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Glycogen Synthase Kinase 3 β in the ventral hippocampus is important for cocaine reward and object location memory.

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Abstract

The ventral hippocampus is a component of the neural circuitry involved with context-associated memory for reward and generation of appropriate behavioral responses to context. Glycogen synthase kinase 3 beta (GSK3 β) has been linked to the maintenance of synaptic plasticity, contextual memory retrieval, and is involved in the reconsolidation of cocaine-associated contextual memory. In this study, the effects of targeted downregulation of GSK3 β in the ventral hippocampus were examined on a series of behavioral tests for assessing drug reward-context association and non-reward related memory. The Cre/loxP site-specific recombination system was used to knockdown GSK3 β through bilateral stereotaxic delivery of an adeno-associated virus expressing Cre-recombinase (AAV-Cre) into the ventral hippocampus of adult mice homozygous for a floxed GSK3 β allele. GSK3 β floxed mice injected with AAV-Cre had a loss of 56–75 % of GSK3 β in the ventral hippocampus and displayed diminished development of cocaine conditioned place preference, but not morphine place preference as compared with wild-type mice injected with AAV-Cre or GSK3 β floxed mice injected with a control virus, AAV-GFP. Impaired object location memory was observed in mice with GSK3 β downregulation in the ventral hippocampus, but novel object recognition remained intact. These results indicate that GSK3 β signaling in the ventral hippocampus is differentially involved in the formation of place-drug reward association dependent upon drug class. Additionally, ventral hippocampal GSK3 β signaling is important in detection of discrete spatial cues, but not recognition memory for objects.

Keywords

novel object recognition; morphine; conditioned place preference

Introduction

Relapse to drug use is a significant challenge in the treatment of substance use disorder and is frequently triggered by exposure to cues and contexts previously associated with the

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effects of the drug. The hippocampus integrates environmental information into temporal and spatial representations and is necessary for tasks that require cue-stimuli associations (Rolls and Xiang, 2005; Ongur et al., 2005; Komorowski et al., 2013; McDonald et al., 2018; Contreras et al., 2018). The conditioned place preference procedure is frequently used to evaluate appetitive properties of drugs of abuse in rodents (Sanchis-Segura and Spanagel, 2006; Cunningham et al. 2011). In this procedure, animals acquire preference for a specific context (conditioned stimulus) where exposure to a drug of abuse (unconditioned stimulus) is repeatedly experienced, compared to a context where the drug stimulus is not experienced. Hippocampal neuronal activity is associated with acquisition and expression of drug-induced conditioned place preference (Tan, 2008; Takano et al., 2010; Alvandi et al., 2017), and lesions or inactivation of the hippocampus, particularly the dentate gyrus sub-region, inhibits place preference acquisition and cue-induced drug-seeking behavior (Meyers et al., 2006; Hernández-Rabaza et al., 2008; Keleta and Martinez, 2012; Sikora et al., 2016).

Glutamatergic ventral hippocampal projections to the nucleus accumbens shell contribute to drug-context memory formation and subsequent reinstatement of drug-seeking behavior (Taepavarapruk et al., 2014; Pascoli et al., 2014, Sikora et al., 2016). Further, ventral hippocampal activity is necessary for drug primed-, cue- or context-induced reinstatement of drug-seeking behavior (Vorel et al., 2001; Taepavarapruk et al., 2003, 2014; Sun and Rebec, 2003; Atkins et al., 2008; Lasseter et al., 2010). In addition, it has been demonstrated that acquisition of sign-tracking behavior, in which individuals are largely motivated by reward cues, is blocked by lesions of the ventral hippocampus (Fitzpatrick et al., 2016), expanding upon the significance of the ventral region of the hippocampus in reward cue valuation and the circuitries that drive cue-induced drug-seeking behaviors.

The molecular mechanisms underlying ventral hippocampal-regulated, cue-induced drug-seeking behavior are not well understood. Dopaminergic neurotransmission in the hippocampus is involved in the acquisition and reinstatement of psychostimulant and opioid conditioned place preference as blockade of hippocampal dopamine receptors prevents both (Keleta and Martinez, 2012; Kramar et al., 2014; Assar et al., 2016). One mechanism by which dopamine influences cellular processes is through regulation of glycogen synthase kinase 3 beta (GSK3 β) activity. GSK3 β is a multifunctional serine/threonine kinase with a critical role in memory retrieval (Hong et al., 2012). GSK3 β is constitutively active and is inhibited by phosphorylation of the N-terminal serine-9 by several kinases including Akt (protein kinase B, Beaulieu et al., 2011). Expression of GSK3 β is high in the mammalian brain including in the frontal cortex, nucleus accumbens, caudate putamen, hippocampus and amygdala (Leroy and Brion, 1999). Drugs affecting dopaminergic transmission directly or indirectly, such as cocaine (Miller et al., 2014) and morphine (Muller and Unterwald, 2004), regulate the activity of Akt and GSK3. Inhibition of GSK3 blocks psychostimulant-induced hyperactivity and sensitization (Beaulieu et al., 2004; Miller and Unterwald, 2009; Enman and Unterwald, 2012) and morphine analgesic tolerance (Parkitna et al., 2006; Liao et al., 2014). Systemic administration of the selective GSK3 inhibitor, SB 216763, prior to cocaine conditioning sessions blocked the development of cocaine place preference, without altering the acquisition of a contextual fear conditioning (Miller et al., 2014). Hippocampal GSK3 β has been linked to the maintenance of synaptic plasticity in the hippocampus and contextual memory retention (Peineau et al., 2008; Sintoni et al., 2013). Hippocampal

GSK3 β is activated by memory retrieval, and systemic administration of a GSK3 inhibitor before memory reactivation impairs memory reconsolidation in mice (Hong et al., 2012; Kimura et al., 2008). Moreover, the GSK3 β signaling pathway in the nucleus accumbens and hippocampus is activated by recall of cocaine reward memory induced by re-exposure to the drug-paired context, and systemic inhibition of GSK3 activity following memory retrieval abolishes an established cocaine place preference by interfering with the reconsolidation process (Shi et al. 2014). Taken together, these prior studies suggest that GSK3 β signaling may be an important component of the function of the ventral hippocampus in associating contextual cues with drug reward and subsequent behavioral responses.

In this study, we investigated the importance of GSK3 β in the ventral hippocampus to the formation of drug-associated contextual memories and non-drug related memory. Specifically, we tested whether knockdown of GSK3 β selectively in ventral hippocampus interfered with the development of cocaine or morphine conditioned place preference. Novel object and location memory tests were used to determine the impact of GSK3 β knockdown on non-drug reward memory performance. Results from this study demonstrate that ventral hippocampal GSK3 β signaling is differentially involved in the formation of context-drug reward association dependent upon drug class, and is important in detection of discrete spatial cues, but not recognition for objects.

Experimental procedures

Animals, drugs and stereotaxic delivery of AAV into the ventral hippocampus

Male and female heterozygous floxed GSK3 β (GSK3 $\beta^{lox/wt}$) mice were generously provided by James R. Woodgett, PhD (Patel et al., 2008) and bred in our animal facility. Cocaine and morphine were obtained through the NIDA drug supply program, dissolved in sterile saline, and injected in a volume of 3 ml/kg and 5 ml/kg body weight respectively. Adult mice (N=59) were anesthetized with isoflurane and placed within a stereotaxic frame (Stoelting Co., Wood Dale, IL). Stereotaxic coordinates for the ventral hippocampus were derived from the Paxinos and Franklin Mouse Brain Atlas (2001; AP 3.5 mm anterior and $\pm$ 2.3 mm lateral from Bregma, 4.2 mm ventral). A 26-gauge Hamilton syringe (Hamilton Company, Reno, NV) was inserted through a craniotomy site to deliver 0.5 μ l of virus (AAV1-Cre-GFP or AAV1-GFP, CMV promoter, 1.48 $\times$ 10¹³ VG/mL, Signagen Laboratories, Rockville, MD) at 0.1 μ l/min with an infusion pump (Harvard Apparatus, Holliston, MA). The syringe needle was left in place for 5 min, withdrawn and the injection repeated in the opposite hemisphere. Behavioral testing was performed as described below at least three weeks following viral delivery. Fluorescence microscopy was used to confirm accurate bilateral targeting of the ventral hippocampus (GFP) and GSK3 β knockdown as described below. The experimental time-line is shown in Figure 1.

Conditioned Place Preference

The mouse place preference chambers (45 $\times$ 20 $\times$ 20 cm) had two distinct compartments separated by a removable door. One compartment had a smooth floor with white and black vertical stripes at the walls. The neighboring compartment had a rough floor and white walls

with black circles. Illumination was equivalent for both compartments. The doses of cocaine and morphine were chosen based on preliminary studies in wild-type mice to produce the highest place preference scores using the identical conditioning procedures described herein.

Cocaine-conditioned place preference: Mice were injected with saline or cocaine (10 mg/kg, ip.), and immediately placed into a compartment of the conditioning chamber for 30 minutes. This was repeated once daily in alternating compartments for 4 days. On the testing day, the mice had free access to both compartments of the conditioning chamber for 30 minutes in a drug-free state. The study used an unbiased design in which drug-paired compartment was counterbalanced across animals, and animals were arbitrarily assigned to receive cocaine in the compartment with the smooth or rough floor (Miller et al., 2014). Preference scores were determined by subtracting the amount of time spent in the saline-paired compartment from the cocaine-paired compartment.

Morphine-conditioned place preference: Mice were confined for 40 minutes to one compartment twice daily (09:00 and 15:00) for 4 days with morphine (5 mg/kg, sc.) injected immediately prior to being placed in one compartment and saline prior to exposure to the other compartment. On testing day, the mice had access to both sides of the conditioning chamber for 20 minutes in a drug-free state. Preference scores were determined as described above.

Mouse numbers include for cocaine place conditioning: N=10 wild-type (WT) receiving AAV-Cre (5 male, 5 female); N=9 GSK3 $\beta^{lox/lox}$ receiving AAV-Cre (5 male, 4 female), N=12 GSK3 $\beta^{lox/lox}$ receiving control AAV-GFP (6 male, 6 female) and for morphine place conditioning: N=9 WT receiving AAV-Cre (4 male, 5 female); N=11 GSK3 $\beta^{lox/lox}$ receiving AAV-Cre (3 male, 8 female).

Novel object recognition and object location memory testing

Novel object recognition and object location memory tests were used to assess non-conditioned memory and were performed as described by Silverman et al (2013) with some modifications. The same mice were used for both tests with a one-week interval between tests, and unique objects utilized for each test (Figure 1). *Novel object recognition:* The experiment consisted of habituation, familiarization, and testing stages. On day 1 (habituation), each animal was placed into an open field arena (a plastic chamber, 25 × 20.5 × 30 cm, with a smooth floor and four nontransparent red walls) for 30 minutes in the absence of objects. Twenty-four hours later (day 2, familiarization and testing), mice were placed individually into the open field arena in the presence of two identical objects (A + A) for 10 minutes. Objects were cleaned with 70% ethanol between trials to remove olfactory cues. After familiarization, animals were placed individually into a clean home cage with bedding only. One familiar and one novel object (A + B) were placed into the chamber in the same locations as the proceeding familiarization phase. Thirty minutes after the end of the familiarization session, each animal was returned to its arena for a recognition test (10 min), during which it could freely explore the familiar and novel object.

Object location memory: The habituation and familiarization phase were performed as described for the novel object recognition test. The same objects were used for the test phase, but one object was moved to a novel location while the other object remained in the same location as during training. The arena was cleaned with 70% ethanol between sessions. For both novel object and novel placement tests, the familiarization phase and the recognition testing trials were video-recorded for subsequent analysis. Object exploration during the sessions was defined as time spent sniffing the object when the nose was oriented toward the object at a distance ≥ 1 cm. Exploration of each object (in seconds) during sessions was scored by an experimenter blind to experimental group. Recognition memory was defined as spending significantly more time on the novel object or novel location than the familiar object or familiar location. A discrimination index (DI) was calculated as follows: $[\text{time exploring the novel object or novel location} - \text{time exploring the familiar object or familiar location}] / [(\text{time exploring both objects}) \times 100]$. N=9 WT receiving AAV-Cre (4 male, 5 female); N=11 GSK3 $\beta^{\text{lox/lox}}$ receiving AAV-Cre (3 male, 8 female).

Immunofluorescence

Following behavioral testing, all mice were transcardially perfused (4% paraformaldehyde) and brains were post-fixed for twenty-four hours. Vibratome sections (40 μm) spanning the entire ventral hippocampus were then incubated in goat anti-GFP (1:500, ab6673 Abcam, Cambridge, MA) and rabbit anti-GSK3 β (1:1000, D5C5Z XP mAb #12456, Cell Signaling, Danvers, MA) primary antibodies for 24 hours at 4°C. The sections were rinsed three times in 0.1M phosphate-buffered saline (PBS) and incubated with donkey anti-goat (1:500, Invitrogen Alexa Fluor 488 Carlsbad, CA) and donkey anti-rabbit (1:500, Invitrogen Alexa Fluor 594) secondary antibodies for 2 hours at room temperature. Immunofluorescence controls consisted of sections in which the primary antibody was omitted. Co-labeling of GFP and GSK3 β was visualized to confirm spatial distribution of AAV-infected cells in ventral hippocampus. To quantify the changes in GSK3 β immunostaining, slides were quantified using a Nikon epi-fluorescent microscope with an analog camera and a quantification software system (Bioquant TCW 98, Nashville, TN). The assessment and analysis of the data were carried out as described previously (Miller et al., 2014). Briefly, analyses occurred on every 3rd section between 3–3.8 mm posterior to Bregma (80 μm between sections) for a total of six sections per region per animal. For GSK3 β quantification, the bilateral ventral hippocampus was outlined using the irregular region of interest (ROI) of the Bioquant software and staining intensity was measured for the outlined area (20X magnification). Threshold values were selected based on mean levels of background immunostaining from wild-type mouse tissue (6 sections $\times$ 6 mice) exposed to secondary antibody in absence of primary. The threshold values were stored in the computer program for consistent auto-measurement of the immunostained slides. Camera settings were maintained at a constant level for each acquired image. Positive immunostaining signal was defined as area of ROI containing pixels above the defined background threshold. In sections that contain both dorsal and ventral hippocampus the ventral hippocampus was defined as occurring below 3.25 mm on the dorsal-ventral scale (adapted from Barr et al., 2010). Staining intensity per section was normalized to the mean value of the wild-type control group. Group means plus standard error of the means were plotted against genotype. Differences in immunoreactivity between groups were analyzed by a one way ANOVA with

the level of significance set at $p < 0.05$ (GraphPad Prism 5, La Jolla, CA). Brains from an additional 8 mice, 4 WT and 4 GSK3 β lox/lox receiving AAV-Cre-GFP, were obtained and processed four weeks following AAV-Cre-GFP injection and sagittally sectioned to visualize viral spread.

Statistics

Statistical tests were completed using either Prism 8 (GraphPad Prism, La Jolla, CA) or SigmaPlot v12.5 (Systat Software Inc., San Jose, CA, USA) with alpha level set at 0.05 throughout. Data were analyzed using a two-tailed Student t test, one-way analysis of variance (ANOVA) or two-way ANOVA followed by Bonferroni test for multiple comparisons as required by study design. Data were tested and found to be normally distributed with equal variances.

Results

Knockdown of GSK3 β in the ventral hippocampus following AAV-GFP-Cre injections

We examined the efficiency of AAV-Cre knockdown of GSK3 β by injecting the viral vector into the ventral hippocampus of adult WT (Figure 2A–D) and homozygous GSK3 β floxed mice (Figure 2E–H). To verify the knockdown of GSK3 β in the ventral hippocampus, brains were sectioned and immuno-stained for Green Fluorescence Protein (GFP) and GSK3 β . Expression of GFP was detected within the ventral hippocampus 28 days post intra-ventral hippocampal injection of AAV-GFP-Cre. Fluorescence imaging on brain slices from GSK3 β floxed mice showed that GFP expressing cells overlapped with GSK3 β knockdown (Figure 2G) compared to WT mice (Figure 2C). Quantification of immunostaining following behavioral testing determined that AAV-Cre reduced the expression of GSK3 β in homozygous GSK3 β floxed mice but not wild-type mice 28 days post injection (Figure 3). Ventral hippocampal sections from the brains of homozygous GSK3 β floxed mice showed significantly lower levels of GSK3 β in dentate gyrus compared to wild-type controls injected with AAV-GFP-Cre or GSK3 β floxed mice injected with AAV-GFP ($F(2, 49) = 3.071.0$, $P < 0.001$, $\eta^2 = 0.9194$; Figure 3). GSK3 β levels were reduced overall by 52–74 percent (mean = 64.82%). Level of knockdown were similar between mice tested for cocaine place preference (65.03%) and morphine place preference (64.7%) ($t(18) = 0.1330$, $p = 0.8957$). Thus, AAV-Cre efficiently reduced GSK3 β levels in vivo.

Knockdown of ventral hippocampal GSK3 β attenuated the development of cocaine, but not morphine conditioned place preference

The role of GSK3 β activity in the ventral hippocampus in the development of cocaine conditioned place preference was evaluated 28 days following injection of AAV-GFP-Cre into wild-type and GSK3 β lox/lox mice or control vector (AAV-GFP) into GSK3 β lox/lox (experimental time-line shown in Figure 1A). One-way ANOVA of the place preference scores shown in Figure 4A revealed significant group effects ($F(2, 28) = 10.96$, $P = 0.0003$, $\eta^2 = 0.4392$). GSK3 β lox/lox mice injected with AAV-GFP-Cre into the ventral hippocampus showed lower preference scores as compared to GSK3 β lox/lox mice injected with AAV-GFP or wild-type mice injected with AAV-GFP-Cre ($P < 0.05$). Further analysis demonstrated that the Cre+WT and GSK3 β lox/lox (no Cre) mice showed a significant place preference (Cre

+WT: $t(9) = 9.693$, $p < 0.0001$; GSK3 $\beta^{lox/lox}$: $t(11) = 6.094$, $p < 0.0001$; two-tailed one-sample t -test), whereas the Cre+GSK3 $\beta^{lox/lox}$ did not ($t(8) = 0.3716$, $p = 0.7198$). Thus, knockdown of GSK3 β in the ventral hippocampus during cocaine conditioning prevented the development of a significant cocaine conditioned place preference. Further, the results demonstrate that the phenotype of GSK3 $\beta^{lox/lox}$ transgenic mice in the absence of AAV-Cre is similar to wild-type mice in development of cocaine place preference. These data were expected because previous studies show no impact of the loxP site insertion in this mouse line on expression levels of GSK3 β protein and several behavioral endpoints (e.g., psychostimulant-induced hyperlocomotion, tests for anxiety and metabolic profiles (Patel et al., 2008; Urs et al., 2012; Liu et al., 2010). Therefore, to minimize animal numbers, this group was excluded in subsequent experiments.

To test if ventral hippocampal GSK3 β activity is necessary for the conditioned rewarding effects of opioids, morphine (5 mg/kg) conditioned place preference was performed 28 days following injection of AAV-Cre into the ventral hippocampus of wild-type and GSK3 $\beta^{lox/lox}$ mice. Preference scores were not significantly different between genotypes that received AAV-GFP-Cre prior to morphine conditioning (Figure 4B, t -test, $P = 0.4654$, $\eta^2 = 0.03176$). Both the Cre+WT and Cre+GSK3 β mice showed a significant place preference (Cre+WT: $t(8) = 3.631$, $p = 0.0067$; GSK3 β flx+Cre $t(9) = 3.219$, $p = 0.0105$; two-tailed one-sample t -test). Thus, downregulation of GSK3 β in the ventral hippocampus did not affect the development of morphine place preference.

Knockdown of ventral hippocampal GSK3 β diminished object location memory but did not affect novel object recognition.

To assess if GSK3 β in the ventral hippocampus is required for memory of objects or location, the novel object recognition test and the object location memory test were performed. During the familiarization phase of the novel object recognition test, both groups of mice had similar exploratory time for both objects: (GSK3 $\beta^{lox/lox}$ mice ($N=9$) left object = 20.2 ± 4.1 , right object = 24.55 ± 5.3 ; wild-type mice ($N=11$) left object = 15.6 ± 2.8 , right object = 18.1 ± 2.7 ; seconds mean $\pm$ SEM). During the test phase of the novel object recognition test, ANOVA showed no interaction ($F(1, 36) = 0.09870$, $P = 0.7552$), no effect of genotype ($F(1, 36) = 0.1357$, $P = 0.7148$) and a significant effect of object exploration ($F(1, 36) = 26.03$, $P < 0.0001$) (Figure 5A). Mice of both genotypes preferentially explored the novel object ($P < 0.05$). There were no significant differences in the discrimination ratios between wild-type and GSK3 β -floxed mice (Figure 5B, t -test, $P = 0.5823$, $\eta^2 = 0.01713$). Both groups of mice spent more time exploring the novel object as represented by the positive discrimination ratios.

During the familiarization phase of the object location test, both groups of mice had similar exploratory time for both objects (GSK3 $\beta^{lox/lox}$ mice - left object = 18.5 ± 4.3 , right object = 17 ± 2.9 ; wild-type mice - left object = 19.7 ± 4.2 , right object = 16 ± 1.9 (sec, mean $\pm$ SEM)). During the test phase, exploration time of the two objects was determined (Figure 6). ANOVA revealed no interaction ($F(1, 36) = 0.7956$, $P = 0.3783$), no effect of genotype ($F(1, 36) = 0.0027$, $P = 0.9582$) and a significant effect of object placement ($F(1, 36) = 11.15$, $P = 0.0020$). There was a significant difference between the discrimination ratios of wild-type

and GSK3 $\beta^{lox/lox}$ mice (t -test, $P=0.0339$, $\eta^2=0.2265$) with GSK3 $\beta^{lox/lox}$ mice showing a significantly lower discrimination ratio (Figure 6B).

Discussion

We have demonstrated previously that acute administration of cocaine activates GSK3 β and that systemic administration of the selective GSK3 inhibitor SB 216763 prior to cocaine conditioning sessions blocked the development of cocaine place preference (Miller et al., 2014). The results of the present study indicate that GSK3 β signaling in the ventral hippocampus plays a discrete role in the modulation of responses to contextual cues related to cocaine reward and place memory. The development of cocaine conditioned reward, likely involving communication between the ventral hippocampus and nucleus accumbens, relies upon activation of GSK3 β in the ventral hippocampus. This result agrees with the role of the ventral hippocampus in spatial processing associated with reward value information and reward-related decision making (Kirk et al., 2017). However, this same mechanism does not appear to be necessary for the development of morphine-induced place preference.

Repeated drug-context pairings induce a conditioned place preference for both cocaine and morphine. Cocaine and morphine both could potentially activate GSK3 β through dopamine neurotransmission and activation of dopamine receptors in the hippocampus which are involved in the acquisition and reinstatement of morphine and cocaine place preference (Assar et al., 2016; Wang et al., 2019). However, there are differences in the pharmacological and behavioral effects of opiates and psychostimulants. Psychostimulant administration activates GSK3 β by decreasing its inhibitory serine-phosphorylation via Akt inactivation (Miller et al., 2014). Conversely, studies deviate on whether opiates activate or inactivate GSK3 (Li et al., 2010; Polakiewicz et al., 1998; Dobashi et al., 2010; Xie et al., 2010). Cocaine also indirectly increases glutamate transmission via increased activation of D1 dopamine receptors (Pierce et al., 1996b; Wolf et al., 2003; Gao and Wolf, 2007; Dunah and Standaert, 2001; Ladepeche et al., 2013). Conversely, extracellular glutamate in hippocampus is decreased following either acute or chronic administration of morphine (Guo et al., 2005). In addition, activation of mu opioid receptors reduces glutamate transmission via a presynaptic mechanism while postsynaptic NMDA-mediated glutamate transmission is increased via protein kinase C which can also inhibit GSK3 β activity (Chen and Huang, 1991; Martin et al., 1997; Rodriguez-Muñoz et al., 2012; De Montigny et al., 2013). Consequently, cocaine and morphine may differentially impact GSK3 β signaling in part through differential activation of glutamate receptors in hippocampus. Rapid NMDA-induced activation of GSK3 β in ventral hippocampus may play a greater role in formation of cocaine reward-related contextual memories whereas separate signaling pathways, such as extracellular signal-regulated protein kinases (ERK), may be initiated following morphine exposure. The lack of an effect on morphine place preference suggests no impairment in the ability of GSK3 β knockdown mice to form and consolidate contextual memories in general. This agrees with our previous results demonstrating that systemic administration of the GSK3 inhibitor SB216763 attenuates development of cocaine place preference but does not impact contextual fear conditioning (Miller et al., 2014). However, it remains to be determined whether GSK3 β knockdown mice will fail to complete reconsolidation when this memory is reactivated (Kimura et al., 2008; Shi et al., 2014). Indeed, recent evidence

suggests that acquisition of morphine conditioned place preference involves activity of the ERK-CREB pathway but not GSK3 in the ventral hippocampus whereas reinstatement is associated with GSK3 signaling via Akt inhibition in the ventral hippocampus (Wang et al., 2019). These results suggest that GSK3 β signaling is differentially involved in the acquisition and retrieval of morphine contextual memory, whereas GSK3 β signaling is important for both the acquisition of cocaine-induced place preference and retrieval of cocaine-associated contextual memories (Miller et al., 2014, Shi et al., 2014).

The role of ventral hippocampal GSK3 β signaling in non-reward and non-conditioned memory was investigated using the novel object recognition and object location memory tests. Novel object recognition depends upon the dorsal hippocampus when a delay greater than 10 min occurs between the sample and test session (Cohen and Stackman, 2015). In the present study, a hippocampal-dependent test was used with a delay of thirty minutes between familiarization and test sessions. Constitutively active GSK3 β has been shown to impair novel object recognition, as well as other hippocampal-dependent cognitive tasks such as spatial navigation and inhibitory avoidance (Hernandez et al., 2002; Dewachter et al., 2009; Pardo et al., 2015; 2016) indicating that excessive GSK3 β activity impairs spatial learning and memory. Likewise, inhibitory phosphorylation of GSK3 β improves memory in cognitive disorders (Hong et al. 2012; Llorens-Martin et al. 2013). In the present study, knockdown of GSK3 β in the ventral hippocampus had no effect on performance in the novel object recognition task. This result indicates that object memory is independent of GSK3 β activity in ventral hippocampus or depends more upon other regions such as dorsal hippocampus or perirhinal cortex. In contrast, knockdown of GSK3 β in the ventral hippocampus reduced object location memory, a hippocampus-dependent memory.

The influence of the hippocampus on the performance of these memory tasks may depend on the modulation by the hippocampus of target regions involved in short-term memory. Performance on both memory tasks is influenced by dopamine transmission in the nucleus accumbens. For instance, dopaminergic lesions of the nucleus accumbens core specifically impairs object recognition, whereas lesion of the shell of the nucleus accumbens impairs novel object location memory, suggesting differential regulation of these tasks within the accumbens (Nelson et al., 2010). While the dorsal and ventral hippocampus both influence accumbal dopaminergic activity indirectly via multi-synaptic projections to the ventral tegmental area (VTA) (Floresco et al., 2001; Luo et al., 2011; Kahn and Shohamy, 2013), the dorsal and ventral hippocampus have different direct innervations of the nucleus accumbens (Floresco, 2014; Pecina and Berridge, 2013). The ventral hippocampus projects to the medial shell of the nucleus accumbens whereas the dorsal hippocampus projects to the core and more lateral accumbens (Strange et al., 2014; Groenewegen et al., 1987; Sesack and Pickel, 1990). Therefore, knockdown of GSK3 β in the ventral hippocampus may have altered ventral hippocampal regulation of dopaminergic transmission in the nucleus accumbens shell which is poised to have a greater effect on the object location task, as lesions of the nucleus accumbens shell region impairs novel object location memory but not object recognition (Nelson et al., 2010). Indeed, while no significant differences were found previously in the discrimination ratios in the object location memory test between control and mice with GSK3 β reduction in the dorsal hippocampus (Chew et al., 2015), the current findings demonstrate an impairment in object location memory following knockdown of

GSK3 β in the ventral hippocampus. This may indicate that reduction of GSK3 β alters ventral hippocampal regulation of dopamine in the nucleus accumbens shell. Future studies will determine this possibility. We have demonstrated previously that repeated cocaine administration increases NMDA-mediated ventral hippocampal-stimulated dopamine efflux in the nucleus accumbens of rats and produces an associated reduction in ventral hippocampal NR2A:NR2B subunit ratio that suggests increased NR2B-containing NMDA receptors (Barr et al., 2014). Previous reports have connected NMDA receptor signaling to dephosphorylation of phospho-Ser9-GSK3 β , indicating that GSK3 β is activated by NMDA receptor signaling (De sarno et al., 2006). In fact, cocaine-induced activation of GSK3 β is attenuated by NMDA receptor antagonists in the striatum (Miller et al., 2014). NMDA-triggered GSK3 β activation is mediated by NMDAR that contain the NR2B subunit (Szatmari et al., 2005; Tackenberg et al., 2013). Increased activation of GSK3 β by NMDAR in the ventral hippocampus may be involved in greater evoked accumbal dopamine release following repeated cocaine administration. The inability of cocaine to activate GSK3 β via NMDA and subsequent dopamine release in the accumbens shell may underlie the observed impairment of the object location memory task and cocaine-induced place preference behavior in the GSK3 β floxed mice.

No differences were observed between male and female on any of the endpoints measured in this study. However, the current study was not designed and therefore not adequately powered to detect sex differences. Relevantly, estrogen has been shown to regulate GSK3 activity in the hippocampus (Cardona-Gomez et al., 2004) and sex differences in the mechanisms underlying memory formation have been reported (Mizuno and Giese, 2010). Therefore, future studies will need to determine whether there are distinct sex specific mechanisms of memory formation involving drug-reward contextual memory formation. Future studies should also parse the role of ventral hippocampal GSK3 β in the formation of long-term memory through consolidation and updating reactivated memory via re-consolidation in drug reward-context memories and whether different sub-regions or cell-types are responsible for the observed effects.

In summary, these data point to the engagement of the GSK3 β pathway in the development of cocaine conditioned place preference over development of place preference for morphine. Furthermore, location memory was found to be contingent on ventral hippocampal GSK3 β while novel object recognition was not. Taken together, the results highlight the importance of GSK3 β signaling in the cocaine-reward memory and suggest that regulation of hippocampal GSK3 β signaling is important in spatial memory but not incentive motivation in general or non-spatial memory formation.

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Highlights:

- Knockdown of GSK3 β in the ventral hippocampus diminished acquisition of cocaine conditioned place preference.
- Acquisition of place preference for morphine was not altered by knockdown of ventral hippocampal GSK3 β
- Object location memory but not novel object recognition was impaired by knockdown of ventral hippocampal GSK3 β

Figure 1: Timelines of experimental procedures.

(A) Wild-type (Cre+WT) and $GSK3\beta^{lox/lox}$ mice were injected with AAV-Cre-GFP (Cre + $GSK3\beta^{lox/lox}$), and $GSK3\beta^{lox/lox}$ mice were injected with AAV-GFP ($GSK3\beta^{lox/lox}$) bilaterally into the ventral hippocampus on Day 1. Mice underwent place conditioning with cocaine (10 mg/kg ip) and saline using a 4-day conditioning procedure. On test day (day 32), preference scores were determined during 30 min test session. Following behavioral tests, the brains of all mice were analyzed for efficiency of GSK3 knockdown. (B) Wild-type (Cre + WT) and $GSK3\beta^{lox/lox}$ mice were injected with AAV-Cre-GFP (Cre + $GSK3\beta^{lox/lox}$) bilaterally into the ventral hippocampus on Day 1. Mice underwent place conditioning with morphine (5 mg/kg sc.) or saline using a 4-day conditioning procedure. On test day (day 32), preference scores were determined during 20 min test session. Novel-object recognition (NORT) and object location memory (OLM) tests were carried out with a one-week interval between each of the tests. Following behavioral tests, mouse brains were analyzed for efficiency of GSK3 knockdown.

Figure 2: Efficiency of viral knock down of ventral hippocampal GSK3 β .

Representative images from a WT (left) and GSK3 $\beta^{lox/lox}$ (right) mouse injected with AAV-Cre-GFP showing infection (green=GFP) in ventral hippocampus (A, E). Expression of GFP was restricted to the hippocampus. DAPI staining (B, F). The ventral hippocampus of GSK3 $\beta^{lox/lox}$ mice with AAV-Cre infusion shows reduced levels of GSK3 β (red) as compared with GSK3 β immunoreactivity in wild-type mice with AAV-Cre infusions (C, G). Merged image of GFP, GSK3 β , and DAPI staining (D, H).

Figure 3: Efficiency of viral knock down of ventral hippocampal GSK3β.

Representative images (60X) from a WT (top) and GSK3β^{lox/lox} (bottom) mouse injected with AAV-Cre-GFP showing infection (green=GFP) in ventral hippocampus (**A, D**). DAPI staining (**B, E**). The ventral hippocampus of GSK3β^{lox/lox} mice with AAV-Cre infusion shows reduced levels of GSK3β (red) as compared with GSK3β immunoreactivity in wild-type mice with AAV-Cre infusions (**C, F**). Quantification of GSK3β fluorescence in the ventral hippocampus of wild-type (Cre+WT, N=19) and GSK3β^{lox/lox} mice injected with AAV-Cre-GFP (Cre+GSK3β^{lox/lox}, N=20) or AAV-GFP (GSK3β^{lox/lox}, N=12). (**G**). Following behavioral testing, all mouse brains were analyzed for GSK3β immunoreactivity. Data are represented as means ± SEM. * =significantly different from Cre+WT and GSK3β^{lox/lox}.

Figure 4: Knockdown of GSK3 β in the ventral hippocampus prevented the development of cocaine but not morphine-induced conditioned place preference.

(A) Wild-type mice injected with AAV-Cre (Cre+WT) and GSK3 $\beta^{lox/lox}$ mice injected with AAV-GFP (GSK3 $\beta^{lox/lox}$) showed significantly greater cocaine-induced place preference scores compared to GSK3 $\beta^{lox/lox}$ mice injected with AAV-Cre-GFP (Cre+GSK3 $\beta^{lox/lox}$). (N = 10 Cre+WT; N = 9 Cre+GSK3 $\beta^{lox/lox}$; N = 12 GSK3 $\beta^{lox/lox}$). (B) Cre+WT and Cre+GSK3 $\beta^{lox/lox}$ mice conditioned with morphine showed similar place preference scores. (N = 9 Cre+WT; N = 11 Cre+GSK3 $\beta^{lox/lox}$). Data are represented as means $\pm$ SEM of preference scores in seconds (time spent on drug-conditioned side minus time spent on saline-conditioned side) on left y-axis and as % time in drug compartment on right y-axis. * = significantly different from Cre+WT and GSK3 $\beta^{lox/lox}$ groups (*p<0.05).

Figure 5: Effect of knockdown of GSK3β in the ventral hippocampus on novel-object recognition.

Wild-type mice injected with AAV-Cre (Cre+WT) and GSK3β^{lox/lox} mice injected with AAV-Cre (Cre+ GSK3β^{lox/lox}) into the ventral hippocampus spent more time exploring the novel object than the familiar object. **(A)** Time spent exploring each object during the test phase was not different between genotypes. **(B)** Discrimination index in the test phase was also not different between groups. The discrimination index (DI) was calculated by dividing the difference between the time spent exploring objects by the total time exploring the objects $\times 100$. Data are expressed as means $\pm$ SEM. * = significantly different from time spent exploring the familiar object (*p<0.05). N=9 Cre+WT; N=11 Cre+GSK3β^{lox/lox}.

Figure 6: Effect of knockdown of GSK3β in the ventral hippocampus on object-location memory. (A) Time spent (in seconds) exploring the familiar and novel locations for wild-type mice injected with AAV-Cre (Cre+WT) and GSK3β^{lox/lox} mice injected with AAV-Cre (Cre+GSK3β^{lox/lox}) into the ventral hippocampus. (B) Discrimination index in the test phase was significantly lower for GSK3β^{lox/lox} mice injected with AAV-Cre in the ventral hippocampus than for wild-type mice. *= significantly different from Cre+WT. The discrimination index (DI) was calculated by dividing the difference between the time spent exploring locations by the total time exploring both locations $\times 100$. (* $p < 0.05$). Data are expressed as means $\pm$ SEM. N=9 Cre+WT; N=11 Cre+GSK3β^{lox/lox}.