuroD1 virus and subjected to the CPP paradigm, we confirmed that morphine's negative effect on adult neurogenesis could be restored by the overexpression of NeuroD1. The decrease in numbers of EGFP-labeled adult-born granule cells was detected in the control-virus group (saline-CPP: 991±56.1; morphine-CPP: 795±42.4; N=8, $p<0.05$) but not in NeuroD1-virus group (saline-CPP: 963±29.5; morphine-CPP: 879±53.8; N=8, $p>0.05$) (Figure 2.12A, left panel; Figure 2.12B; two-way ANOVA, effect of virus: $F_{1, 28}= 0.36, p=0.55$; effect of drug: $F_{1, 28}= 8.972, p<0.05$). Similarly, the difference in the survival of BrdU-labeled neural progenitors was observed in the control-virus group (saline-CPP: 223±18.2; morphine-CPP: 158±17.1; N=8, $p<0.05$), but not in the NeuroD1-virus group (saline-CPP: 207±21.1; morphine-CPP: 183±10.3; N=6,

p>0.05) (Figure 2.12A, left panel; Figure 2.12C; two-way ANOVA, effect of virus: $F_{1, 24}= 0.083, p=0.78$; effect of drug: $F_{1, 24}= 7.057, p<0.05$). Furthermore, we also found that the number of DCX (Figure 2.12A, right panel; Figure 2.12D; two-way ANOVA, effect of virus: $F_{1, 24}= 5.658, p<0.05$; effect of drug: $F_{1, 24}= 4.613, p<0.05$) and Tuj-1 (Figure 2.12A, right panel; Figure 2.12E; two-way ANOVA, effect of virus: $F_{1, 24}= 2.164, p=0.16$; effect of drug: $F_{1, 24}= 11.67, p<0.05$) labeled immature neurons decreased in the control-virus group (DCX: saline: 1493±78.5; morphine: 1275±64.2; Tuj-1: saline: 106±6.8; morphine: 82±5.2; N=8, p<0.05) the same as in naive mice without the lentivirus injection (Figure 2.5 I-J), and the decrease was compensated in the NeuroD1-virus group (DCX: saline: 1487±54.9; morphine: 1517±49.1; Tuj-1: saline: 98±7.1; morphine: 94±2.6; N=6, p>0.05). These results indicated that morphine's regulation on adult neurogenesis was dependent on NeuroD1 activity, and the overexpression of NeuroD1 could compensate for morphine's negative effect on neurogenesis, but it could not enhance neurogenesis when compared to the control-virus group. In conclusion, morphine indirectly deactivated the transcriptional factor NeuroD1, which controlled a key step of neural progenitor differentiation in hippocampal neurogenesis, resulting in a reduction of the early progenitors differentiating into immature neurons.

2.4 Discussion

2.4.1 Morphine exerts actions on adult neural stem/progenitor cells (NSPCs) in the pre-differentiation stage

Adult neurogenesis is a multi-step dynamic process that is manipulated by many intrinsic and extrinsic factors. The neuron stem cells are defined as primary multifunctional progenitor cells that resemble radial glia and are characterized by the expression of GFAP and nestin, which are known as Type-I or B-type cells (Ihrie & Alvarez-Buylla 2008). Type-I cells are thought to divide to produce intermediate stage progenitors (Type-II, Type-III or D-type cells), which then undergo further rounds of cell division to generate post-mitotic immature granule neurons (Zhao et al. 2008). Previously, we reported that opioid agonists such as morphine and fentanyl differentially regulated adult neurogenesis by altering NeuroD1 activity differently (Zheng et al. 2013). Although both fentanyl and morphine produced similar CPP responses after 4 days of training, only morphine treatment resulted in a decrease in adult neurogenesis(Zheng et al. 2010)(Zheng et al. 2013). So the effect on neural progenitors is morphine related, but not just related to learning a context-association.

NeuroD1 is one of the classic B basic Helix-Loop-Helix transcriptional factors that promotes premature cell cycle exit and differentiation in neural precursor cells (Miyachi et al. 1999). NeuroD1 has been reported to be essential for late-stage progenitors to differentiate into hippocampal granule neurons (Gao et al. 2009), and it exhibits a pronounced neuron-inductive effect that leads to a granule neuron commitment (Roybon et al. 2009). Hence, by regulating NeuroD1 activities, opiate agonists such as

morphine can modulate the newborn granule neurons' differentiation into mature neurons.

The POMC-EGFP transgenic model allows the visualization of adult-generated granule cells in a restricted time window. Thus far, our study has demonstrated that the analgesic dose (5 mg/kg) of morphine injection during a contextual memory behavior task can regulate adult neurogenesis, and morphine probably interfered with the late phase of development of neural precursors rather than affecting the early proliferation stage. Our current study shows that CPP training alone is sufficient to enhance adult hippocampal neurogenesis, as shown by increased EGFP and BrdU labeled cells (Figure 2.3). This observation is supported by many behavior studies demonstrating that both physical exercise and environmental enrichment can increase adult neurogenesis (Kempermann et al. 1997; van Praag et al. 1999; Kobilo et al. 2011). Morphine treatment, on the other hand, blocks such enhancement by impeding the differentiation of early neural progenitors (type-I and type-II cells) into more mature stages of development (type-III and immature neurons), which are stages that are known to have NeuroD1 expressed (Hevner et al. 2006; von Bohlen Und Halbach 2007). However, this process did not lead to an accumulation of NeuroD1 expressing progenitors because a large portion of these progenitors die within a few days following their birth (Dayer AG, 2003). It is reasonable to assume that the neural precursors that fail to differentiate into functional immature neurons would go through apoptosis. The massive cell death of adult-born granule neurons may serve as a natural selective mechanism since it has been demonstrated that cell survival and death are both important during learning and memory

(Dupret et al. 2007). However, our TUNEL and Caspase-3 staining did not show morphine accelerating apoptosis in the dentate gyrus area (Figure 2.10).

An alternative explanation for the negative effect on adult neurogenesis is that morphine alters the fate of early progenitors indirectly, inducing more neural stem cell differentiation into glia rather than neurons. Our recent *in vitro* study with the isolated progenitor cells from the mouse hippocampus indicates that morphine alters the lineage of the progenitors during differentiation by regulating the Prox1/Notch1 pathway via the drug's control of miR-181a (Xu et al. 2014). Similar results were observed with our current *in vivo* study. Among all BrdU-labeled cells, morphine increased BrdU/GFAP co-localization and decreased BrdU/DCX co-localization (Figure 2.9). Whether morphine regulation of the Prox1/Notch1 pathway *in vivo* also contributes to the eventual decrease in the newborn progenitors differentiated into immature neurons during the CPP paradigm remains to be determined.

Our conclusion is further supported by the observation that the ratio of EGFP+/MOR+ cells was not altered by morphine (Figure 2.10) and that the over-expression of NeuroD1 could rescue the decrease in EGFP+ cells in the DG (Figure 2.12). If the morphine action were via the inhibition of early progenitor's proliferation, then one would expect a decrease in the presence of MOR in the EGFP+ cells that survived. This was not the case. Because NeuroD1 activities are normally observed in the intermediate stages of progenitors, type II and type III cells, an increase in NeuroD1 expression should not rescue the morphine inhibitory effect if the drug's action was on the regulation of early progenitors' proliferation. Our previous studies indicated that

morphine inhibited the CaMKIIα activity, thus attenuating the phosphorylation and activation of NeuroD1 (Zheng et al. 2010). The ability of NeuroD1 over-expression to block the morphine decrease in a number of EGFP+ cells in the SGZ suggested that one of morphine's actions is via the regulation of NeuroD1-mediated maturation of the newborn granule neurons.

2.4.2 Correlation between drug-experienced memory and the levels of hippocampal neurogenesis

Our study also indicates that morphine-induced contextual memory is related to the manipulation of adult hippocampal neurogenesis. When over-expressing NeuroD1 in the dentate gyrus to counteract morphine's inhibition on adult neurogenesis, the animal exhibited much longer memory of their drug experience, which was represented by a prolongation of the CPP extinction time. On the other hand, when knocking down NeuroD1 with a RNA interference method, it has an opposite effect. These results suggest that the progenitors at a certain stage of development may be associated with contextual memory formation. This observation is concurrent with the reports that adult-born granule cells are preferentially incorporated into spatial memory networks in the dentate gyrus (Kee N, 2007). In addition, it is reported in literature that reactive glial cells in the cortex of stab-injured or Alzheimer's disease (AD) model mice can be directly reprogrammed into functional neurons in vivo using retroviral expression of NeuroD1 (Guo et al. 2014). Thus, it is also possible that NeuroD1 overexpression enhances adult neurogenesis and prolongs animal's contextual memory by reprogramming of glial cells into functional neurons.

However, morphine's negative effect on modulating the differentiation and maturation of neural progenitors may not be considered to be the only mechanism that affects an animal's drug experienced memory. Our lab has also reported that morphine and fentanyl have different effects on dendritic spine stability through different modulations of NeuroD1 activity (Zheng et al. 2010). Such synaptic changes are considered essential for memory formation and retention (Yang et al. 2009). A genome-wide prediction of NeruroD1 direct target genes includes multiple Eph receptors (Seo et al. 2007), which are reported as key players in synapse formation and plasticity (Lai & Ip 2009). Therefore, the fact that NeuroD1 overexpression prolonged morphine-induced CPP could be due to its effects in maintaining spine stability and synapse formation. How to distinguish the contribution of the synaptic effect of NeuroD1 on an animal's drug memory formation from the contribution of its influence on progenitors' development and fate choice in adult neurogenesis needs to be further established.

2.4.3 Possible molecular mechanisms of morphine's modulation and future prospects

In our previous study, we reported that the extinction of the fentanyl-trained CPP response could be shortened by the injection of the miR-190 lentivirus within the DG (Zheng et al. 2013). We surmised that such a miR-190 effect was due to its decrease in the NeuroD1 level. However, miR-190 has multiple targets, one of which is Pax6 that is associated primarily with the Type 1 progenitors and directly activates the *Neurogenin2 (Ngn2)* gene (Scardigli et al. 2003). To confirm our initial observation that NeuroD1 activity is the key to the extinction of drug associated memory, we re-examined the

extinction of morphine-associated memory by using siRNA to specifically decrease the NeuroD1 activity. Similar to previously reported observations, over-expressing NeuroD1 in the DG counteracted morphine's inhibition on adult neurogenesis and extended the drug-associated memory (Figure 2.11). In contrast, the expression of the NeuroD1 siRNA clearly shortened the extinction of the morphine-associated memory and decreased the number of ND+ cells in the SGZ (Figure 2.11). These results clearly indicated the central role NeuroD1 exhibits in adult neurogenesis and subsequent drug-associated memory. Some studies indicated that NeuroD1 could be the upstream activator of the Pax6 gene and a downstream target of Ngn1 and Ngn2 (Chae et al. 2004). These transcriptional factors are able to define a cascade with two other factors, T-box brain genes 1 and 2 (Tbr1 and Tbr2), in a sequence of Pax6-Ngn2-Tbr2-NeuroD-Tbr1, which plays a crucial role in regulating the adult SGZ NSPCs (Englund et al. 2005) (Hodge et al. 2008). Furthermore, one of NeuroD1's targets, Notch1, is known to up-regulate Hes and Herp (Iso et al. 2003), which are basic helix-loop-helix (bHLH) transcription factors that antagonize proneural genes such as Mash1 and Ngn (Bertrand et al. 2002). Cells with higher Notch1 levels will continue to divide, while those with low levels differentiate (Sommer & Rao 2002). Hence, whether the effects of morphine on adult neurogenesis are due to its control of this closed, positive-feedback loop of gene regulation (i.e., Pax6→Ngn2 →NeuroD1→Pax6) at the NeuroD1 level only or at multiple points of this gene circuit needs to be investigated.

However, it is clear that the morphine control of adult neurogenesis and subsequent contextual memory can trace back to the agonist regulation of various

miRNAs. Activation of MOR by morphine resulted in an elevation of the miR-181a level (Xu et al. 2014), while the miR-190 level was not altered (Zheng, 2010; Zheng, 2013). Such differential regulation of the miRNA resulted in changes in the activities of the transcription factors that are associated with neurogenesis such as Prox1, Notch1 and NeuroD1. If the miRNA(s) central to the regulation of these transcription factors' activities can be identified, one would expect that adult neurogenesis' contribution to drug-associated memory could be manipulated by altering the miRNA(s) level(s) and subsequently the drug relapse. Whether such a scenario can be achieved remains to be determined.

Figure 2.1

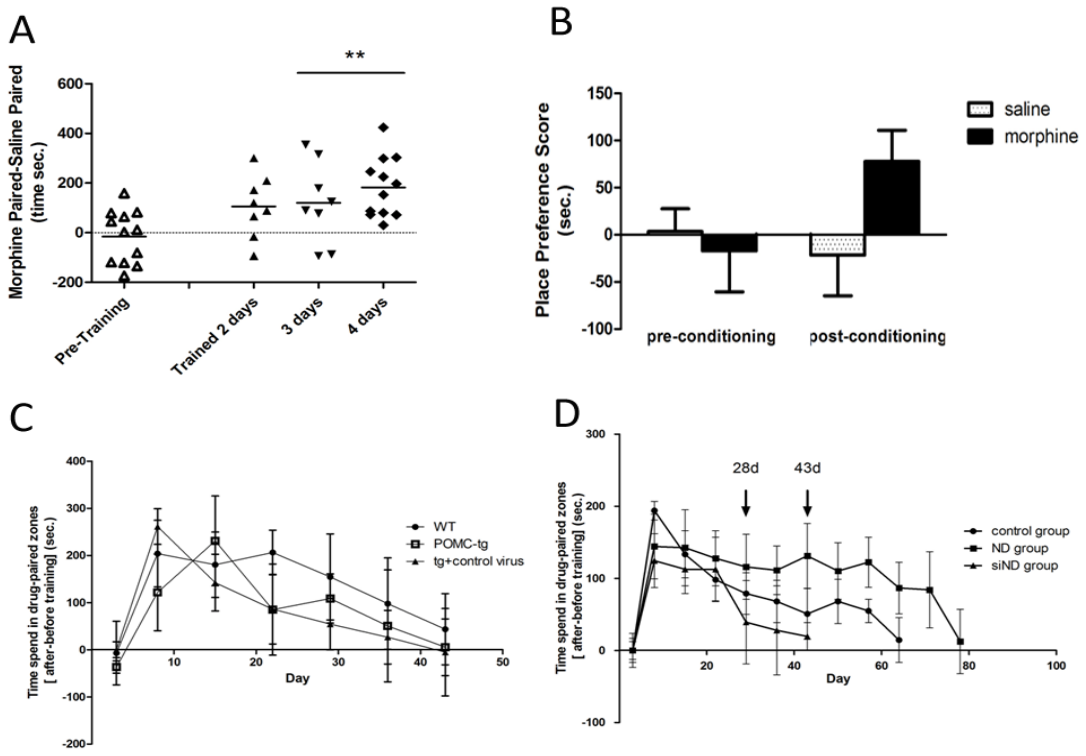


Figure 2.1 Preliminary CPP test.

(A) WT mouse trained by morphine (5mg/kg) for 3 days started to show place preference. When trained for 4 days, the amplitude of CPP reach maximum. (B) On day 8, mice injected with 5mg/kg morphine acquired strong place preference to drug-paired chamber. (C) The WT mouse, POMC-EGFP transgenic mouse and mouse injected with control virus did not show any significant differences in CPP training and extinction. (D) Measurement of CPP extinction for 3 groups of mouse injected with different lentivirus. Data represent mean ± SEM of 6 to 10 animals in separate experiments. Statistical significance was determined by one-way ANOVA with Bonferroni test as post hoc comparisons.

Figure 2.2

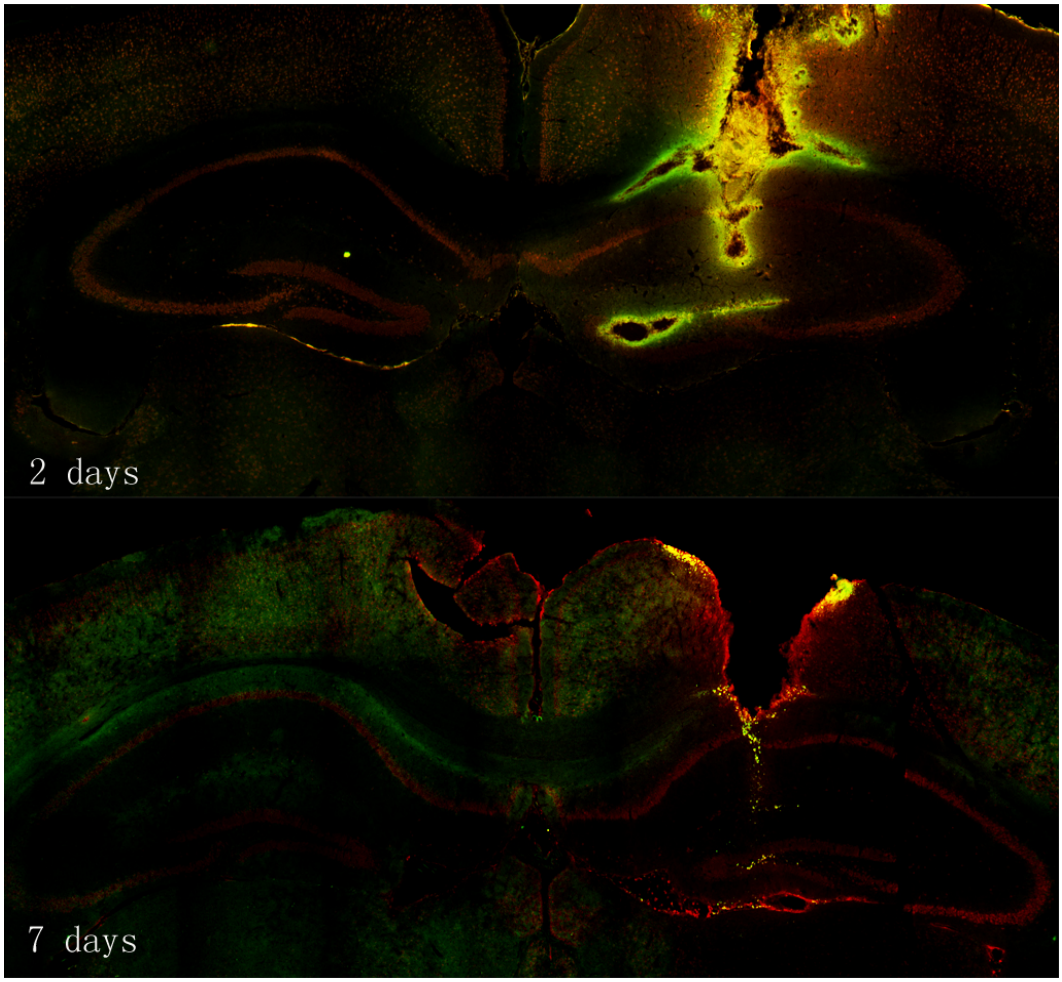


Figure 2.2 Injection site of NeuroD cDNA lentivirus.

Histological sections showing a needle track to confirm the site of the lentivirus injections (Red: NeuroN; Green: NeuroD).

Figure 2.3

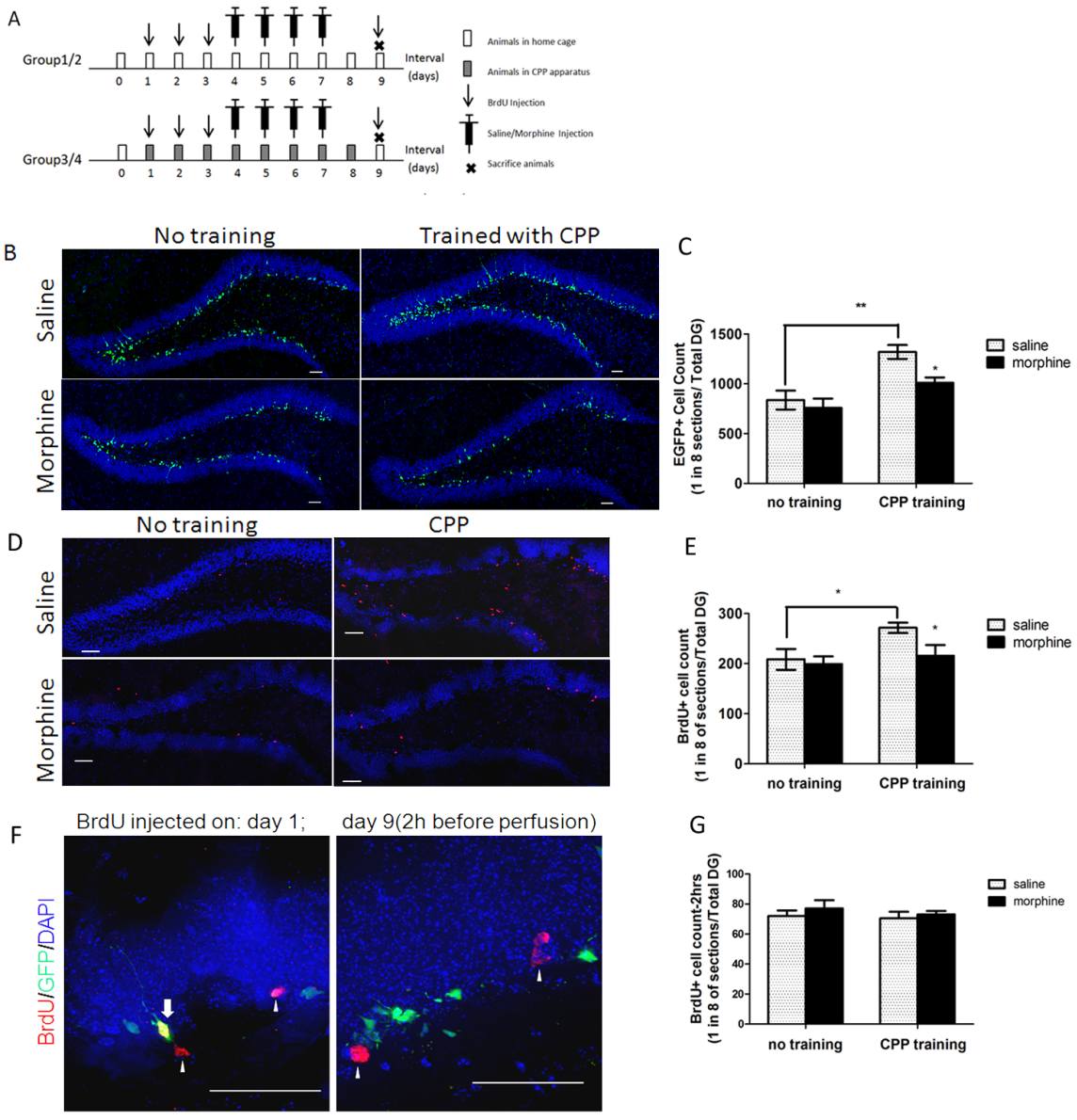

A
Group1/2
Group3/4
Interval (days)
Animals in home cage
Animals in CPP apparatus
BrdU Injection
Saline/Morphine Injection
Sacrifice animals
B
No training
Trained with CPP
Saline
Morphine
C
EGFP+ Cell Count (1 in 8 sections/ Total DG)
saline
morphine
no training
CPP training
D
No training
CPP
E
BrdU+ cell count (1 in 8 of sections/Total DG)
F
BrdU injected on: day 1;
day 9(2h before perfusion)
BrdU/GFP/DAPI
G
BrdU+ cell count-2hrs (1 in 8 of sections/Total DG)

Figure 2.3 Morphine treatment decreases adult-born GCs labeled with EGFP only after going through a CPP paradigm.

(A) Experiment design. (B) Comparison of the images of EGFP fluorescent from four groups of mice. (C) Fluorescent images of BrdU labeled newborn cells (Red) from four groups (Antigen-retrieval with acid often disrupted morphology of the granular layer). (D) Neural proliferation measured by average number of EGFP labeled newborn granule cells (count every one in eight brain sections, add together) in total dentate gyrus (N=10/per group, *$p<0.05$, **$p<0.01$). (E) Neural progenitors' survival was measured by the average number of BrdU (injected three days prior to CPP) labeled granule cells in the mouse dentate gyrus (N=6/per group, *$p<0.05$). (F) Comparison of the fluorescent images of BrdU-labeled cells when injected on day 1-3 or day 9. (White arrow: BrdU-labeled cells colocalized with EGFP fluorescent; White arrow head: BrdU-labeled cells did not colocalize with EGFP positive cells). (G) Neural proliferation measured by average number of BrdU (injected 2 h before perfusion) labeled granule cells in the mouse dentate gyrus (N=4/per group, $p>0.05$).

Data represent mean ± SEM of 6 to 10 animals in separate experiments. Statistical significance was determined by two-way ANOVA with Bonferroni test as post hoc comparisons.

Figure 2.4

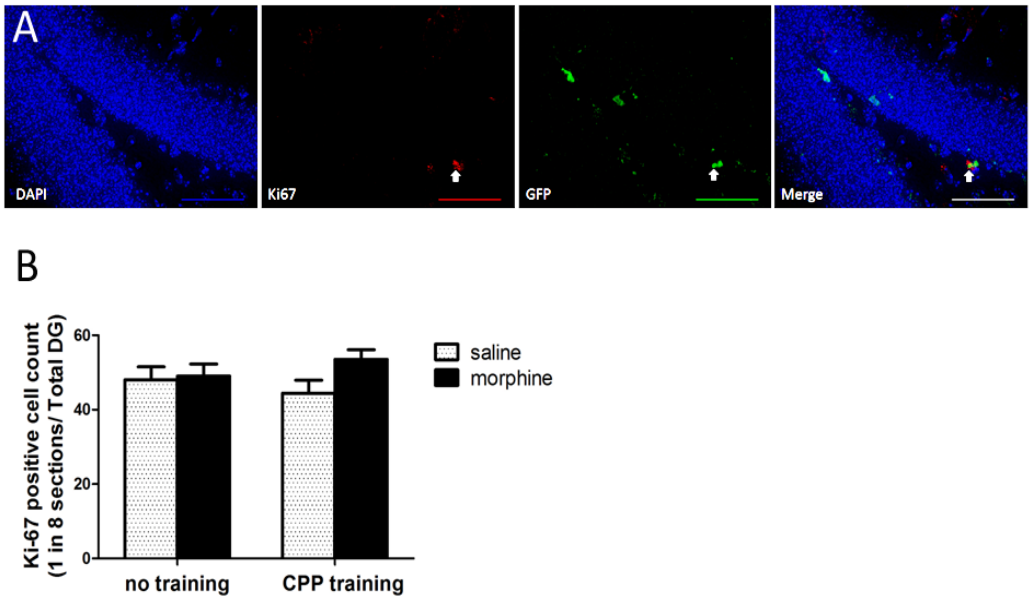


Figure 2.4 Morphine injections have no effect on proliferating neuron progenitors.

(A) Fluorescent images of EGFP labeled cells and cell proliferation marker ki-67 (colocalization marked by white arrows. Scale bar: 50μm). (B) Measurement of the numbers of ki67 positive neural progenitors in SGZ. Data represent mean ± SEM of 6 to 8 animals in separate experiments. No significant differences were found.

Figure 2.5

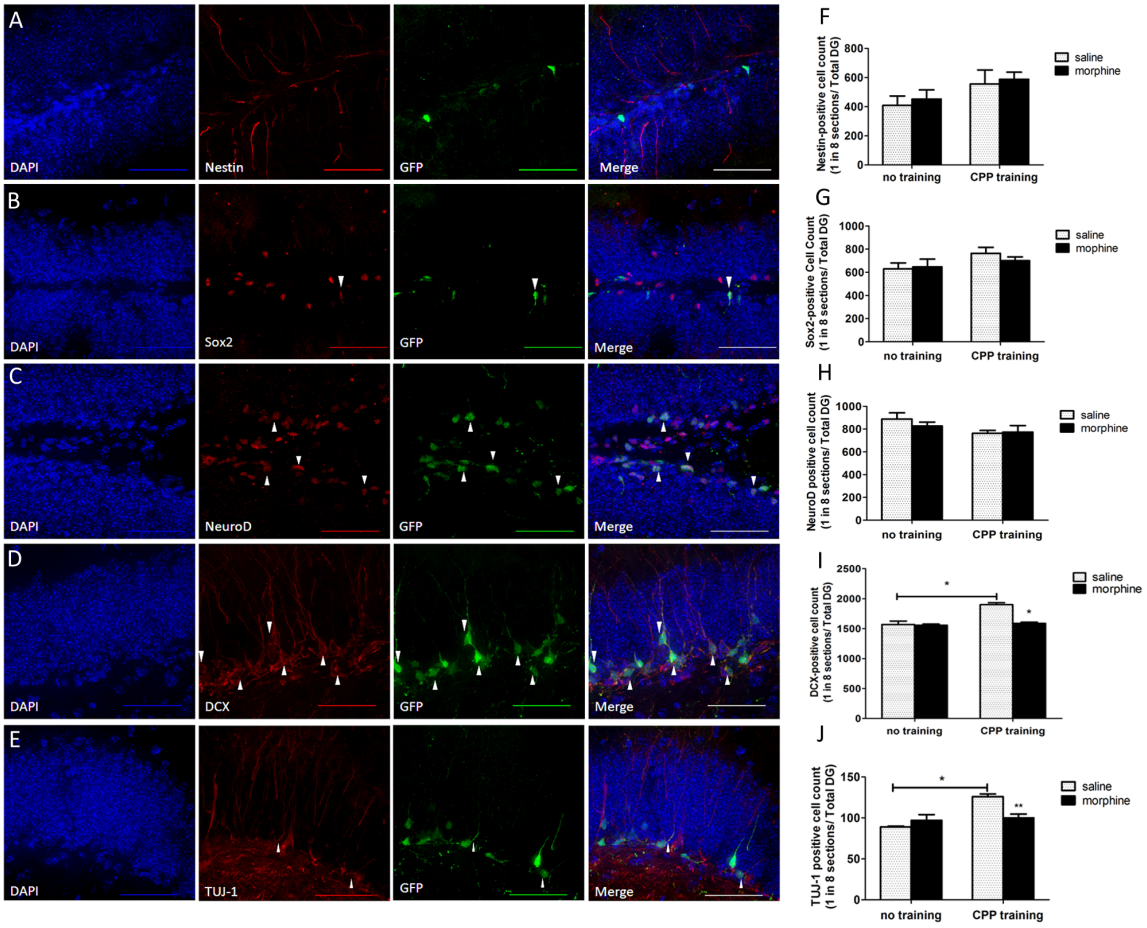

A
DAPI
Nestin
GFP
Merge
B
DAPI
Sox2
GFP
Merge
C
DAPI
NeuroD
GFP
Merge
D
DAPI
DCX
GFP
Merge
E
DAPI
TUJ-1
GFP
Merge
F
Nestin-positive cell count (1 in 8 sections/ Total DG)
800
600
400
200
0
no training
CPP training
saline
morphine
G
Sox2-positive Cell Count (1 in 8 sections/ Total DG)
1000
800
600
400
200
0
no training
CPP training
saline
mophine
H
NeuroD positive cell count (1 in 8 sections/ Total DG)
1000
800
600
400
200
0
no training
CPP training
saline
morphine
I
DCX-positive cell count (1 in 8 sections/ Total DG)
2500
2000
1500
1000
500
0
*
*
no training
CPP training
saline
morphine
J
TUJ-1 positive cell count (1 in 8 sections/ Total DG)
150
100
50
0
*
**
no training
CPP training
saline
morphine

Figure 2.5 Morphine decreased later stage progenitors and immature neurons.

(A-E) Fluorescent images of EGFP labeled cells and multiple neurogenesis markers: Nestin, Sox2, NeuroD, DCX, TUJ-1. There were no or very little Nestin or Sox2 positive neurons that were also EGFP positive (<5%), but there was a high percentage of NeuroD (20%-40%) or DCX (60%-80%) positive cells colocalized with EGFP neurons (colocalization marked by white arrow heads. Scale bar: 50 μm). (F-H) Measurement of the numbers of nestin, Sox 2 or NeuroD marked early neural precursors (N=6/per group, no significant differences). (I-J) Measurement of the numbers of DCX or Tuj-1 labeled type III cells or immature neurons (N=8/per group, *$p<0.05$). Data represent mean ± SEM of 6 to 8 animals in separate experiments. Statistical significance was determined by two-way ANOVA with Bonferroni test as post hoc comparisons.

.

Figure 2.6

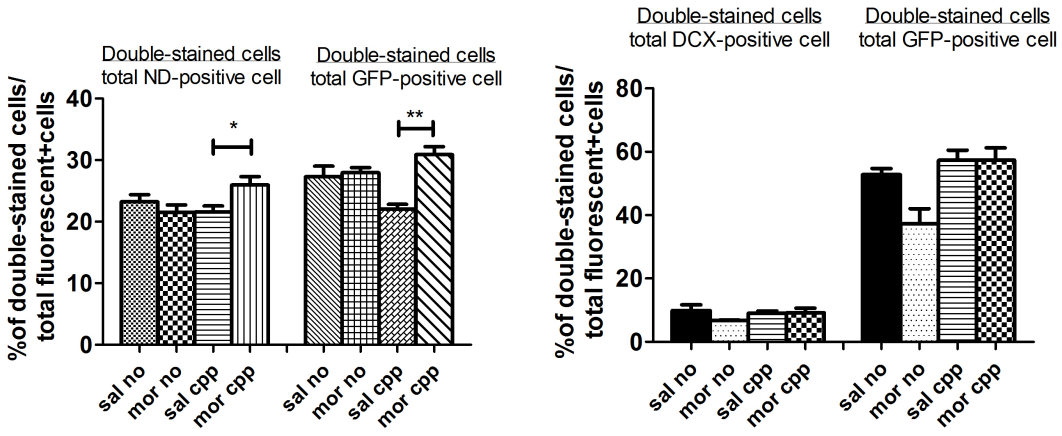
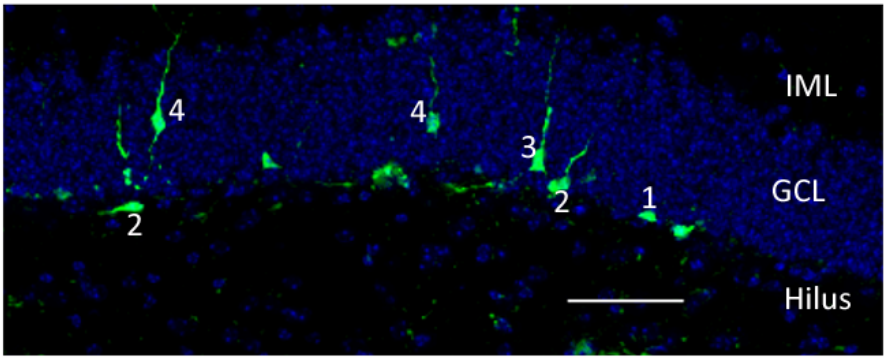


Figure 2.6 Morphine decelerates the differentiation of neural progenitors.

(A) Measurement of the colocalization between EGFP and NeuroD between groups. (B) Measurement of the colocalization between EGFP and DCX between groups.

Data represent mean ± SEM of 4 to 6 animals in separate experiments. Statistical significance was determined by two-way ANOVA with Bonferroni test as post hoc comparisons.

Figure 2.7


IML
GCL
Hilus
4
4
3
2
1
2

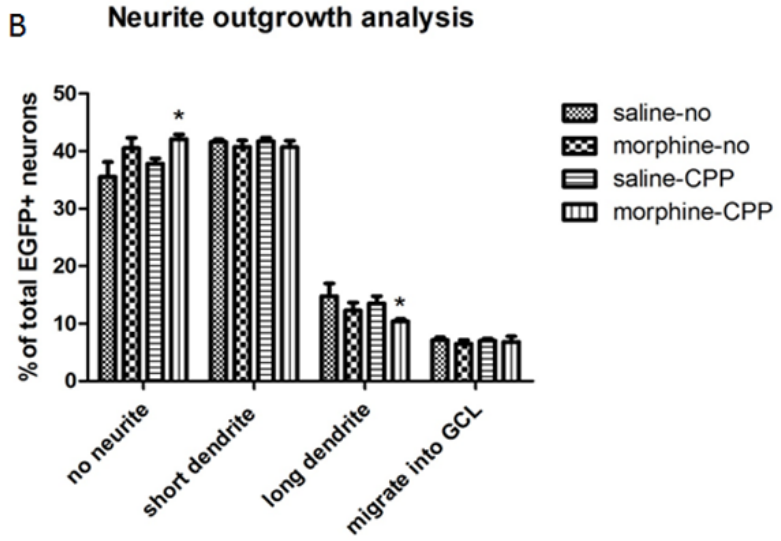

B
Neurite outgrowth analysis
% of total EGFP+ neurons
50
40
30
20
10
0
*
*
saline-no
morphine-no
saline-CPP
morphine-CPP
no neurite
short dendrite
long dendrite
migrate into GCL

Figure 2.7 Morphine decelerates the maturation process of newborn granule neurons.

(A)Example of EGFP-labeled granule cells with different morphology in dentate gyrus: ① progenitors without noticeable neurite growth; ② progenitors with short dendrite (single dendrite did not reach molecular layer) ③ progenitors with long dendrite (dendrite reached inner molecular layer (IML) or with branching) ④ progenitors migrate into granule cell layer(GCL). (B)EGFP-labeled cell morphology analysis; measured by percentage of each defined group of progenitors in total number of EGFP+ cells (N=6/per group, *$p<0.05$). Mice trained with morphine showed more percentage of cells without noticeable neurite while less percentage of cells with long or branching dendrite.

Data represent mean ± SEM of 6 to 8 animals in separate experiments. Statistical significance was determined by two-way ANOVA with Bonferroni test as post hoc comparisons.

Figure 2.8

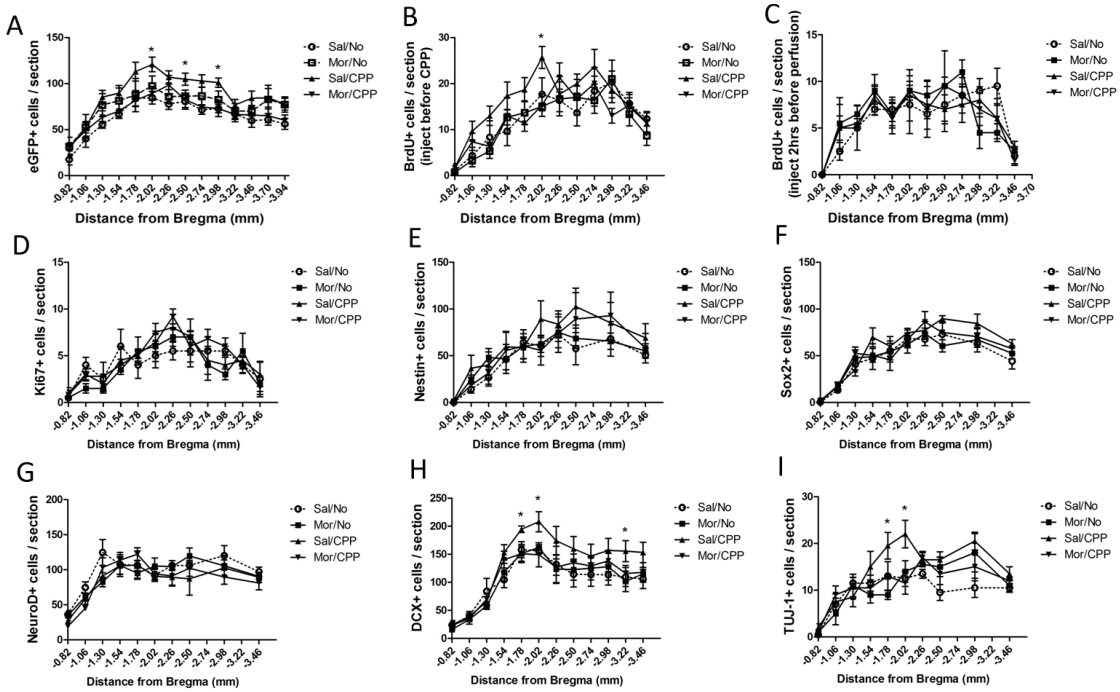


Figure 2.8 Stereotaxic quantification for each neurogenesis markers.

Data represent mean ± SEM of 6 to 8 animals in separate experiments. Statistical significance was determined by repeated one-way ANOVA with Bonferroni test as post hoc comparisons.

Figure 2.9

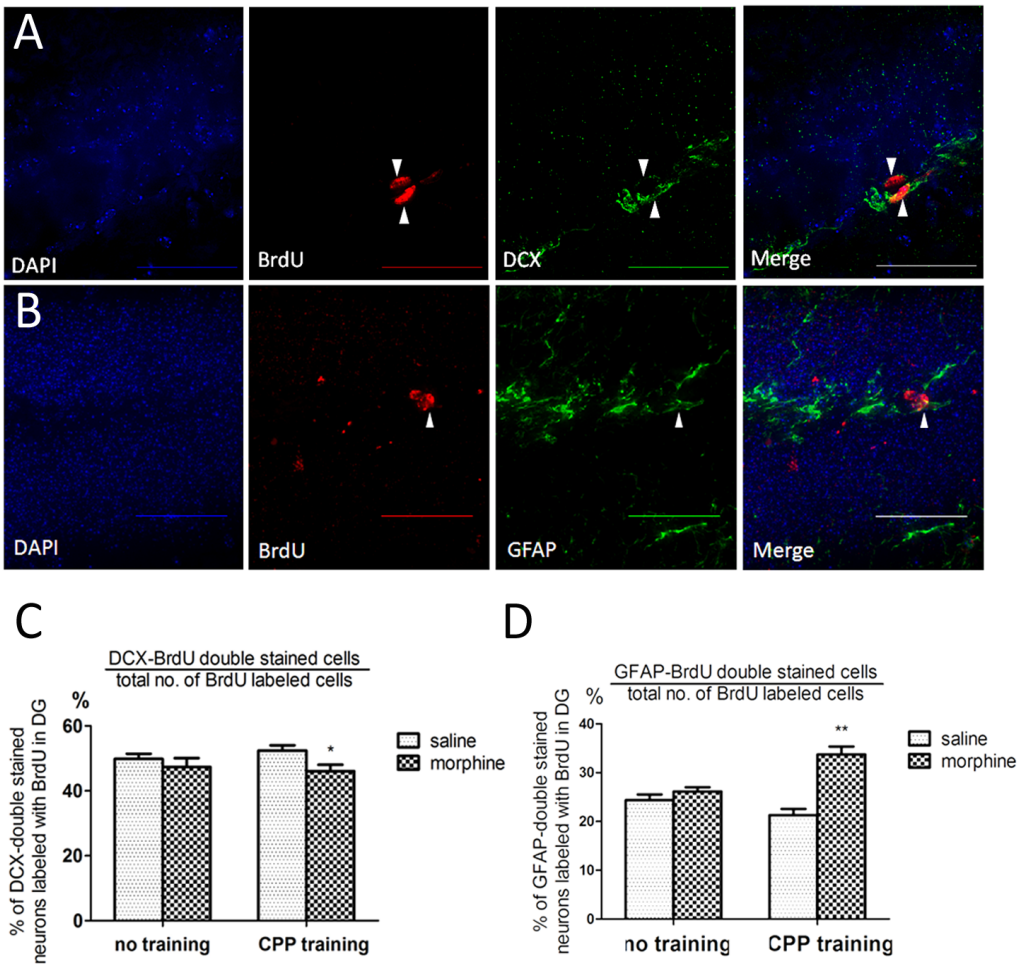

A
DAPI
BrdU
DCX
Merge
B
DAPI
BrdU
GFAP
Merge
C
DCX-BrdU double stained cells
total no. of BrdU labeled cells
% of DCX-double stained neurons labeled with BrdU in DG
%
60
40
20
0
*
no training
CPP training
saline
morphine
D
GFAP-BrdU double stained cells
total no. of BrdU labeled cells
% of GFAP-double stained neurons labeled with BrdU in DG
%
40
30
20
10
0
**
no training
CPP training
saline
morphine

Figure 2.9 Morphine alters the lineage of neural progenitors' differentiation.

(A-B) Fluorescent images of BrdU labeled cells colocalized with immature neuron marker DCX and glia marker GFAP (colocalization marked by white arrow heads. Scale bar: 50 μm). (C) Measurement of the colocalization between DCX and BrdU between groups (N=6/per group, *$p<0.05$). (D) Measurement of the colocalization between GFAP and BrdU between groups. Morphine decreased co-localization between BrdU and DCX labeled immature neurons in CPP training group, while it increased co-localization between BrdU and GFAP labeled glia. (N=6/per group, **$p<0.01$).

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 2.10

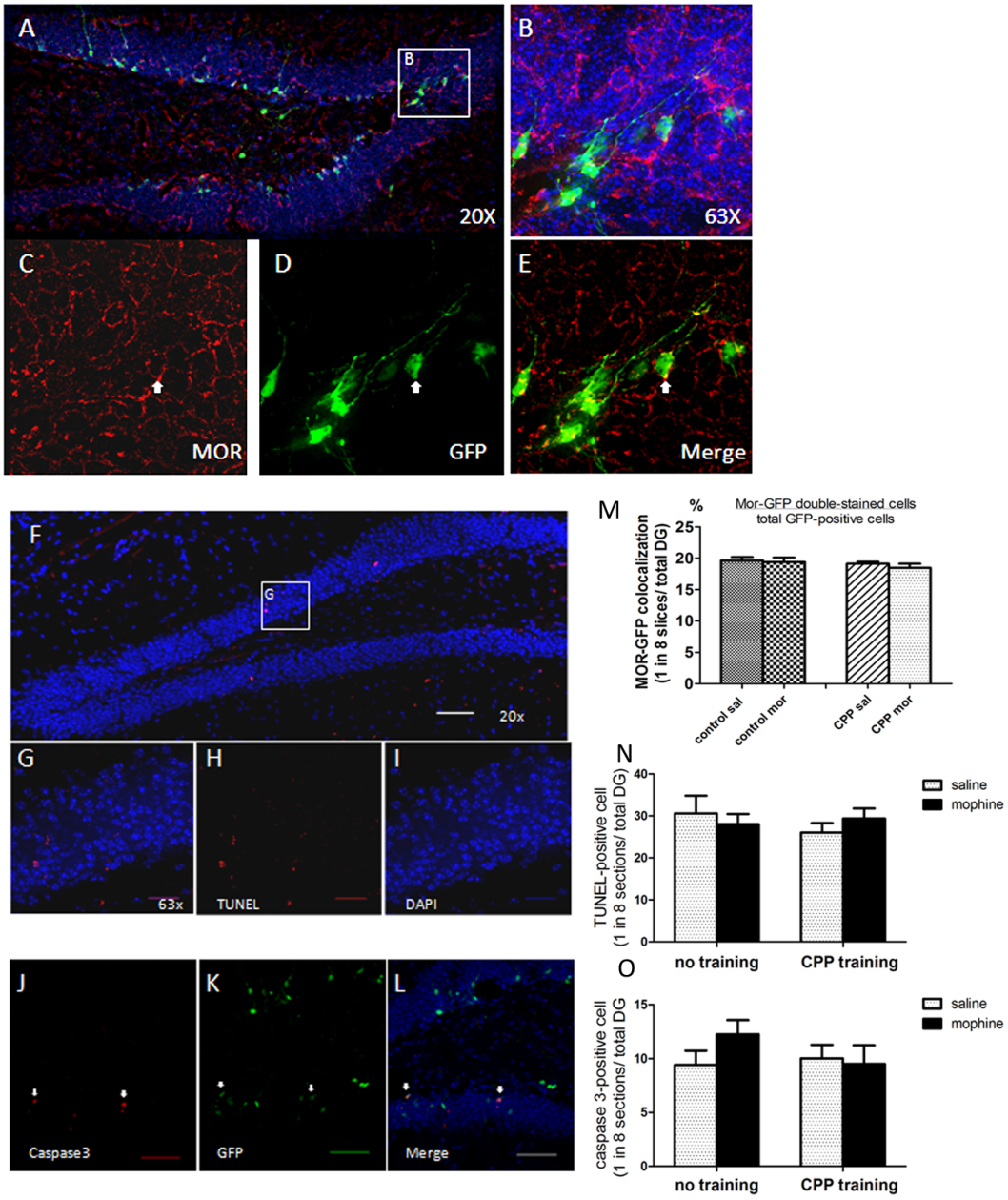

A
B
20X
B
63X
C
MOR
D
GFP
E
Merge
F
G
20x
G
63x
H
TUNEL
I
DAPI
J
Caspase3
K
GFP
L
Merge
M
% Mor-GFP double-stained cells
total GFP-positive cells
MOR-GFP colocalization
(1 in 8 slices/ total DG)
25
20
15
10
5
0
control sal
control mor
CPP sal
CPP mor
N
TUNEL-positive cell
(1 in 8 sections/ total DG)
40
30
20
10
0
saline
mophine
no training
CPP training
O
caspase 3-positive cell
(1 in 8 sections/ total DG
15
10
5
0
saline
mophine
no training
CPP training

Figure 2.10 Expression of the μ-opioid-receptor protein (MOR) and apoptosis in the DG of the hippocampus

(A-B) Photo image of MOR staining in the dentate gyrus of the hippocampus (Red: MOR; Green: GFP; Blue: DAPI; A. 20x objective lens, 200x magnification. scale bar: 50 μm; B. 630x magnification, oil-lens). (C-E) Fluorescent images of DG cells stained for GFP (Green) and MOR (Red). White arrow indicates GFP labeled cells colocalized with MOR. (F-I) Fluorescent images of DG cells stained for apoptosis (Red: TUNEL –positive, Blue: DAPI; F. 20x objective lens, 200x magnification. scale bar: 50 μm; G-I. 63x oil-lens, scale bar: 20 μm). (J-L) Fluorescent images of DG cells stained for apoptosis (Red: caspase 3, Green: GFP, Blue: DAPI; 20x objective lens, 200x magnification. scale bar: 50 μm). (M) Analysis of the colocalization between EGFP and MOR double-staining between groups. Control groups received no training, only injections; CPP groups were trained as in Figure1A (N=6, no significant differences). (N-O) Measurement of the numbers of TUNEL+ or Caspase3+ apoptosis cells (N=6, no significant differences).

Data represent mean ± SEM of six animals in separate experiments.

Figure 2.11

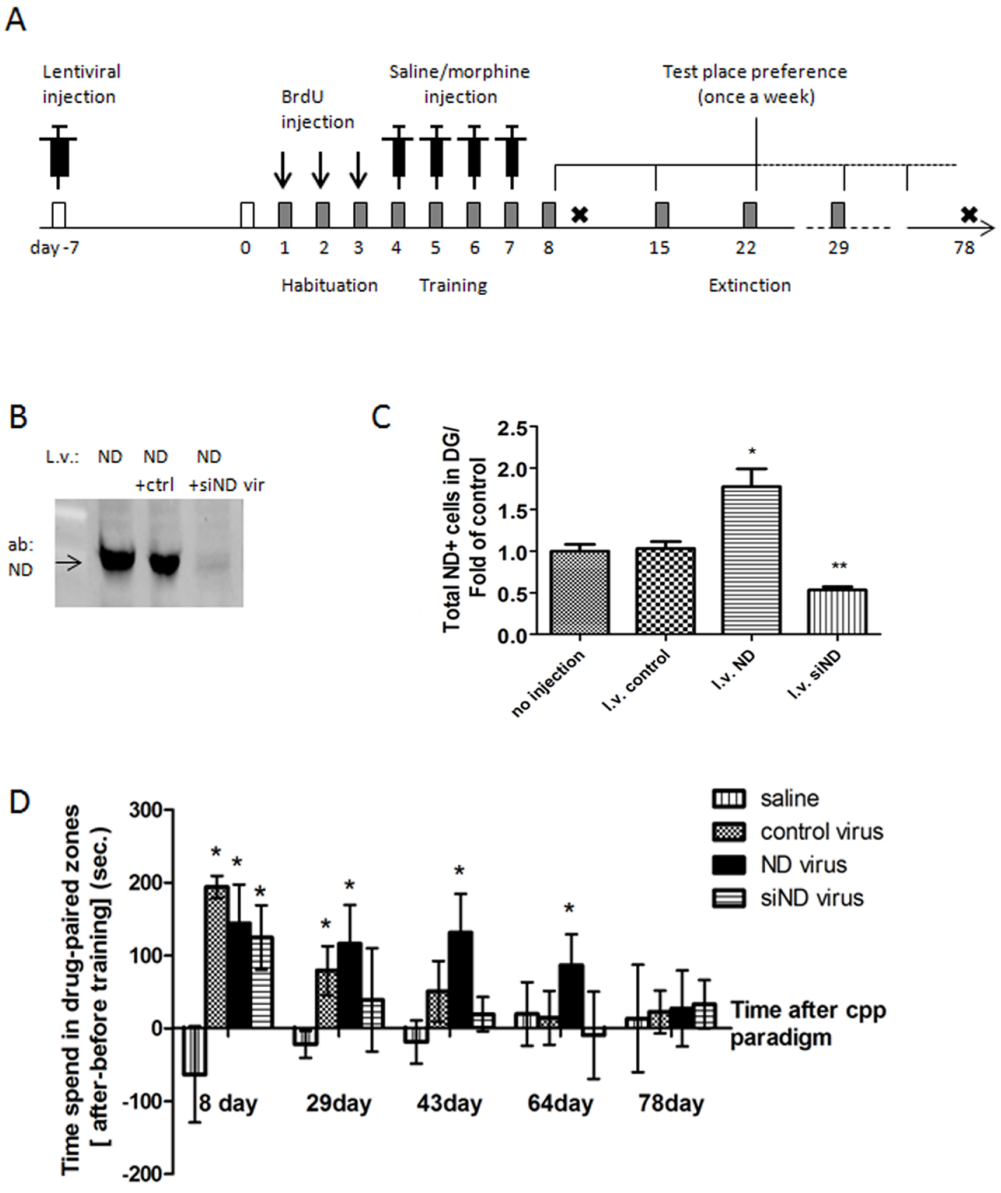

A
Lentiviral injection
BrdU injection
Saline/morphine injection
Test place preference (once a week)
day -7
0 1 2 3 4 5 6 7 8 15 22 29 78
Habituation
Training
Extinction
B
L.v.: ND ND +ctrl ND +siND vir
ab: ND
C
Total ND+ cells in DG/ Fold of control
2.5
2.0
1.5
1.0
0.5
0.0
*
**
no injection
l.v. control
l.v. ND
l.v. siND
D
Time spend in drug-paired zones [after-before training] (sec.)
300
200
100
0
-100
-200
saline
control virus
ND virus
siND virus
8 day
29day
43day
64day
78day
Time after cpp paradigm

Figure 2.11 Overexpression or knock down of NeuroD1 protein in the system.

(A) Experiment design: Lenti-virus of control oligonucleotide (control), NeuroD1 cDNA (ND) or NeuroD1 siRNA (siND) was injected one week prior to the CPP study. A subset of mice from the control and ND groups was sacrificed on day 9 for the following staining analysis. (B) Western blot test of the NeuroD1 virus *in vitro*. (C) Immunoactivity test of the NeuroD1 and NeuroD1 siRNA virus *in vivo*. The NeuroD1 cDNA virus increases the total number of NeuroD1 expressing cells in the DG by 1.8-fold compared to the no-injection control, while NeuroD1 siRNA decreases the number of NeuroD1 expressing cells in the DG to 0.53-fold of the control ($N=4$,$^{*}p<0.05$, $^{**}p<0.01$). (D) The CPP extinction test measured by time spent in the drug-paired chamber in different groups ($N=6$-8, $^{*}p<0.05$). On day 8, all groups injected with the virus developed CPP to the same extent. However, the group injected with siRNA of NeuroD1 lost their drug-chamber preference on day 29, while the group injected with the control virus and NeuroD1 virus lost their preference on day 43 and 78, respectively.

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by one-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 2.12

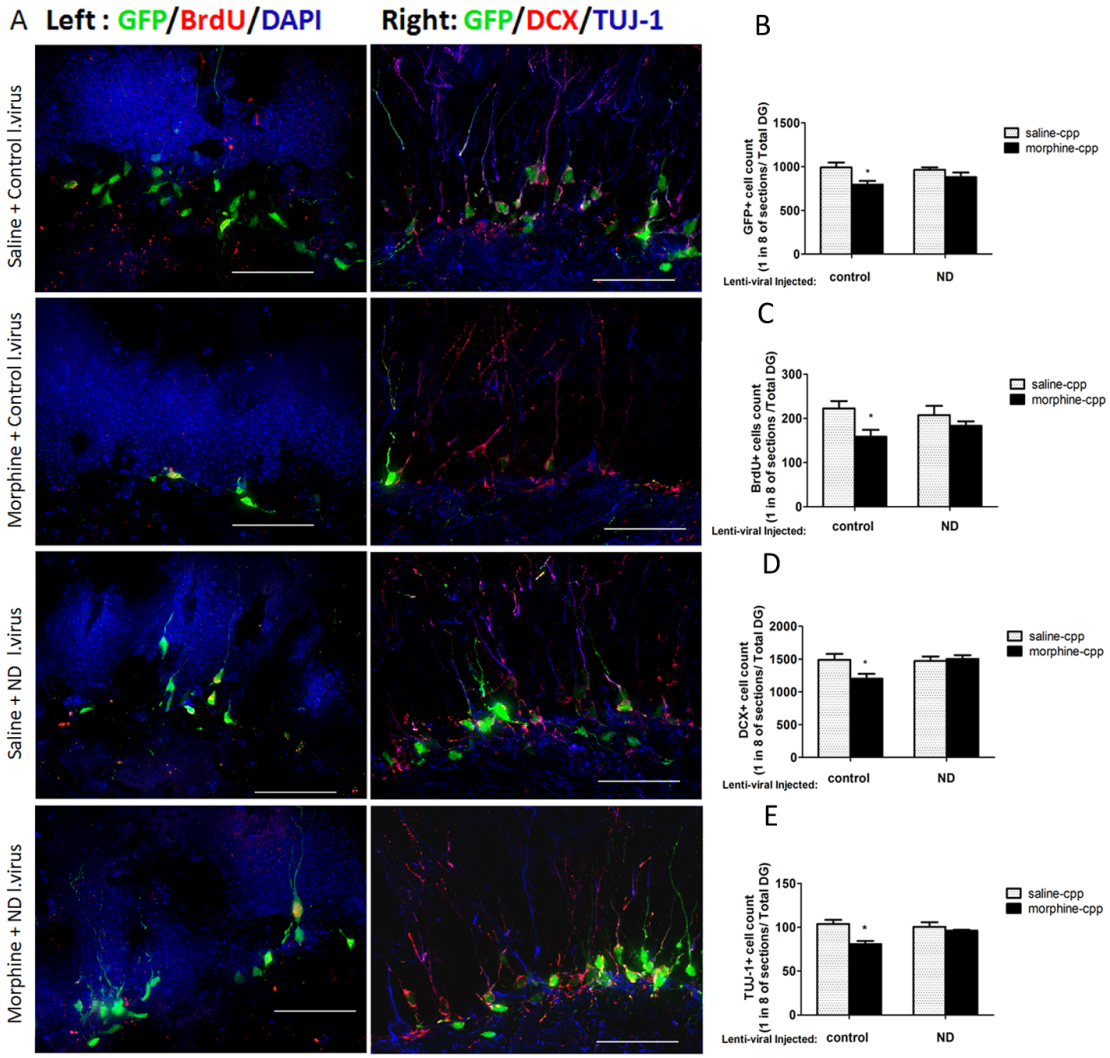

A
Left : GFP/BrdU/DAPI
Right: GFP/DCX/TUJ-1
Saline + Control I.virus
Morphine + Control I.virus
Saline + ND I.virus
Morphine + ND I.virus
B
GFP+ cell count (1 in 8 of sections/ Total DG)
1500
1000
500
0
saline-cpp
morphine-cpp
Lenti-viral Injected:
control
ND
C
BrdU+ cells count (1 in 8 of sections /Total DG)
300
200
100
0
saline-cpp
morphine-cpp
Lenti-viral Injected:
control
ND
D
DCX+ cell count (1 in 8 of sections/ Total DG)
2000
1500
1000
500
0
saline-cpp
morphine-cpp
Lenti-viral Injected:
control
ND
E
TUJ-1+ cell count (1 in 8 of sections/ Total DG)
150
100
50
0
saline-cpp
morphine-cpp
Lenti-viral Injected:
control
ND

Figure 2.12 Overexpression of NeuroD1 by lenti-virus injection in mouse hippocampus

(A) Fluorescent images of four groups of mice that received different lenti-virus injections (control/ND) and CPP training (saline/morphine injection) (Figure 2.10). Left panel: GFP-BrdU double staining; Right panel: DCX-Tuj-1 double staining (scale bar: 50 μm). (B-C) Measurement of the numbers of EGFP and BrdU labeled neural precursors (N=8, *$p<0.05$). (D-E) Measurement of the numbers of DCX and Tuj-1 labeled immature neurons (N=6, *$p<0.05$). Data represent mean ± SEM of 6-8 animals in separate experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni test for post hoc comparisons.

CHAPTER 3

MANIPULATION OF ADULT NEUROGENESIS AFFECTS DRUG CONTETUAL MEMORY

3.1 Introduction

It is well established that hippocampus plays a key role in the processing of contextual cues associated memories, and in the modulation of the reinforcing actions of opioids and the reinstatement of drug-seeking behavior (McDonald & White 1993; Nestler 2002; Floresco et al. 2001; Luo et al. 2011). As we summarized in Chapter One, previous studies suggest that adult hippocampal neurogenesis is implicated in the expression and reconsolidation of multiple forms of associative memories (Shors et al. 2001; Saxe et al. 2006; Dupret et al. 2007); thus it is intriguing to investigate whether adult neurogenesis also contribute to the formation of drug-context associations. There is circumstantial evidence to support this assumption, for instance, suppression of adult neurogenesis via cranial irradiation before drug-taking significantly increased cocaine self-administration, while suppression of adult hippocampal neurogenesis after drug-taking significantly enhanced the resistance to extinction (Noonan et al. 2010). It was reported that hippocampal neurogenesis was enhanced by physical exercise or enriched environment (EE)(Praag et al. 1999; Pereira et al. 2007; Nilsson et al. 1999; Olson et al. 2006). However, these positive regulators of neurogenesis may have a different impact on the rewarding effect of addictive drugs. Prior experience of forced exercise for 8 days attenuated conditioned place preference (CPP) to morphine (Lett, Virginia L. Grant, et al. 2002). In contrast, three weeks of voluntary exercise before drug exposure enhanced the learning of CPP to morphine in rats (Eisenstein & Holmes 2007). Rodents housed in EE from weaning to adulthood before any drug exposure showed a reduced CPP to heroin (El Rawas et al. 2009a) and a decreased cue-induced reinstatement to methamphetamine

(METH)(Hofford et al. 2014), while 30 days of EE during abstinence significantly reduced cue- and stress-induced reinstatement in mice trained with cocaine self-administration (Chauvet et al. 2009).

One of the class B basic Helix-Loop-Helix transcriptional factors, neurogenic differentiation1 (NeuroD1), plays an important role in specifying neuronal fate during adult hippocampal neurogenesis (M. Liu et al. 2000; Roybon et al. 2009). NeuroD1 promotes premature cell cycle exit and differentiation in neural precursor cells (Miyachi et al. 1999), and it has multiple transcriptional targets involved in neurogenesis, synapse formation, neuronal differentiation and migration (Seo et al. 2007). A recent study shows that NeuroD1 is required for late-stage progenitors to differentiate into hippocampal granule neurons (Gao et al. 2009). Previous studies in our lab have shown that overexpressing NeuroD1 by lentivirus injection before opioid exposure enhanced adult neurogenesis, and strengthened the animal's memory of their drug experiences during a CPP paradigm (Zheng et al. 2013). However, since the expression of NeuroD1 is constant after lentivirus injection, it is hard to tell whether the manipulation of NeuroD1 expression act on the acquisition, reconsolidation, or retrieval of a CPP paradigm.

To investigate the temporal effect of NeuroD1 overexpression and avoid stereotaxic surgery, we utilized a synthetic small molecule, KHS101, which is reported in the literature to induce NeuroD1 overexpression in cultured neural stem/progenitor cells(NSPCs) and neural progenitor differentiation in rat hippocampus (Wurdak et al. 2010). We use subcutaneous injection of 3mg/kg KHS101 for 7 days before morphine-primed CPP training to mimic the effect of NeuroD1 lentivirus injection 1 week prior to

CPP, and got similar effect in prolonging the CPP extinction. On the contrary, when same dose of KHS101 was administrated after training with morphine, the duration of CPP extinction was significantly reduced. The effect of KHS101 on CPP extinction was abolished by small interference RNA of NeuroD1. We also utilized an inducible tetracycline system to precisely control the timing of NeuroD1 overexpression. Lentivirus containing a Tet-On 3G transactivator gene (Clontech, CA, http://www.clontech.com/), and a NeuroD1 cDNA under the control of a Tet-response element (TRE) promoter gene was injected into mouse hippocampus. NeuroD1 expression was induced only with feeding of doxycycline. Similar to the KHS101 treatment, we were able to show that overexpression of NeuroD1 by doxycycline feeding for 7 days before morphine condition training strengthen the memory of drug experience and prolong CPP; on the other hand, such regulation after conditioning could facilitate the extinction of CPP.

3.2 Methods

3.2.1 Drugs

Morphine sulfate (Merck &Co. Rahway, NJ) was dissolved in 0.9% saline and administered at a dose of 5 mg／kg via subcutaneous (s.c.) injections to mice. KHS101 was dissolved in 22.5% (2-Hydroxypropyl)-beta-cyclo-dextrin (CDEX) (Sigma Aldrich, St Louis, MO, USA) and administered at a dose of 3 mg／kg via subcutaneous injections. The dose of KHS101 was chosen on the basis of the literature and has been tested in vivo (Wurdak et al. 2010). Doxycycline (Dox) diet at a concentration of 625 mg/kg was feed to animals no longer than 7 days.

3.2.2 Tet-on Expression System, Lentivirus construction

The pLVX-Tet3G (Tet), pLVX-TRE3G (TRE) vector construct were purchased from Clontech (Cat. No: 631191, 631193) as part of Lenti-X™ Tet-On® Advanced Inducible Expression System (Clontech, CA, http://www.clontech.com/). The NeuroD-cDNA was prepared as described previously (Zheng et al. 2010) and cloned into pLVX-TRE3G Vector with a In-Fusion HD Cloning Kit (Cat. No: 639650) to make a pLVX-TRE3G-ND (TRE-ND) construct. High titer virus particles were obtained by mixing these constructs (Tet, TRE, TRE-ND) with the Lenti-X HTX Packaging Mix (Cat. No: 631250) and co-transfected into 293T cells using the Xfect^{TM} Transfection System (Cat. No: 631317) following manufacturer's instructions (Clontech, CA).

Virus particles were assembled in 293FT cells by transfecting control construct or shRNA of NeuroD1 (siND) construct together with pLP1, pLP2 and pLP-VSVG construct from the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen, Grand Island, NY) following manufacturer's instructions. Viral titers (~5.0×10^7 TU/mL) were determined by assessment of DNA sequences in transduced 293FT cells (Sastry et al. 2002).

3.2.3 Condition Place Preference (CPP).

Experiment was carried out in a three chamber apparatus supplied by Panlab (Harvard Apparatus, Holliston, MA) according to the unbiased CPP protocol (Cunningham, C LPatel & Milner 2006) with slight modification, as described in Chapter 2. Mice with strong bias (>60% time spend) to one of the chambers were rejected from the experiment. During the extinction stage, the place preference of each mouse was

tested once in two days, or once a week until no significant preference was detected in two continuous tests. AnyMaze software (Stoelting, Wood Dale, IL) was used to track and record the position of mice in the apparatus during the testing period.

3.2.4 Morris Water Maze.

MWM was performed as reported (Vorhees & Williams 2006). In the acquisition section, the mice were placed in the water maze (122cm diameter) with the platform (10cm diameter) submerged in the SW quadrant. The mice were trained for four trails per day for 6 days with a semi-random set of start positions as previously published (Kibaly et al. 2014). Any-Maze software was used to track the mice. Probe tests were performed every 3 days and the amount of time the mice spent in each quadrant searching for the platform was recorded.

3.2.5 Primary Cell Culture.

Primary culture of mouse hippocampal neurospheres was carried out as previously described(Xu et al. 2015). Briefly, cells were cultured as neurospheres in proliferation medium containing 10 μg/ml EGF and 10 μg/ml FGF2. Mouse NeuroCult neural stem cell proliferation medium was purchased from STEMCELL Technologies (Vancouver, Canada, http://www.stemcell.com). EGF and FGF2 were obtained from Sigma-Aldrich (St Louis, MO).

3.2.6 Real-time PCR

The total RNAs were extracted and reverse transcribed with the miScript system (Qiagen, Germany, www.qiagen.com). Real-time polymerase chain reaction (PCR) was performed according to the instructions in the miScript system, which included a SYBR

Green PCR kit (Qiagen). GAPDH was used as an internal control. Primer sets used in real-time PCR assays are as listed:

NeuroD forward, 5'-CTTCCCGGTGCATCCCTACTCCTACC-3';

NeuroD reverse, 5'-AGGAAGGGCTGGTGCAATCAGTTAGG-3'.

GAPDH forward, 5'-CCTGCACCACCAACTGCTTAGC-3';

GAPDH reverse, 5'-GCCAG TGAGCTTCCCGTTCAGC-3'.

3.2.7 Immunohistochemistry and Microscopy.

Following behavioral testing, all animals were anesthetized with 100 mg / kg intraperitoneal sodium pentobarbital and then perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. The preparation of samples with a microtome and immunohistochemistry were carried out as described before. The following primary antibodies were used in our studies: Mouse monoclonal anti-GFP (1:2000; Life technologies, Grand Island, NY), Goat NeuroD antibodies (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) Secondary antibodies were used as follows: Alexa Fluor 488 donkey anti-mouse IgG, Alexa Fluor 594 donkey anti-goat IgG, Alexa Fluor 647 donkey anti-goat IgG (Life technologies). The dilution of all secondary antibodies is 1:2000.

Coronal sections were visualized using a CCD camera connected to a Leica DM5500 B upright microscope (Leica, Germany). In order to identify cells with positive markers (EGFP, NeuroD1) within the DG, maximum intensity z-axis projections of series of sequential fluorescent images were constructed using Leica Application Suite (Leica, Germany).

3.2.8 Statistical analysis.

Data were analyzed with the GraphPad Prism 5.0 software. Data represent mean ± SEM of 6 to 12 animals in separate experiments. Statistical significance was determined by one-way or two-way ANOVA with Bonferroni test as post hoc comparisons.

In addition, the CPP data were analyzed separately within each group, using standard methods. All treatment groups are fully counterbalanced for stimulus–drug assignment(Cunningham et al. 2006). The duration spent on morphine-paired chamber was compared between pre-conditioning (day3) and post-conditioning days, using repeated paired t-test .A difference of $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 KHS101 administration induces NeuroD1 overexpression

A former study from our lab already confirmed the potential of regulating NeuroD1 to modulate mice's adult neurogenesis and CPP extinction by lentivirus (Zheng et al. 2013). Thus, we want to use a chemical compound known to regulate NeuroD1 expression to achieve the similar effect on animal's drug contextual memory. The synthetic molecule KHS101, is reported in the literature to induce NeuroD expression and neural differentiation *in vitro* and *in vivo* (Wurdak et al. 2010). We treated cultured neural stem/progenitor cells (NSPCs) with different concentration of KHS101 dissolved in 0.1% DMSO for 24 hours, and used the cell lysate to perform a quantitative real-time PCR. As reported in the literature, 1μM KHS101 increase the relative mRNA level of NeuroD1 to about 1.5 fold of control (DMSO treated NSPCs), and 5μM KHS101 increase the relative mRNA level to about 2.5 fold ($F_{4,15}$= 25.93,$P < 0.0001$) (Figure 3.1A). For the in vivo test, one group of mice (N=7) were injected with 3mg/kg of KHS101 subcutaneously (s.c.) for seven days, and the other group (N=6) were injected with solvent vehicle only. Administration of KHS101 increases the total number of NeuroD1 positive cells by 26.9% in mouse dentate (Figure 3.1B-D) (t=4.590, df=11, $p<0.001$). Stereotaxic quantification from anterior to posterior of the hippocampus sections indicated that the KHS101 induced increases of NeuroD positive cells are distributed homogenously through the dentate (Figure 3.1E).

3.3. 2 KHS101 prolongs or shortens CPP extinction via regulation of NeuroD1

So we were able to use KHS101 injection to induced NeuroD1 overexpression in mouse brain instead of lentivirus. Then we test the CPP expression and extinction after KHS101 administration, to see whether this small molecule could modulate animal's performance in morphine CPP the same way as NeuroD1 lentivirus. Mice were divided into 3 groups, group 1 mice were injected with vehicle for 7 days, group 2 mice were injected with 3mg/kg KHS101 for 7 days prior to CPP conditioning, while group 3 mice were injected with 3mg/kg KHS101 for 7 days after CPP conditioning (Figure 3.2A). All three group developed preferences to the morphine-paired zone after 4 days of CPP training and no difference was observed between groups in the magnitude of the CPP expression ($F_{2,21}$= 0.03398,P>0.05). When testing their extinction preference once every 2 days, the three groups showed significant differences in the time they took to extinguish their drug-related preference. Group1 mice (control) took about 38 days for their preference to morphine-paired chamber drop to basal level (compare day 3 to day 38, group1: t=1.978, df=7, P>0.05; group2: t=2.972 df=7, P<0.05;) and group 2 mice took about 56 days (compare day 3 to day 56, group2: t=1.788, df=7, P> 0.05), indicating that KHS101 injection 1 week before drug training could prolong CPP extinction just like NeuroD1 lentivirus (Figure 3.2B). In contrast, when KHS101 is injected after the morphine-primed CPP training, it will shorten the period of CPP extinction. The group of mice injected with KHS101 on 8-14 day during CPP (group3) took about 29 days (compare day 3 with day 29, group 1: t=5.152 df=7, P<0.01; group2, t=7.515, df=7, P<0.001; group3: t=2.350, df=7, P>0.05) to extinct their morphine-related CPP, much

shorter than the control group (Figure 3.2B). After all the extinction trails, when no significant preference was detected in two continuous tests, each mouse were given a single morphine injection (5mg/kg; s.c) and put back into the CPP apparatus to test their drug-induced CPP performance. All groups of mice show a strong preference to the former morphine-paired chamber, indicating the reinstatement of CPP-related contextual memory. But no significant differences in reinstatement CPP score was detected between group 1(CDEX), group 2 (KHS -6-0day) and group3 (KHS 8-14day) mice (Figure 3.3), indicating that KHS101 did not influence the animal's memory of drug-induced relapse.

To determine if the effect of KHS101 on mice CPP memory is due to KHS101's regulation on NeuroD1 level, we used RNA interference method to block NeuroD1 expression in the dentate, and then test CPP extinction of animals under the same condition as in the former CPP experiment. Mice were divided into 5 groups. We first injected group1, group 2 and group 4 mice with control lentivirus (ctrl vir), and injected group 3 and group 5 mice with lentivirus containing small interference RNA of NeuroD1 (siND vir). Next, group 1 mice were injected with vehicle (CDEX) for 7 days, group 2 and 3 mice were injected with 3mg/kg KHS101 for 7 days prior to CPP conditioning, while group 4 and 5 mice were injected with 3mg/kg KHS101 for 7 days after CPP conditioning (Figure 3.4A) Group 1 mice (control) took about 36 days when their preference to morphine paired chamber drop to basal level (compare time spend of day 3 with day 36, group 1: $t=2.281$, $df=6$, $P>0.05$; group2, $t=2.972$, $df=7$, $P<0.05$), while group 2 mice took about 54 days (compare time spend of day 3 with day 54, group2: $t=1.788$, $df=7$, $P>0.05$), but group 3 mice only took about 27 days (compare time spend

of day 3 with day 27, group 1: t=7.403, df=6, P<0.001; group2, t=5.492, df=7, P<0.001; group 3: t=1.304, df=5,P>0.05) (Figure 3.4 B). These results show that KHS101 administration before CPP training significant prolongs CPP extinction, but siND injection diminish such enhancement.

We also found that control virus injection and KHS101 administration after CPP training facilitate CPP to extinct, as we know in the former experiment, but siND injection also block this effect. Group 4 mice took about 29 days when their preference to morphine paired chamber drop to basal level, less than group1 (36 days) (compare day 3 with day 29, group 1: t=2.859, df=6, P<0.05; group4, t=1.493 df=7, P>0.05; group 5: t=5.637, df=6,P<0.01), but group 5 mice more than 58 days (compare day 3 with day 58, group 5: t=2.928 df=6, P<0.05) (Figure 3.4C). These results indicated that KHS101's regulation on drug-association memory is depended on its modulation of NeuroD1 expression.

3.3.3 KHS101 enhances adult neurogenesis and spatial learning

After the CPP experiments, two groups of mice received KHS101 injection (3mg/kg) for 7 days show a significant increase in NeuroD1 positive cell number ($F_{2,21}$= 6.677,P<0.01) (Figure 3.5A-B) and EGFP positive cell number ($F_{2,21}$= 10.16,P<0.01) (Figure 3.5C-D) compare to control group of mice, indicating that adult hippocampal neurogenesis was enhanced by KHS101 injection.

To test the effect of KHS101 on hippocampus function, mice were tested in a standard Morris Water Maze. One group of mice was injected with vehicle and another

group was injected with KHS101 for 7 days. Mice with KHS101 treatment show a faster learning as demonstrated by significant shorter latencies and distances on the third and fourth day of training compared with the group injected with vehicle (3^{rd} day: $F_{5,230}$= 54.49, P<0.001; $F_{1,230}$= 18.97, P<0.01; 4^{th} day: $F_{5,230}$= 48.88, P<0.001; $F_{1,230}$= 10.39, P<0.01), indicating that administration of KHS101 facilitate animal's acquisition in spatial learning (Figure 3.6).

3.3.4 Induce NeuroD1 overexpression at different time has different impact on drug contextual memory

To confirm that the effect of KHS101 on drug contextual memory was due to its regulation on NeuroD1 expression, we use a Tet-on system to induce NeuroD1 overexpression in a limited time period by lentivirus injection and feeding with doxycycline (dox). Co-transfection of pLVX-TRE3G Vector (Tet) and pLVX-TRE3G-Luciferase (TRE-Luc) Vector with doxycycline induce high luciferase activity in vitro (Figure 3.7). After transduction of lentivirus contain Tet and pLVX-TRE3G-NeuroD1 vector (TRE-ND), and adding doxycycline to reach final concentration from 100μg/ml to 1000μg/ml in the cell culture media of HEK 293 cell line, western blot result show a strong NeuroD1 immunoactivity in vitro (Figure 3.7). In the transgenic mice model, the EGFP-expressing cells in the sub-granular zone (SGZ) of the dentate gyrus represent adult-born neural progenitors, which are 3-28 days post-mitotic(Overstreet et al. 2004). We injected lentivirus containing both Tet, TRE-ND vector to the mice and fed them with food containing doxycycline for zero day, 3 days and 7days. Only the two groups injected with Tet, TRE-ND lentivirus and fed with doxycycline for 3-7days show a

significant increase in the number of NeuroD1 and EGFP positive cells in their dentate gyrus (DG) ($F_{3,17}$=47.92,P<0.0001; $F_{3,17}$=22.08,P<0.001) (Figure3.8).

After testing the efficiency of Tet-on system, we tried to induce NeuroD1 overexpression by lentiviral injection and feeding with doxycycline during different time period of CPP, to mimic the effect of KHS101 injection. Three groups of mice were injected with either control virus (control) or Tet, TRE-ND lentivirus (group1, group2). Mice in group 1 were fed with dox food one week before CPP conditioning (-6-0 day), while group 2 mice were fed with dox one week after conditioning (8-14day) (Figure 3.9A).

On day 8, all three groups of mice show a significant preference to the drug-paired chamber, and the magnitude of the CPP response at day 8 did not differ between groups ($F_{2, 22}$= 0.01749, P>0.05). However, when testing their extinction preference once in every 2 days, group 2 mice took about 25 days when their preference to morphine-paired chamber drop to basal level (compare day 3 with day 25, control: t=2.942, df=9, P<0.05; group1, t=5.277, df=6, P<0.01; group2: t=2.168, df=10, P>0.05), while control group mice and group 1 mice took about 42 days and 58 days, respectively (compare day 3 with day 42, control: t=1.772, df=9, P>0.05; group1: t=3.858, df=6, P<0.01; compare day 3 with day 58, group1: t=2.340, df=6, P> 0.05) (Figure 3.9B). The result indicate that induce NeuroD1 overexpression before drug training could prolong CPP extinction, while induce NeuroD1 overexpression after training shorten CPP extinction.

The effect of NeuroD overexpression could also be observed in a spatial learning task. Mice were divided into two groups, one group injected with control virus (control)

and another group injected with Tet, TRE-ND virus, and feed doxycycline food for 7 days. After resting for 1 week, two groups of mice were tested in a standard Morris Water Maze. Two-way ANOVAs on swimming latency and distance to the hidden platform during the acquisition between two groups revealed significant (treatment x training day interactions). Mice injected with Tet, TRE-ND virus and fed with dox show a faster learning as demonstrated by significant shorter latencies and distances on the fourth day of training compared with the group injected with control virus ($F_{5,250}$= 38.48, P<0.01; $F_{1,250}$= 3.478, P<0.05), indicating that NeuroD1 over-expression facilitate animal's acquisition in special learning (Figure 3.10).

When we testing the animal's contextual preference at a lower frequency (once in every week), the animals take longer time to lose their drug-association memory, but the effect of time limited regulation of NeuroD1 expression to the long-term memory of drug-contextual association persist. Similar to former experiment, one group of mice were injected with control lentivirus (control), while three groups of mice were injected with lentivirus contain Tet-on system (Tet, TRE-ND) and were fed with dox food at different time of CPP paradigm (Figure 3.11A). Four groups of mice (N=7-8/group) show similar level of CPP expression on day 8 ($F_{3,27}$= 0.5186, P>0.05), but have significant differences in extinction rates. Group 3 mice (dox 8-14day) took about 50 days when their preference to morphine-paired chamber drop to basal level, and the CPP scores of group 3 are significant less compare to other groups on day 50 ($F_{3,27}$= 3.609, P<0.05). Control group mice took about 64 days to extinct their place preference ($F_{3, 26}$= 4.042, P<0.05) and group 2 mice took about 78 days. The drug-associated place

preference for group 1 mice persists even after 10 weeks of extinction test (Figure 3.11B). When we compare the NeuroD1/EGFP expression level after the CPP experiment, group1, group2 and group3 mice all show a significant increase in number of NeuroD1 positive cells ($F_{3,26}$= 28.17,P<0.01) and EGFP positive cells ($F_{3,26}$= 3.171,P<0.05), indicating that adult hippocampal neurogenesis was increased by NeuroD1 inducement(Figure 3.11C).

In addition, after the extinction session, mice were challenged with morphine (5mg/kg) 1 week after the last place preference test. All groups of mice show a strong preference to the former morphine-paired chamber, indicating the reinstatement of CPP-related contextual memory. But these memories were not different between individual groups (Figure 3.12), indicating that NeuroD1 overexpression did not influence on animal's memory of drug-induced relapse.

3.4 Discussion

Thus far, our study has demonstrated that similar to Tet-on lentiviral injection (NeuroD1 overexpression), administration of KHS101 before the training of drug-context association prolong CPP extinction, while administration of KHS101 after training shorten CPP extinction. The POMC-EGFP transgenic model allows the visualization of adult-generated granule cells, and we were able to show that NeuroD1 overexpression significantly increase the number of the EGFP+ adult-born NSPCs in vivo (Figure 3.8). As reported in the literature, adult-born granule cells are more preferential in their incorporation into spatial memory networks in the dentate gyrus (Kee et al. 2007). The immature neurons generated in the sub-granular zone (SGZ) of the dentate gyrus (DG) exhibit lower thresholds for activity dependent synaptic plasticity (Wang et al. 2000) than the mature neurons, and have been hypothesized to display greater ability to induce long-term potentiation(LTP) in response to new experiences (Praag et al. 1999)(Snyder et al. 2001). These studies suggest that NeuroD1 manipulation of contextual memory may be associated with the neural progenitors and immature neurons at certain stage of development.

Our study also implicated that the effect of NeuroD1manipulation is closely related to the experience when animals are tested in a behavior task. Several lines of evidences indicated that the extinction of conditioned learning involves formation of new memory, rather than simple forgetting or erasure of original memory (Bouton 2000) (Santini et al. 2001). We have shown that inducing NeuroD overexpression by lentivirus and KHS101 both accelerate the spatial learning in Morris-Water Maze (Figure 3.6,

Figure 3.10). According to these findings, we hypothesize that the difference in extinction periods of different groups may be due to their distinct abilities to form new place preference memory, as the test for extinction is similar to the training that both compartments are paired with no drug. This hypothesis is agree with a recent study with paradoxical conclusions, for wheel running can accelerate or delay cocaine CPP, whose influence depends critically on the timing of wheel access relative to drug conditioning (Mustroph et al. 2011).

However, there are other mechanisms to explain the effect we observed. NeuroD1 was also proved to modulate spine stability and increase spine density (Zheng et al. 2010). Such synaptic changes are considered essential for memory formation and retention (Yang et al. 2009). A genome-wide prediction of NeruroD1 direct target genes includes multiple Eph receptors (Seo et al. 2007), which are reported as key players in synapse formation and plasticity (Lai & Ip 2009). Therefore, the fact that NeuroD1 overexpression prolonged morphine-induced CPP could be due to its effects in maintaining spine stability and synapse formation. How to distinguish the contribution of the synaptic effect of NeuroD1 on an animal's drug memory formation from the contribution of its influence on progenitors' development and fate choice in adult neurogenesis needs to be further established.

In conclusion, when NeuroD1 overexpression is induced by administration of KHS101 or the Tet-on Expression System after CPP conditioning, both treatments promote the extinction of drug-associate memory (Figure 3.2, Figure 3.9). The lentiviral injection with doxycycline feeding could limit NeuroD1 overexpression in a temporal

time window, but utilizing the compound KHS101 has bigger advantage in avoiding surgery. Manipulation of NeuroD1 expression by chemical compounds like KHS101 after the formation of drug-contextual association represents a potentially therapeutic approach to human drug addiction.

Figure 3.1

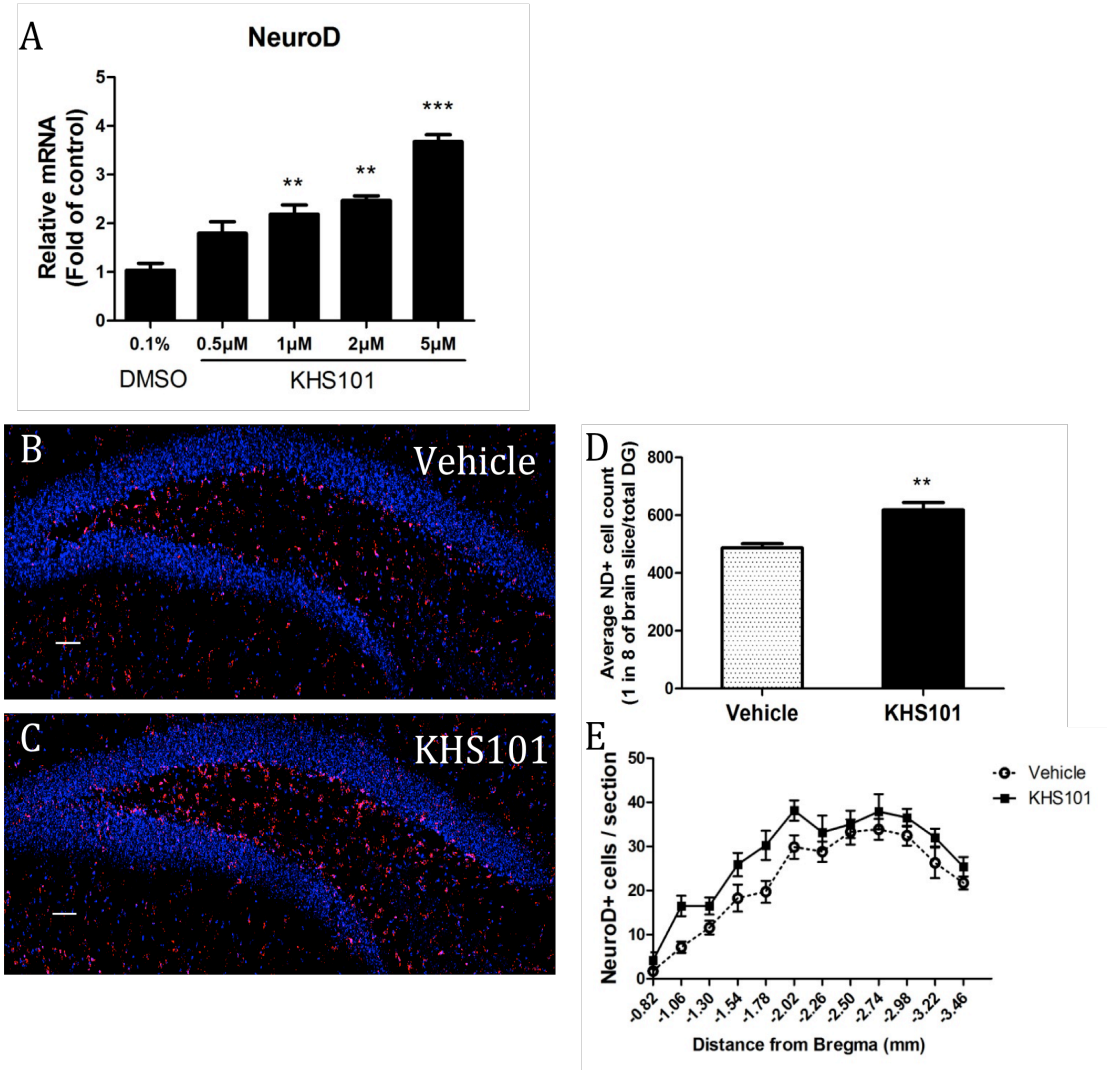

A
NeuroD
Relative mRNA (Fold of control)
0
1
2
3
4
5
**
**
***
0.1%
0.5μM
1μM
2μM
5μM
DMSO
KHS101
B
Vehicle
C
KHS101
D
Average ND+ cell count (1 in 8 of brain slice/total DG)
0
200
400
600
800
**
Vehicle
KHS101
E
NeuroD+ cells / section
0
10
20
30
40
50
-0.82
-1.06
-1.30
-1.54
-1.78
-2.02
-2.26
-2.50
-2.74
-2.98
-3.22
-3.46
Distance from Bregma (mm)
Vehicle
KHS101

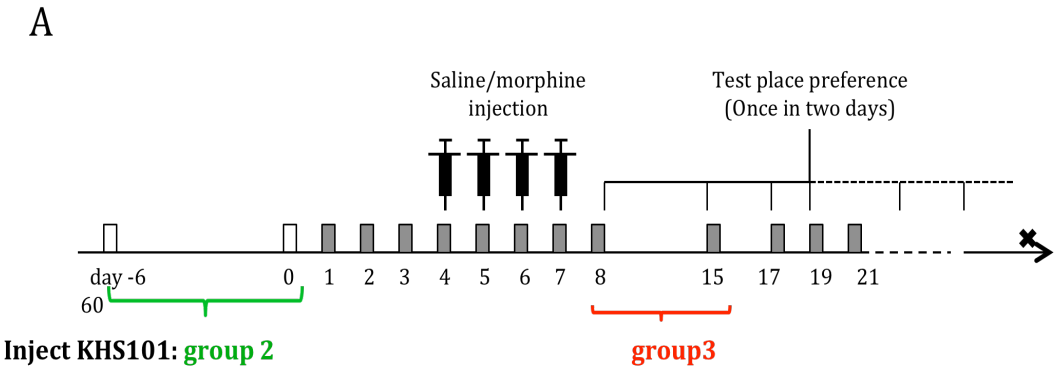
Figure 3.1 KHS101 induce NeuroD over-expression *in vitro* and *in vivo*.

(A) Quantative PCR of NeuroD mRNA level in mouse neural stem/progenitor cells culture incubate with KHS101 for 24 hours (3 replicate, **p<0.01, ***p<0.001) .

(B-C) Immunohistochemistry image of NeuroD (ND) (red) and DAPI(blue) of vehicle and KHS101 treated mice in the SGZ. (Scale bar: 50 μm) (D)Analysis of NeuroD expression level measured by average number of NeuroD positive cells in total dentate gyrus (N=4-6/per group, *p<0.05, **p<0.01). (E) Stereotaxic quantification for NeuroD positive cells through hippocampus (count every 1 in 8 coronal sections) .

Data represent mean ± SEM of 4 to 6 animals in separate experiments. Statistical significance was determined by one-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.2


A
Saline/morphine injection
Test place preference (Once in two days)
day -6
60
0
1
2
3
4
5
6
7
8
15
17
19
21
Inject KHS101: group 2
group3

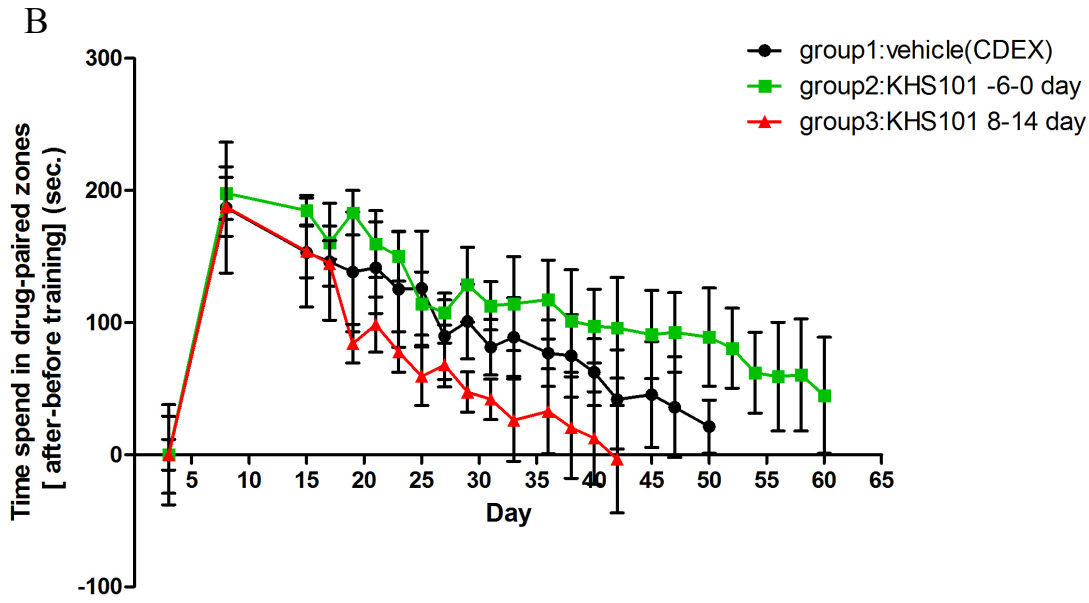

B
group1:vehicle(CDEX)
group2:KHS101 -6-0 day
group3:KHS101 8-14 day
Time spend in drug-paired zones [after-before training] (sec.)
300
200
100
0
-100
5
10
15
20
25
30
35
40
45
50
55
60
65
Day

Figure 3.2 KHS101 Administration influence animal's drug contextual memory.

(A)Timeline of CPP experiment: group 1 mice were injected with vehicle control; group 2 mice were injected with KHS101 from day -6 to day 0, group 2 mice were injected with KHS101 on day 8 to 14. After 4 days of training, mice were tested for their post-conditioning preferences once in 2 days. All animals were sacrificed on day 60 (B) CPP extinction test measured by time spend in drug-paired chamber in different groups (N=8/ group). On day 8, all group injected with vehicle or KHS101 developed CPP to the same extent. However, animal from group 3 showed an accelerated memory loss of their drug experience (29 day) than group 1 (38 day), while group 2 mice showed a prolonged extinction (56 day) than the other groups. Data represent mean ± SEM of six to eight animals in separate experiments.

Figure 3.3

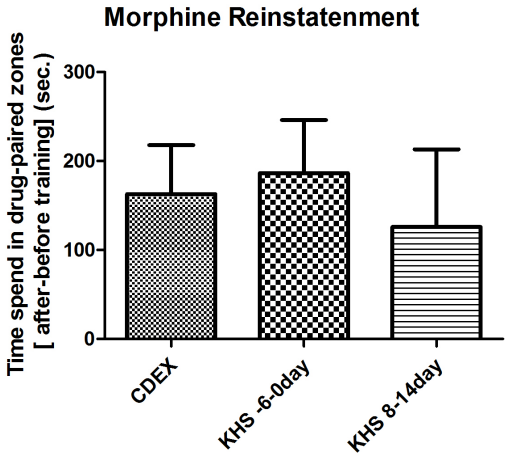


Figure 3.3 KHS101 did not change morphine-primed relapse.

After the extinction session, mice were challenged with morphine (5mg/kg) 1 day after the last place preference test. Recovered place preference was used to determine the reinstatement of CPP-related contextual memory. All groups of mice show a strong preference to the former morphine-paired chamber. No significant differences in reinstatement CPP score was detected between group 1(CDEX), group 2 (KHS -6-0day) and group3 (KHS 8-14day) mice.

Data represent mean ± SEM of six to eight animals in separate experiments.

Figure 3.4

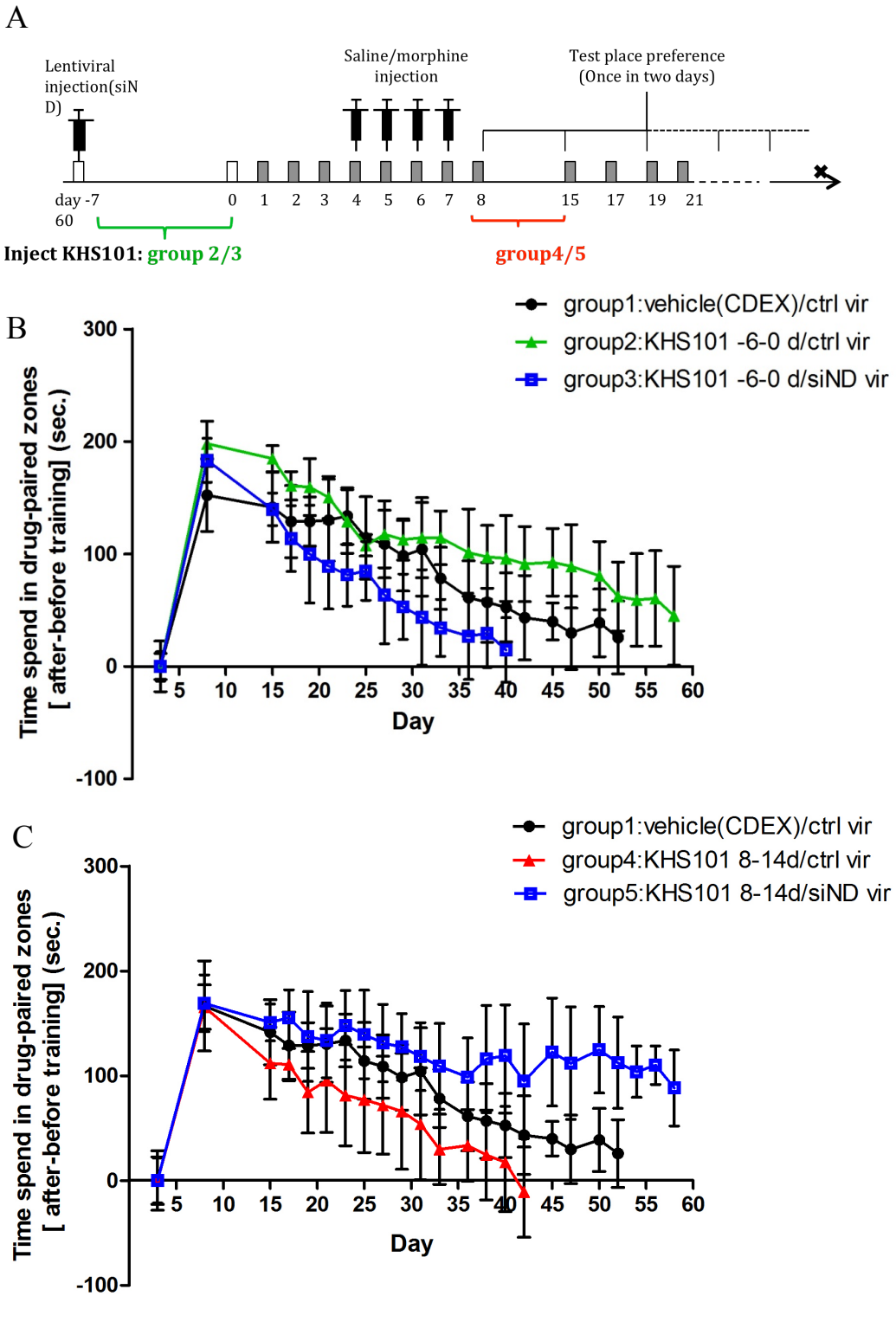

A
Lentiviral injection(siND)
Saline/morphine injection
Test place preference (Once in two days)
day -7
60
0 1 2 3 4 5 6 7 8 15 17 19 21
Inject KHS101: group 2/3
group4/5
B
group1:vehicle(CDEX)/ctrl vir
group2:KHS101 -6-0 d/ctrl vir
group3:KHS101 -6-0 d/siND vir
Time spend in drug-paired zones [after-before training] (sec.)
300
200
100
0
-100
5 10 15 20 25 30 35 40 45 50 55 60
Day
C
group1:vehicle(CDEX)/ctrl vir
group4:KHS101 8-14d/ctrl vir
group5:KHS101 8-14d/siND vir
Time spend in drug-paired zones [after-before training] (sec.)
300
200
100
0
-100
5 10 15 20 25 30 35 40 45 50 55 60
Day

Figure 3.4 lentivirus of NeuroD siRNA blocked KHS101 effect on animal's drug memory.

(A) Timeline of CPP experiment: group 1, group2, and group 4 mice were injected with control virus (ctrl vir) 1 week before experiment, while group 3 and group 5 mice were injected with NeuroD siRNA lentivirus (siNd vir). Group 1 mice were injected with vehicle, group 2 and group 3 mice were injected with KHS101 from day -6 to day 0, while group 4 and group 5 mice were injected with KHS101 from day 8 to 14. Five groups of mice were tested for their CPP score once in 2 days. (B) CPP extinction test measured by time spend in drug-paired chamber in different groups (N=6-8/ group) On day 8, all group injected with virus developed CPP to the same extent. However, Compared group 2 and 3, NeuroD siRNA abolished KHS101's effect of prolonged CPP memory. (B)CPP extinction test measured by time spend in drug-paired chamber in different groups (N=6-8/ group) On day 8, all group injected with virus developed CPP to the same extent. However, Compared group 2 and 3, NeuroD siRNA abolished KHS101's effect of shorten CPP memory. Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by one-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.5

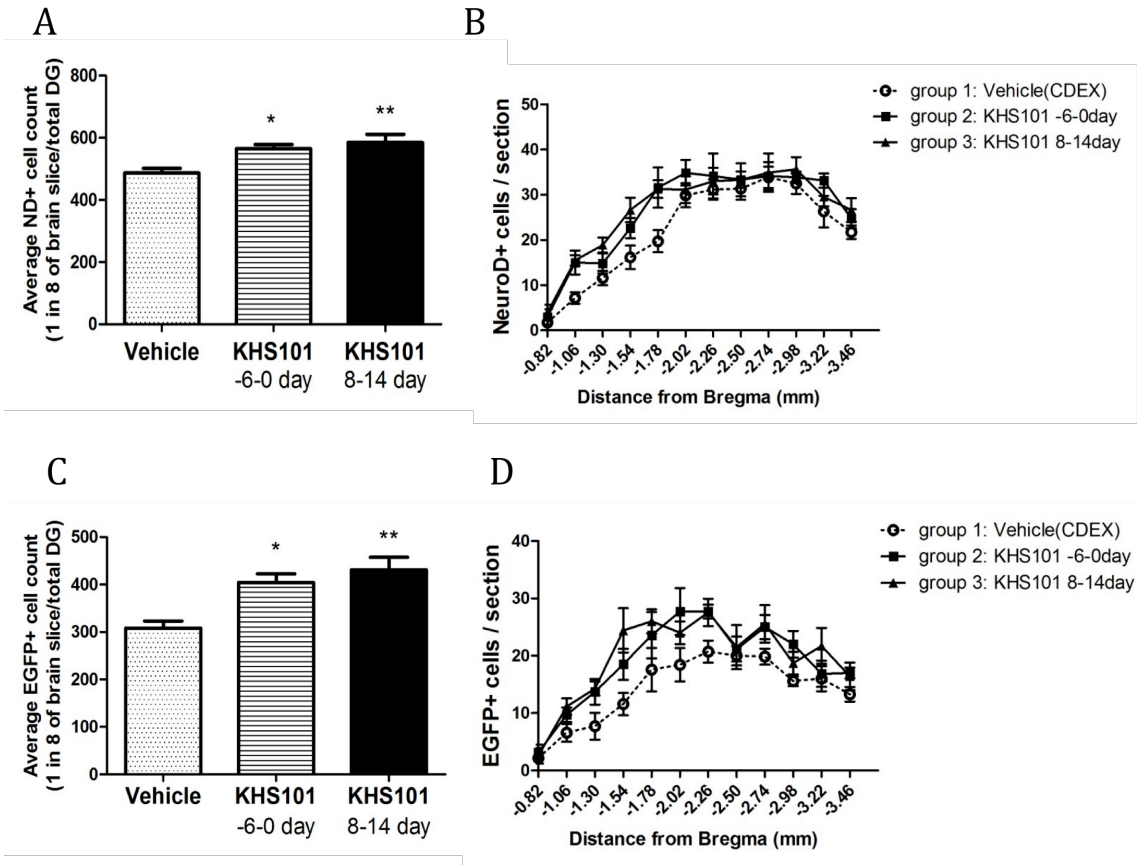

A
B
Average ND+ cell count
(1 in 8 of brain slice/total DG)
800
600
400
200
0
*
**
Vehicle
KHS101
-6-0 day
KHS101
8-14 day
NeuroD+ cells / section
50
40
30
20
10
0
-0.82
-1.06
-1.30
-1.54
-1.78
-2.02
-2.26
-2.50
-2.74
-2.98
-3.22
-3.46
Distance from Bregma (mm)
group 1: Vehicle(CDEX)
group 2: KHS101 -6-0day
group 3: KHS101 8-14day
C
D
Average EGFP+ cell count
(1 in 8 of brain slice/total DG)
500
400
300
200
100
0
*
**
Vehicle
KHS101
-6-0 day
KHS101
8-14 day
EGFP+ cells / section
40
30
20
10
0
-0.82
-1.06
-1.30
-1.54
-1.78
-2.02
-2.26
-2.50
-2.74
-2.98
-3.22
-3.46
Distance from Bregma (mm)
group 1: Vehicle(CDEX)
group 2: KHS101 -6-0day
group 3: KHS101 8-14day

Figure 3.5 KHS101 induce NeuroD over-expression and adult hippocampus neurogenesis.

(A) Comparison of NeuroD expression level measured by average number of NeuroD positive cells in three groups of mice injected with vehicle, KH101 from -6 to 0 day and 8-14 day. After CPP extinction test, all group of mice injected with KHS101 for 7 days show an increase in NeuroD cells number (N=8/per group, *$p<0.05$, **$p<0.01$). (B) Stereotaxic quantification for NeuroD positive cells through hippocampus (count every 1 in 8 coronal sections) (C) Comparison of adult neurogenesis level measured by average number of EGFP positive cells in three groups of mice injected with vehicle, KH101 from -6 to 0 day and 8-14 day. After CPP extinction test, all group of mice injected with KHS101 for 7 days show an increase in EGFP cells number (N=8/per group, *$p<0.05$, **$p<0.01$). (D) Stereotaxic quantification for EGFP positive cells through hippocampus (count every 1 in 8 coronal sections)

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by one-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.6

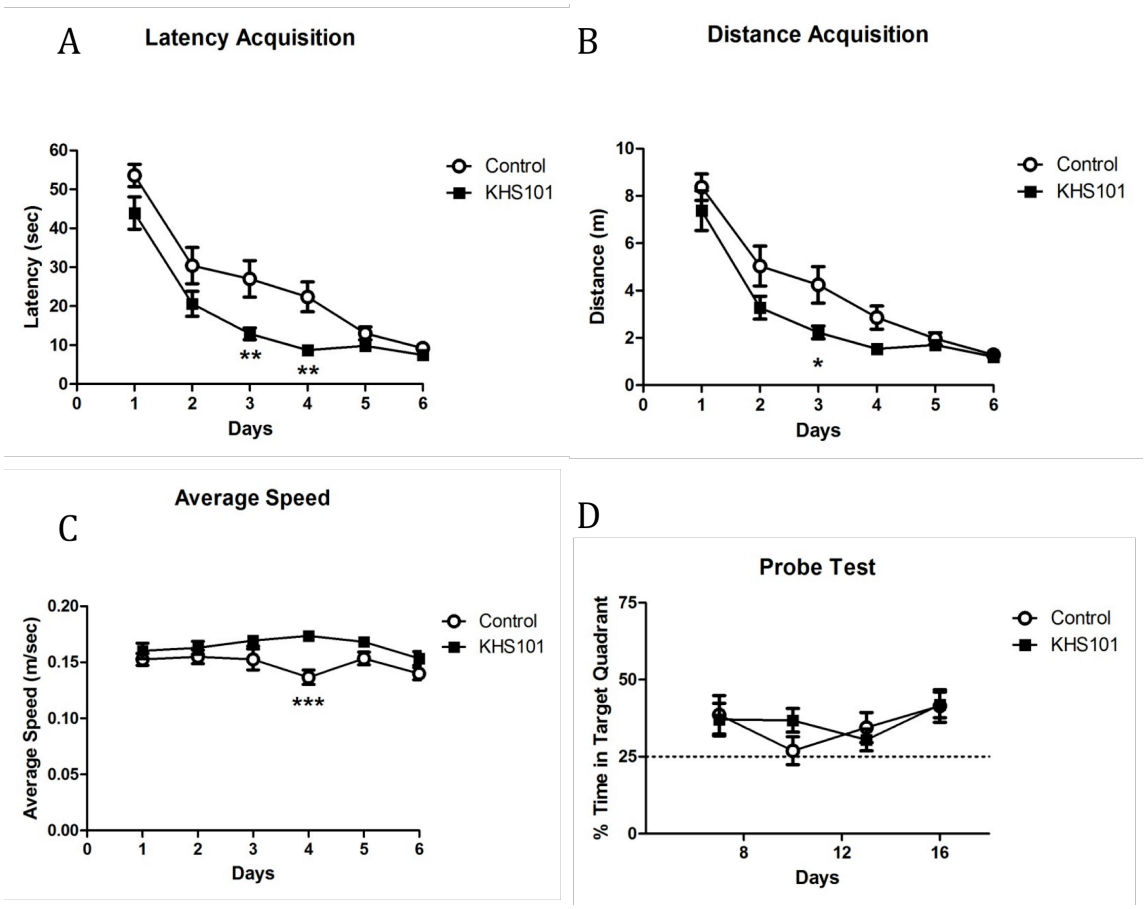


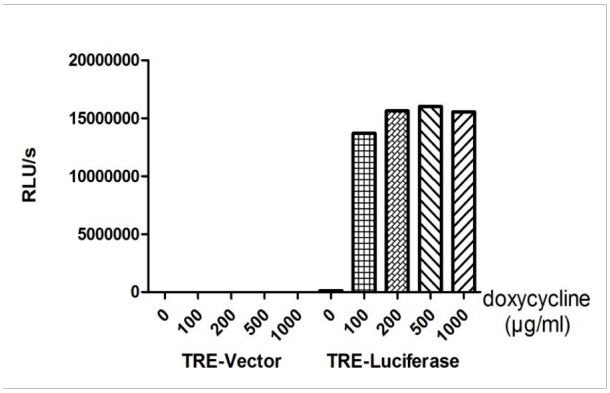
Figure 3.6 KHS101 facilitate animal's spatial learning in Morris Water Maze (MWM).

(A-B) Latency and distance travelled to find the platform in a 6 days water-maze training of different groups of mice. (C) Mice injected with KHS101 for 7 days show higher average velocity in day 4 of the acquisition test. (D) No significant difference was found in two groups of mice in a probe-test, which represent the extinction of their space memory.

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.7

Tet: Tetracycline-controlled transactivator； TRE: Tet response element.



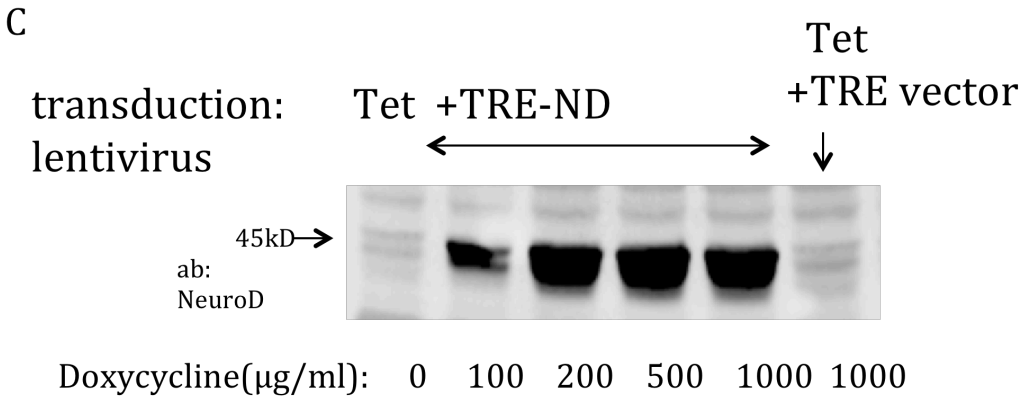


Figure 3.7 Tetracycline-Inducible Expression Systems (Tet-on system) induce NeuroD overexpression only with the presence of doxycycline.

(A) Sketch map of Tet-on gene expression system we used. (B) HEK 293 cells were transiently transfected with response vectors (TRE-Luc, containing luciferase) and regulator vectors (Tet) from the Tet-On 3G Expression Systems. The cells were cultured in the presence and absence of doxycycline for24 hrs, then luciferase expression was measured. (C)Western blot analysis of the efficiency of NeuroD expression in the presence of doxycycline in cultured neurons with the transduction of two Lentivirus containing Tet-on vectors (Tet, TRE-ND).

Figure 3.8

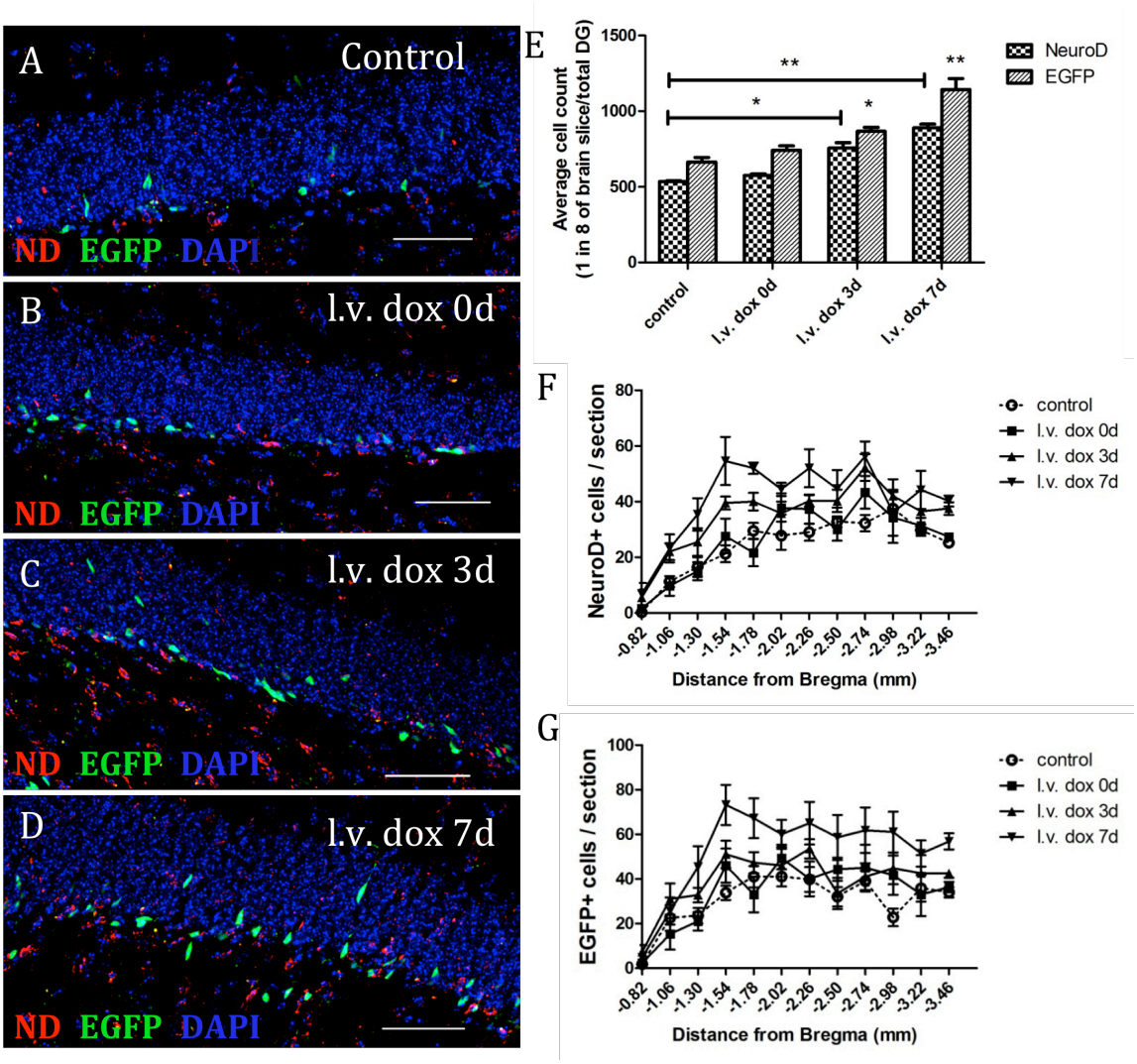
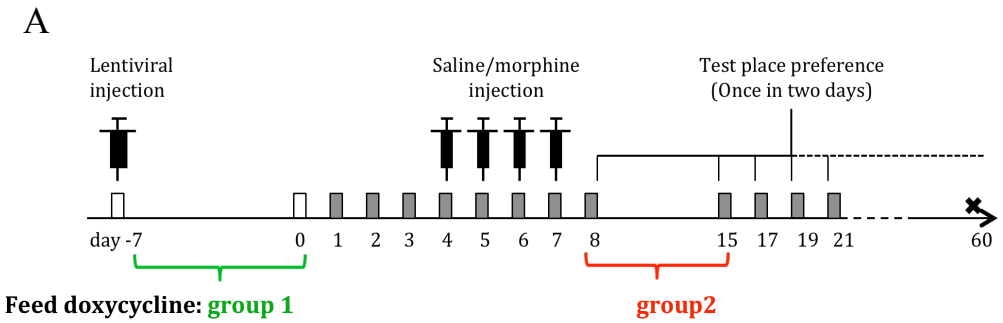

A
Control
ND EGFP DAPI
B
l.v. dox 0d
ND EGFP DAPI
C
l.v. dox 3d
ND EGFP DAPI
D
l.v. dox 7d
ND EGFP DAPI
E
Average cell count (1 in 8 of brain slice/total DG)
1500
1000
500
0
**
*
*
**
NeuroD
EGFP
control
l.v. dox 0d
l.v. dox 3d
l.v. dox 7d
F
NeuroD+ cells / section
80
60
40
20
0
-0.82
-1.06
-1.30
-1.54
-1.78
-2.02
-2.26
-2.50
-2.74
-2.98
-3.22
-3.46
Distance from Bregma (mm)
control
l.v. dox 0d
l.v. dox 3d
l.v. dox 7d
G
EGFP+ cells / section
100
80
60
40
20
0
-0.82
-1.06
-1.30
-1.54
-1.78
-2.02
-2.26
-2.50
-2.74
-2.98
-3.22
-3.46
Distance from Bregma (mm)
control
l.v. dox 0d
l.v. dox 3d
l.v. dox 7d

Figure 3.8 Tet-on Systems induces NeuroD over-expression and adult neurogenesis *in vivo*.

(A-D) Fluorescent images of NeuroD immunostaining in mouse hippocampus. After injection of lentivirus, mice were feed with doxycycline from zero to seven days to induce the overexpression of NeuroD. The green fluorescent cells represented neural progenitors and immature neurons in the SGZ. (E)Analysis of NeuroD and EGFP expression level measured by average number of NeuroD/EGFP positive cells in total dentate gyrus (N=5/per group, *$p<0.05$, **$p<0.01$). (F-G) Stereotaxic quantification for NeuroD/EGFP positive cells through hippocampus (count every 1 in 8 coronal sections) .

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by one-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.9


A
Lentiviral injection
Saline/morphine injection
Test place preference (Once in two days)
day -7
0
1
2
3
4
5
6
7
8
15
17
19
21
60
Feed doxycycline: group 1
group2

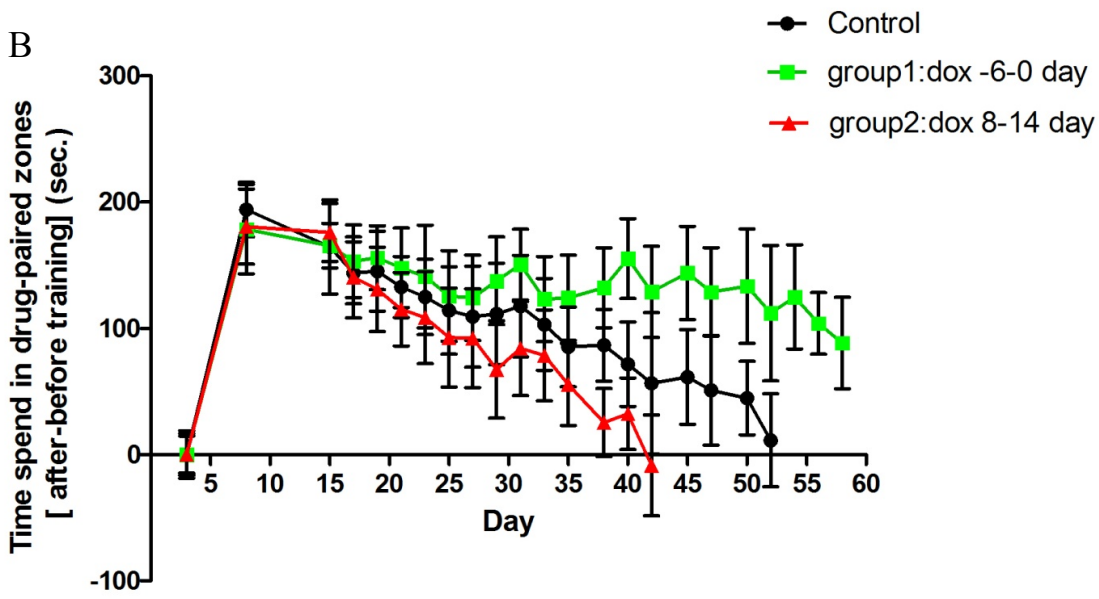

B
Control
group1:dox -6-0 day
group2:dox 8-14 day
Time spend in drug-paired zones [after-before training] (sec.)
300
200
100
0
-100
5
10
15
20
25
30
35
40
45
50
55
60
Day

Figure 3.9 NeuroD over-expression influence on animal's drug contextual memory.

(A) One week before CPP experiment, two groups of mice were injected with lentivirus contain Tet-on vectors (tTA, TRE-ND) and 1 group of mice were injected with control lentivirus (control). Then group 1 mice were fed with doxycycline from day -6 to day 0, while group 2 mice were fed with doxycycline on day 8 to 14. (B)Comparing the CPP extinction period in different groups, group 2 lost their drug-related chamber preferences faster than group injected with control virus, while the memory of drug experiences of group 1 mice were much longer than the control group(N=7-8/group). Data represent mean ± SEM of six to eight animals in separate experiments.

Figure 3.10

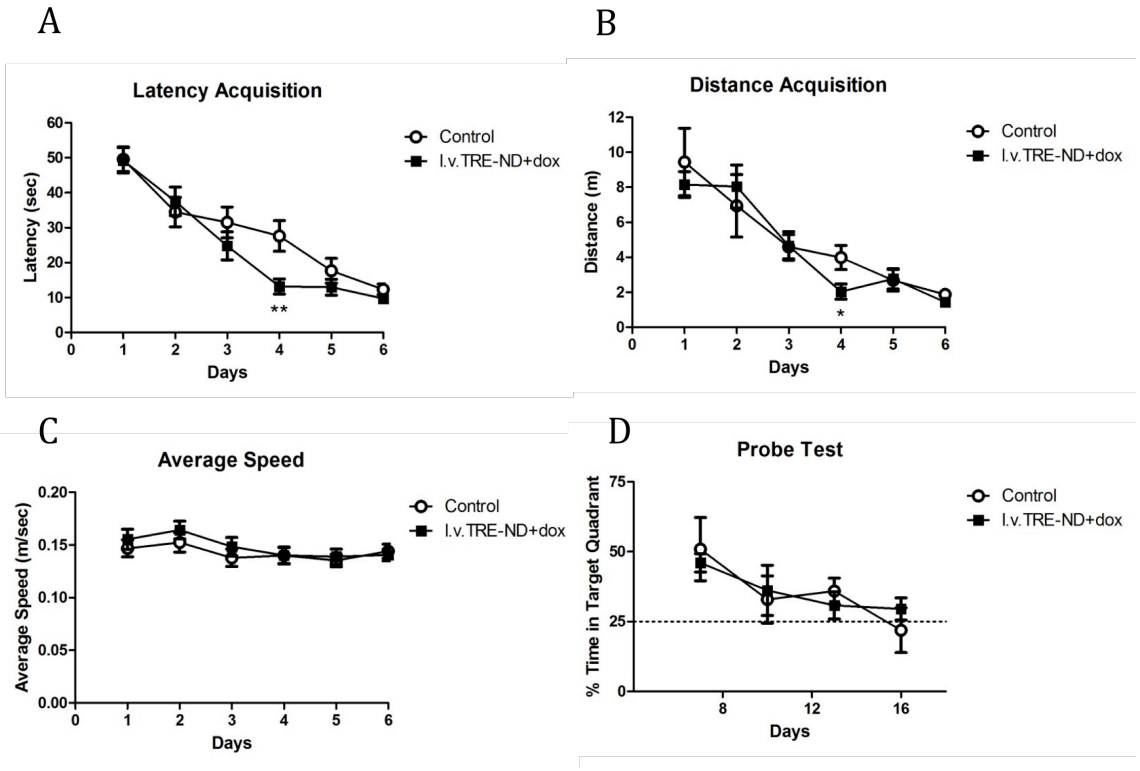


Figure 3.10 NeuroD overexpression facilitates animal's spatial learning in MWM.

(A-B) Latency and distance travelled to find the platform in a 6 days water-maze training of different groups of mice. Compare animals injected with control virus and tTA with TRE-ND virus (feed dox for 7 days), the groups of mice with NeuroD overexpression show faster in learning of the special task. (C) No significant difference was found in average velocity of two groups of mice. (D) No significant difference was detected in two groups of mice in a probe-test, which represent the extinction of their space memory.

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by two-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.11

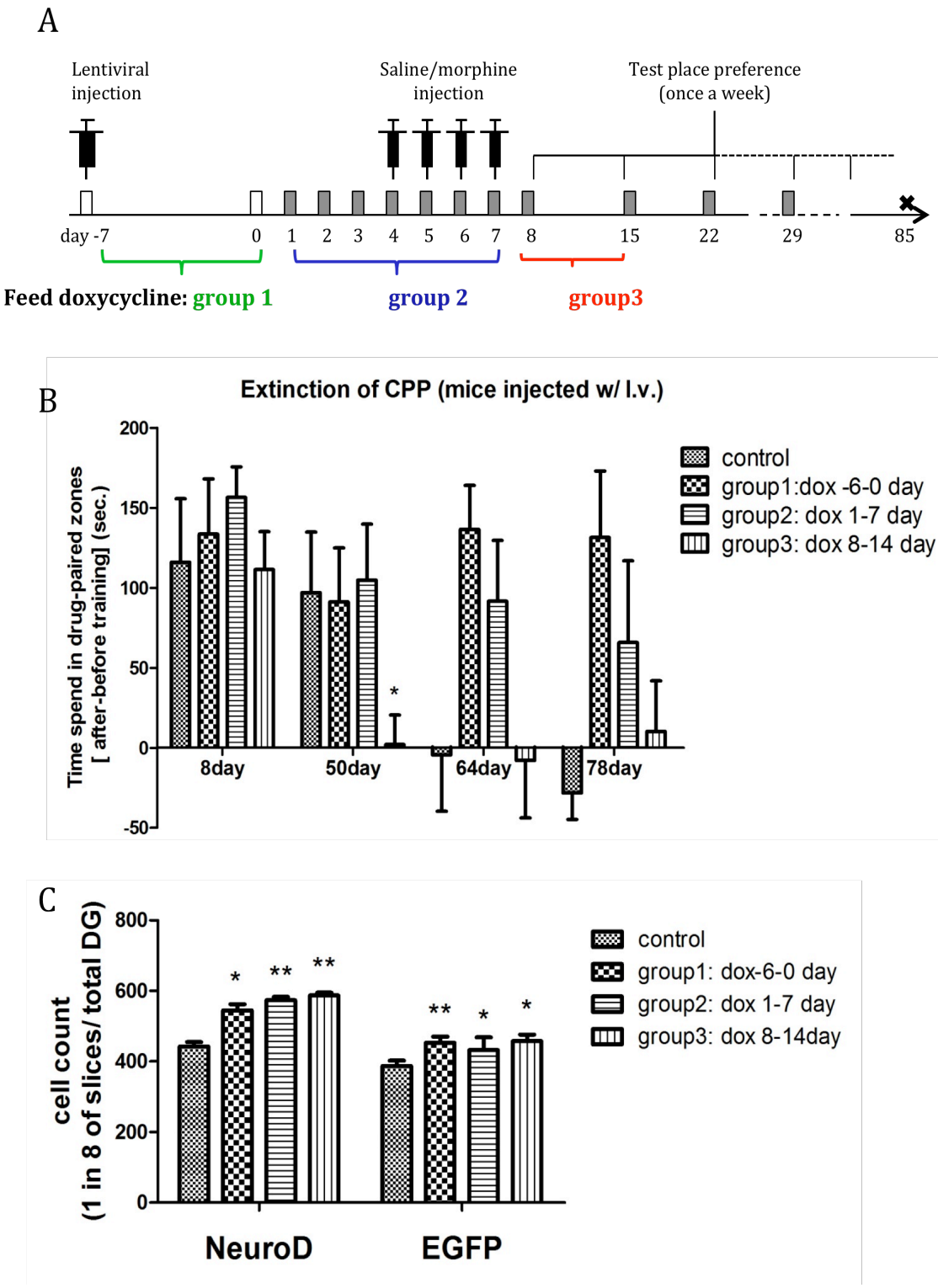

A
Lentiviral injection
Saline/morphine injection
Test place preference (once a week)
day -7
0
1
2
3
4
5
6
7
8
15
22
29
85
Feed doxycycline: group 1
group 2
group3
B
Extinction of CPP (mice injected w/ l.v.)
Time spend in drug-paired zones [after-before training] (sec.)
200
150
100
50
0
-50
8day
50day
64day
78day
control
group1:dox -6-0 day
group2: dox 1-7 day
group3: dox 8-14 day
C
cell count (1 in 8 of slices/ total DG)
800
600
400
200
0
NeuroD
EGFP
control
group1: dox-6-0 day
group2: dox 1-7 day
group3: dox 8-14day

Figure 3.11 NeuroD overexpression at different time has different effect in CPP extinction.

(A)Similar to former CPP, group 1 mice were fed with doxycycline(dox) food from day-6 to day 0, group 2 mice were fed with dox from day 1 to day 7, while group 3 mice were fed with dox on day 8 to 14. After 4 days of training, mice were tested for their post-conditioning preferences in a 15min drug-free post-condition test. Three groups of mice were tested for their CPP score once every week. All animals were sacrificed on day 85. (B)CPP extinction measured by time differences spends in the drug-paired chamber. On day 8, all group injected with virus developed CPP to the same extent. However, on day 50, group 3 completely lost their drug-chamber preference, while group injected with control virus lost their preference on day 64. Even on day 78, mice of group 1 and group 2 retained the preferences to the drug-pair chamber. (C)Analysis of adult hippocampal neurogenesis level measured by average number of NeuroD and EGFP positive cells (count every 1 in 8 brain sections, add together) in total dentate gyrus (N=6-8/per group, *$p<0.05$, **$p<0.01$). After CPP extinction test, all groups of mice injected with tTA and TRE-ND lentivirus and fed with dox show an acceleration in neurogenesis, represented by an increase in NeuroD and EGFP positive cells number. No significant difference was observed between group1, group2 and group 3 mice.

Data represent mean ± SEM of six to eight animals in separate experiments. Statistical significance was determined by one-way ANOVA with a Bonferroni test for post hoc comparisons.

Figure 3.12

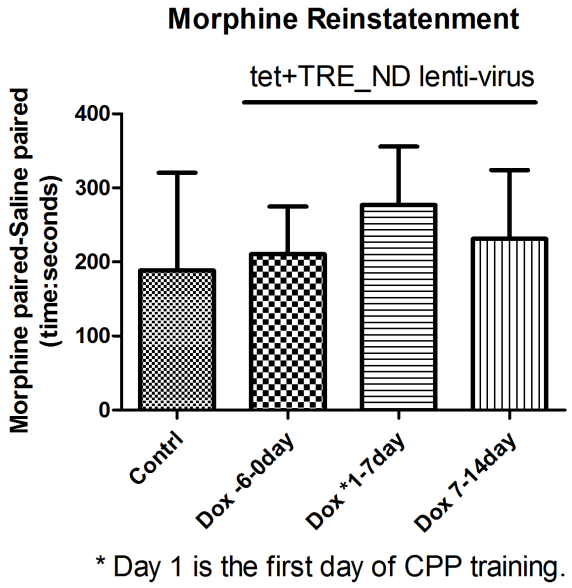


Figure 3.12 Overexpression of NeuroD1 did not change morphine-primed relapse.

After the extinction session, mice were challenged with morphine (5mg/kg) 1 week after the last place preference test. Recovered place preference was used to determine the reinstatement of CPP-related contextual memory. All groups of mice showed a strong preference to the former morphine-paired chamber. No significant differences in reinstatement CPP score were detected between control group (control), group 1(dox -6-0day), group 2 (dox 1-7day) and group3 (dox 7-14day) mice.

Data represent mean ± SEM of six to eight animals in separate experiments.

CHAPTER 4
SUMMARY

My doctoral study examined the neural adaptive changes associated with opioid addition, especially the adaptive changes taken place in the SGZ of DG area in the adult hippocampus. I investigated the details of morphine's effect on neurogenesis in the SGZ of hippocampus in the adult mouse brain, and also the correlation between the manipulations of adult neurogenesis and the drug contextual memory.

My project first studied an important, timely question: how did morphine influence adult neurogenesis in the context of CPP, and on what stage of neural development did morphine exert its action? Using a variety of approaches including reporter mouse, cell counts, morphological analysis, lentiviral infusion and behavioral analysis, I observed a morphine-induced reduction in the number of late stage progenitors and immature neurons in the SGZ of dentate gyrus in the mouse hippocampus. This effect was specific to an increased adult neurogenesis which occurred after mice were exposed to condition place preference training. The effect of morphine was mediated through a disruption in the maturation of type II progenitors and type III neuroblasts, rather than an effect on cell proliferation or apoptosis. Both *in vitro* and *in vivo* studies support the assumption that morphine altered the lineage of the neural progenitors during differentiation via a mechanism involving transcription factor NeuroD1(Xu 2014).

The next step of my study was to explore the relationship between the regulation of neural progenitors' differentiation and drug-associated memories. Previously we had established that the reduction of neural progenitors by morphine treatment was mitigated by introduction of NeuroD1 via a lentiviral construct. And NeuroD1 lentiviral construct could also prolong the animal's memory of their drug experience during a CPP task. Base

on these facts, we want to utilize a small molecule that known to regulate NeuroD1 level in the hippocampus to substitute for lentiviral construct. Such substitution takes the advantage of NeuroD1 modulation in a time-dependent manner, enable us to explorer the role of NeuroD1 during different time period of CPP paradigm, when animals are learning the association between drug-experience and environmental cues, or gradually losing this memory. We hypothesized that when an important development regulator like NeuroD1 was up-regulated during these periods, the new-born granule cells would have consequent neural adaptive changes, and influence the formation of animal's memory in return. Our hypothesis was supported by the results that both lentiviral injection and administration of KHS101 before conditional training, which induced NeuroD1 overexpression and neural progenitor's differentiation, could enhance the retention of drug-related memory and prolong CPP extinction; while the same treatments after training disrupted the drug-contextual associations and shortened the period of CPP extinction. Our findings demonstrate that compounds that inducing NeuroD1 overexpression in adult hippocampus could regulate the rate or efficiency of neural progenitors' differentiation, hence manipulate the length of animal's drug-associate memory.

Combining the two specific aims of my project, I conclude that opioid receptor agonist such as morphine has a deleterious effect on neural progenitors' differentiation and maturation; on the other hand, as a consequence of the regulation on adult neurogenesis, the animal's addictive behavior is also altered due to the role of the hippocampal neurogenesis in the processing of contextual cues associated memories

(Figure 4.1).

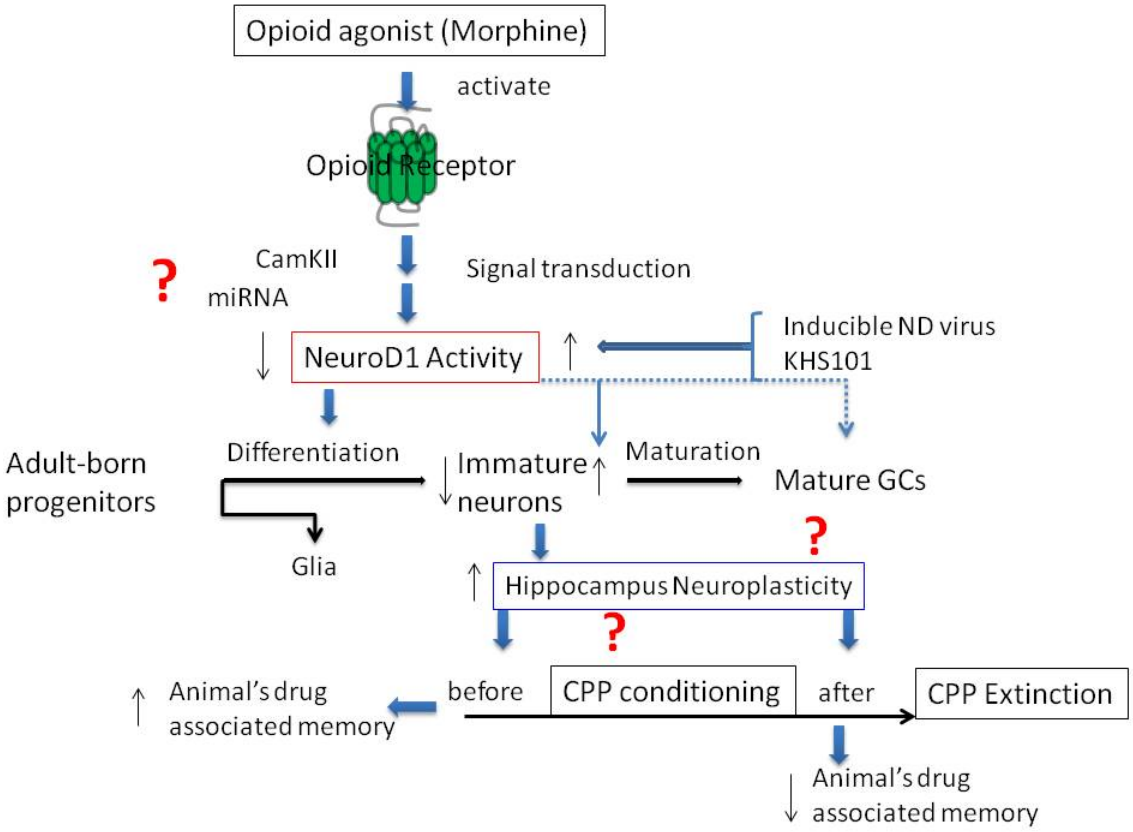


Figure 4.1 Summary of current conclusions and unsolved questions.

The current study spreads our knowledge of the mechanisms of regulating neural progenitor cells *in vivo* by opioid drugs, and the correlation between adult neurogenesis and the memories of drug-context association during the **preoccupation/anticipation** stage of the addiction cycle. Especially, we point out the significant role of NeuroD1 in regulating the neural adaptive changes taken place in the adult hippocampus during a CPP paradigm. More importantly, chemical compounds such as KHS101 which possess the ability to manipulate NeuroD1 level may have potential therapeutical implication in

the treatment of drug addiction.

Although a few questions have been answered, there are still several limitations of this study. First of all, our animal model of CPP procedure is limited in reflecting the animal's memory of the rewarding effect of opioids, instead it measures the memory of a drug-intake experience associated with a defined context. A self-administration model may be an alternate approach to estimate the drug reward and the reinstatement of drug-seeking behavior more directly. Second, both the treatment of KHS101 or NeuroD1 lentivirus fail to change the morphine-primed relapse after the CPP extinction (Figure 3.3, Figure 3.12), which means that such regulation on adult neurogenesis can not reverse the initial neural adaptive changes paired with the drug reward. In addition, the detail mechanisms of NeuroD1 activity influence on progenitors' development and fate choice in adult neurogenesis remain to be determined. There are several possible candidates in the downstream transcriptional targets of NeuroD1 that may serve as regulators of adult neurogenesis or synaptogenesis in the hippocampus (Seo et al. 2007), which could contribute to the mechanisms underlying increased NeuroD1 level associated with the retention or elimination of drug contextual memory.

Future studies need to be conducted to make up the deficiency of the current project. For instance, the detail signaling pathway from the initial activation of the opioid receptor by morphine to the final outcome of regulating NeuroD1 activity remains to be determined. Also, it is necessary to distinguish the effect of NeuroD1 on the existing neural circuits of the hippocampus from its impact on the newly-born progenitors or immature neurons (Figure 4.1). Besides, since we found that the time period of the

regulation on NeuroD1 and adult neurogenesis is essential to control the memory of animal's drug-experience, other positive regulators of adult neurogenesis such as physical exercise or environment enrichment may also have beneficial effect on addict patients, as long as the treatment is conducted in the adequate time window. Thus, the combination of different therapies including compounds like KHS101 and physical exercise to manipulate adult neurogenesis in cooperation may be the future direction of exploring new treatments for opioid addiction.

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Appendix

Abbreviations

AC adenylyl cyclases

ANOVA analysis of variance

BrdU 5-Bromo-2-deoxyuridine

CPP conditioned place preference

CaMKIIa calcium/calmodulin-dependent protein kinase II

DCX doublecortin

DG dentate gyrus

DOR delta-opioid receptor

EGFP enhanced green fluorescent protein

ERK extracellular signal-regulated kinase

GC granular cell

GCL granular cell layer

GFAP glial fibrillary acidic protein

GPCR G-protein coupled receptor

KOR kappa-opioid receptor

MOR mu-opioid receptor

MWM Morris water maze

NeuroD1 neurogenic differentiation1

NSPCs neural stem/progenitor cells

IML inner molecular layer

IR immunoreactive

PCNA proliferating cell nuclear antigen

pHisH3 phosphorylated histone H3

POMC pro-opiomelanocortin

Prox1 Prospero homeobox protein 1

PSA-NCAM polysialylated form of the neural cell adhesion molecule

SGZ sub-granular zone

SVZ sub-ventricle zone

TRBP TAR RNA-binding protein

SOX2 SRY (sex determining region Y)-box 2

TuJ1 β-III Tubulin

TUNEL Terminal deoxynucleotidyl transferase dUTP nick end labeling

VEGF vascular endothelial growth factor