Published in final edited form as:

Neuropharmacology. 2018 October ; 141: 126–138. doi:10.1016/j.neuropharm.2018.08.031.

Morphine regulates adult neurogenesis and contextual memory extinction via the PKC ϵ /Prox1 pathway

Wenxiang Fan¹, Helei Wang¹, Yue Zhang², Horace H. Loh², Ping-Yee Law², and Chi Xu^{1,*}

¹State Key Laboratory of Natural Medicines, School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing, Jiangsu, 210009, People's Republic of China

²Department of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455

Abstract

We have previously reported that the miR-181a/Prox1/Notch1 pathway mediates the effect of morphine on modulating lineage-specific differentiation of adult neural stem/progenitor cells (NSPCs) via a PKC ϵ -dependent pathway, whereas fentanyl shows no such effect. However, the role of the PKC ϵ /Prox1 pathway in mediating drug-associated contextual memory remains unknown. The current study investigated the effect of PKC ϵ /Prox1 on morphine-induced inhibition of adult neurogenesis and drug-associated contextual memory in mice, while the effect of fentanyl was tested simultaneously. By using BrdU labeling, we were able to examine the lineages of differentiated NSPCs in adult DG. PKC ϵ knockout blocked morphine's effects on inducing in vivo astrocyte-preferential differentiation of NSPCs, but did not alter NSPC lineages upon fentanyl treatment. Inhibited adult neurogenesis further resulted in prolonged extinction and enhanced reinstatement of morphine-induced CPP, as well as prolonged extinction of space reference memory indicated by the Morris water maze paradigm. However, after fentanyl administration, no significant changes were found between wild-type and PKC ϵ knockout mice, during either CPP or water maze tasks. When the lentivirus encoding *Nestin*-promoter-controlled *Prox1* cDNA was injected into hippocampi of wildtype and PKC ϵ knockout adult mice to modulate PKC ϵ /Prox1 activity, similar effects were discovered in adult mice injected with lentivirus encoding Prox1, and more dramatic effects were found in PKC ϵ knockout mice with concurrent Prox1 overexpression. In conclusion, morphine mediates lineage-specific NSPC differentiation, inhibits adult neurogenesis and regulates contextual memory retention via the PKC ϵ /Prox1 pathway, which are implicated in the eventual context-associated relapse.

Abstract

*Correspondence to: Chi Xu, PhD., School of Basic Medicine and Clinical Pharmacy, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing, Jiangsu, 210009, People's Republic of China. xuchi@cpu.edu.cn.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

neural stem/progenitor cells; PKCε; Prox1; adult neurogenesis; contextual memory; hippocampus

1. Introduction

The association between drug addiction and maladaptive memories has been widely recognized. A series of addictive drugs, including opioids (Kibaly et al., 2016; Zhang et al., 2016; Zheng et al., 2013), cocaine (Kramar et al., 2014), cannabinoids (Steinmetz and Freeman, 2016) and amphetamine (Marchese et al., 2016), have been reported to not only impair normal functions of learning and memory, but also give rise to the development of strong but aberrant drug memories that contribute to the transition from initial drug exposure to drug dependency (Kutlu and Gould, 2016). The memory of drug addiction is mediated by a number of relatively independent neural systems, such as the amygdala, the dorsal striatum and the hippocampus (Goodman and Packard, 2016). The hippocampus, in particular, plays an essential role in declarative memory, and is therefore a most important region associated with the formation of spatial and contextual memory (Yonelinas, 2013), which may further contribute to the development of maladaptive drug-context associations (Kutlu and Gould, 2016). Recent studies have supported the crucial role of hippocampal dentate gyrus (DG) in drug acquisition, reward and relapse. For instance, NeuroD expression in the DG is responsible for opioid-induced regulation of contextual memory extinction (Zheng et al., 2013). More recently, it was found that Orexin A-mediated signaling in DG promotes the acquisition and maintenance of morphine-induced conditioned place preference (CPP) (Guo et al., 2016). Thus, the hippocampal DG is critical for the association between drug experience and reward.

The crucial role of DG in drug memories and other cognitive functions and disorders is further confirmed by studies of adult neurogenesis, a robust form of neural plasticity observed throughout adulthood (Apple et al., 2017; Goncalves et al., 2016; Kempermann et al., 2015). During adult neurogenesis, neural stem/progenitor cells (NSPCs) in the

subgranular zone (SGZ) of DG proliferate and differentiate into new-born neurons and glial cells via asymmetric cell division (Abrous et al., 2005; Apple et al., 2017; Goncalves et al., 2016). A number of addictive drugs, such as opioids, cocaine, cannabinoids and alcohol, have been shown to regulate adult neurogenesis via a variety of different mechanisms, including the proliferation, differentiation and survival of NSPCs and their offspring (Xu et al., 2016). For example, morphine modulates neuronal differentiation of adult NSPCs in mouse DG via a mechanism involving NeuroD1, which further regulates contextual memory extinction (Zhang et al., 2016). While cocaine, cannabinoids and alcohol were reported to regulate adult neurogenesis via either inhibiting NSPC proliferation or enhancing its apoptosis (Xu et al., 2016).

Despite the various mechanisms that mediate adult hippocampal neurogenesis by addictive drugs, there are common pathways regulated by a series of crucial signaling molecules that lead to NSPC regulation (Xu et al., 2016). These molecules include GPCRs, protein kinases, along with transcription factors and regulators that are essential to NSPC proliferation and differentiation. For example, members of the protein kinase C (PKC) family regulates adult neurogenesis, not only by mediating NF- κ B activation (Bortolotto et al., 2014), but also by activating the CREB-binding protein (CBP) (Wang et al., 2012). Prospero homeobox protein 1 (Prox1), a transcription factor necessary for NSPC proliferation and differentiation, is recently recognized as a key cross-talk point between the diverse signaling pathways that regulate adult neurogenesis, thus representing a central node in NSPC fate machinery (Lavado et al., 2010; Stergiopoulos et al., 2014). Therefore, by thoroughly studying the roles of the crucial signaling molecules including PKC and Prox1, the mechanisms adopted by addictive drugs in controlling hippocampal functions, from adult neurogenesis to contextual memory, can be elucidated.

CPP and Morris water maze (MWM) are two behavioral paradigms that are widely used to assess the effects of addictive drugs. CPP is frequently conducted in rodents to examine not only the abuse liability, but also neurochemical and neuropharmacological mechanisms and consequences underlying the action of clinical used drugs of abused (Nader, 2016). In the past decade, the CPP paradigm has also been used to determine crucial factors, either environmental or social, that influence vulnerability to drug abuse, thus supporting the clinical studies of drug addiction (Trezza et al., 2010; Watanabe, 2011). MWM is an extensively used paradigm for investigating spatial learning and memory, due to the relationship between performance in the MWM and drug effects (Vorhees and Williams, 2006). In the context of drug addiction, MWM performance is an essential index of clinical effects of addictive drugs, such as morphine, heroin and cocaine, on alternation of spatial memory (Kibaly et al., 2016; Mendez et al., 2008; Tramullas et al., 2008; Zheng et al., 2013). Therefore, in the present study, we used CPP and MWM as two major behavioral paradigms to assess the effects of morphine and fentanyl, and to reveal the underlying mechanisms.

Previously, we have focused on the effects of two μ -opioid receptor (OPRM1) agonists, morphine and fentanyl, on both NSPC differentiation and neurogenesis in the DG of adult mice, and demonstrated that morphine, but not fentanyl, was capable of inhibiting adult neurogenesis by facilitating astrocyte-preferential differentiation NSPCs (Xu et al., 2014).

This effect was mediated by the miR-181a/Prox1/Notch1 pathway, which is a result of the different mechanisms of the two agonists in ERK activation. Morphine activates ERK via the PKC ϵ -dependent pathway, thus leading to the phosphorylation of TAR RNA-binding protein (TRBP) and stabilization of the TRBP/Dicer complex, which in turn contributes to the maturation of miR-181a-5p. On the other hand, fentanyl activates ERK via the β -arrestin-dependent pathway, and therefore shows no effect on lineage-specific differentiation of NSPCs (Xu et al., 2015). The critical roles of PKC ϵ and Prox1 in mediating morphine's effect on NSPC fate determination suggest their involvement in drug behaviors of addicted animals. To test this hypothesis, we used CPP paradigm to assess the acquisition, extinction and reinstatement of drug behavior, and MWM to examine the spatial learning of mice. Also, in correspondence with previous in vitro studies, we compared the in vivo effects of both morphine and fentanyl in signaling molecule expression, NSPC differentiation and behavioral tasks.

2. Materials and methods

2.1 Animals

Adult C57BL/6J wild type, PKC ϵ KO and heterozygous mice were bred from pairs of hybrid (50% C57BL/6, 50% 129S4) mice heterozygous for the mutant PKC ϵ gene as previously described (Khasar et al., 1999; Schuster et al., 2013), and were gifts from Dr. George Wilcox. PKC ϵ KO and wildtype littermates were used in individual experiments. All mice were maintained on a 12 h light/dark cycle with food and water available *ad libitum*. Experiments were conducted on eight-week-old male mice (30-35 g). Animal maintenance and procedures were conducted according to the National Institutes of Health guidelines and the Institutional Animal Care and Use Committee (IAUCUC) policies at the University of Minnesota.

2.2 Quantitative Reverse Transcription PCR (qRT-PCR)

The total RNAs were extracted and reverse transcribed with the miScript system (Qiagen, Germany). qRT-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 qRT-PCR assays are listed in the supporting information Table S2.

2.3 Western-blot assay

The western-blot assay was performed as described previously (Kibaly et al., 2016). Briefly, hippocampal protein samples of 8-week-old adult male mice were immunoblotted with antibodies listed in Table S1. Chemifluorescence was detected by using the ECF Reagent (GE Healthcare, UK) and the fluorescence intensity was measured with Storm 860 Molecular Imager (GE Healthcare). The intensity of individual bands was determined with ImageQuant software (GE Healthcare).

2.4 Lentivirus construction

A Lentiviral construct expressing *Prox1* cDNA was prepared as described previously (Zhang et al., 2016; Zheng et al., 2010b), with some modifications. The lentiviral vector pLenti-Nestin was a gift from Dr. Hui Zheng (Li et al., 2014), and the Prox1 cDNA was inserted

into the multiple cloning site downstream of the *Nestin* promoter. A corresponding negative control construct was obtained from University of Minnesota Genomics Center. Virus constructs were assembled in HEK293FT cells using the BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen, Grand Island, NY). Viral titers were determined in primary NSPCs.

2.5 Stereotaxic surgery

The lentivirus was injected by stereotaxic surgery as described previously (Zhang et al., 2016; Zheng et al., 2013). Mice were anesthetized with isoflurane inhalation and a hole of 0.5 mm was drilled (AP: -1.95 mm; ML: ± 1.00 mm, DV: -2.00 mm). Virus microinjection was made through a 28-gauge needle connected to a flexible tube filled with virus. 2 μ l of virus was infused using a micro-syringe pump controller, at a constant rate of 0.2 μ l/min.

2.6 Bromodeoxyuridine (BrdU) labeling and immunohistochemistry

BrdU labeling and immunohistochemistry were performed as previously described (Xu et al., 2014; Zhang et al., 2016), with slight modifications. Briefly, mice were habituated for one day (day 0) and injected with BrdU (200 mg/kg, i.p.) for three consecutive days (day 1-3) and experienced CPP training for four days (day 4-7). Mice were sacrificed after CPP training (day 8) (Fig. 3A), deeply anesthetized with pentobarbital sodium, and perfused with 0.9% saline followed by 4% paraformaldehyde. 30 μ m coronal sections were cut through the dentate gyrus of the hippocampus (-0.82 to -3.70 mm post bregma) with a cryostat and every eighth section across the hippocampus was stained with antibodies listed in Table S1. Sections were mounted on slides with DAPI Fluormount G (SouthernBiotech, Birmingham, AL) and visualized using a CCD camera connected to a Leica DM5500 B upright microscope (Leica, Germany).

2.7 Cell quantification

To quantify the total number of fluorescent-positive cells in the DG, every 8th sections was mounted on the same slide, and the images of every section on the same slide throughout the dentate were captured (8-10 sections in total). The number of BrdU⁺DCX⁺ and BrdU⁺GFAP⁺ double positive cells from each section were counted by using the “Cell Counter” plugin of the ImageJ software (NIH, MA), which outputs the counter results of each marker in each section. The results of all sections were added together to represent the total number of cells of one individual sample.

2.8 Conditioned place preference (CPP)

The CPP paradigm was carried out in a three-chamber apparatus supplied by Panlab (Harvard Apparatus, Holliston, MA) according to the unbiased CPP protocol (Cunningham et al., 2006) and modified as previously described (Zhang et al., 2016). 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, the 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 rejected from the subsequent study, whereas the rest were trained with saline or morphine for four consecutive days (day 4-7), received a saline injection subcutaneously in

the morning before spending 30 min in one chamber (black or white, randomly chosen), and then received either 5 mg/kg morphine or an equal volume of saline injection (s.c.) in the afternoon, before spending 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 every 7 days until no significant preference was detected in two continuous tests (Fig. 3A). ANY-maze software (Stoelting, Wood Dale, IL) was used to analyze the behavior of mice in the apparatus. The CPP score of each individual mouse was calculated by subtracting the pre-conditioning preference score (time spent in the drug-paired chamber before training) from the post-conditioning preference score (time spent in the drug-paired chamber after training).

2.9 Morris water maze (MWM)

The MWM task was carried out as previously reported (Vorhees and Williams, 2006). In the acquisition session, mice were trained for 5 days in the water maze (122-cm diameter) with the platform (10-cm diameter) submerged in the SW quadrant. ANY-maze software was used to analyze the behavior of mice. Probe tests were performed every 4 days until the mice spent an equal amount of time in each quadrant.

2.10 Statistical analysis

Data represent mean $\pm$ SEM of at least three separate experiments (non-behavioral tests) or eight individual animals (behavioral tests). Statistical differences between values from two groups were determined using unpaired Student's *t* tests, whereas statistical differences between three or more groups were analyzed using analyses of variance (ANOVAs) followed by Newman-Keuls post hoc comparisons. Data of three- and four-way ANOVAs were analyzed using SPSS 24 (IBM, NY), and all other statistical and curve-fitting analyses were performed using Prism 7.0 (Graphpad Software, La Jolla, CA). A difference of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Morphine modulates *in vivo* expression of crucial molecules in NSPC lineage determination via a PKC ϵ -mediated pathway.

In our earlier studies, we have demonstrated that morphine was able to induce cell lineage changes during NSPC differentiation by modulating the expression of a series of crucial regulators and markers, including Tuj1, GFAP, Prox1, Notch1 and miR-181a-5p, during NSPC differentiation, whereas fentanyl had no such effects (Xu et al., 2014; Xu et al., 2015). However, these results were not thoroughly tested by *in vivo* studies. Therefore, in the current work, we first examined whether the *in vivo* expression of these molecules in mouse hippocampus, on both mRNA and protein levels, are differentially regulated by morphine and fentanyl via the same pathway. Doublecortin (DCX) was used instead of Tuj1 to indicate immature neurons, due to its *in vivo* expression of extended periods (Xu et al., 2016). As shown in Fig. 1, in hippocampi of wildtype mice, morphine significantly downregulated both mRNA and protein levels of DCX and Prox1, whereas increased the levels of GFAP, Notch1 and miR-181a-5p. However, these effects of morphine were eliminated in PKC ϵ knockout mice. On the other hand, injection of fentanyl showed no significant effect. These

results indicate a similar mechanism by which morphine regulates in vivo differentiation of hippocampal NSPCs via PKC ϵ -mediated pathway, as those observed in our reported in vitro studies.

3.2 Morphine promotes in vivo astrocyte-preferential differentiation of mouse hippocampal NSPCs via a PKC ϵ -mediated pathway.

Our previous study has shown that morphine, but not fentanyl, promotes astrocyte-preferential differentiation of primary NSPCs via PKC ϵ -mediated ERK phosphorylation (Xu et al., 2015). For further confirmation, we next examined the lineages of differentiated hippocampal NSPCs after injection of morphine and fentanyl, both in wildtype and PKC ϵ knockout mice, by using bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) labeling assay. As shown in Fig. 2A, animals received BrdU injection (200 mg/kg, i.p.) on days 1-3 and a following CPP training with morphine, fentanyl or an equal volume of saline on days 4-7, and the fates of BrdU-positive cells were analyzed by immunohistochemistry (Fig. 2, B and C). In wildtype mice, morphine treatment resulted in a significant decrease in the number of new-born immature (BrdU⁺DCX⁺) neurons and a correspondent increase in astrocyte (BrdU⁺GFAP⁺) population, compared to the saline group. In PKC ϵ knockout mice, however, the numbers of BrdU⁺DCX⁺ and BrdU⁺GFAP⁺ cells restored to similar levels as those of saline-treated mice. Fentanyl showed no significant effect in modulating the NSPC lineage. For clearance, a three-color figure was provided by staining BrdU, DCX and GFAP, which showed similar results (Fig. S1). Together, these results indicate that morphine induces astrocyte-preferential differentiation via PKC ϵ -mediated pathway.

3.3 PKC ϵ knockout results in prolonged extinction phase and enhanced reinstatement of morphine- but not fentanyl-induced CPP.

Since the differentiation of adult hippocampal NSPCs may contribute to the formation of contextual memory, which is in turn related to drug acquisition and relapse (Zhang et al., 2016; Zheng et al., 2013), we therefore infer that morphine and fentanyl may also regulate CPP response in an agonist-selective way, due to their different effects in NSPC differentiation. Wildtype and PKC ϵ knockout mice were trained for 4 consecutive days, during which they received injection of either morphine or fentanyl, and an equal volume of saline (Fig. 3A). As shown in Fig. 3B, although mice of all groups showed no significant difference in CPP acquisition on day 8 (two-way ANOVA, effect of treatment: $F_{1,28}=2.79$, $p>0.05$; effect of genotype: $F_{1,28}=0.31$, $p>0.05$), a dramatic difference between extinction rates of morphine- and fentanyl-induced CPP was discovered in wildtype mice ($F_{1,56}=144.1$, $p<0.001$). In PKC ϵ knockout mice, the extinction rate morphine-induced CPP was significantly decreased ($F_{1,56}=77.73$, $p<0.01$), while the extinction rate of fentanyl-induced CPP remained constant between mice of both genotypes ($F_{1,154}=0.04$, $p>0.05$).

As the reinstatement of extinguished CPP response is a widely-accepted measurement for craving and relapse of addictive drugs (Shalev et al., 2002; Zheng et al., 2013), we tested drug-primed CPP reinstatement of all mice, 7 days after total extinction of each group. As shown in Fig. 3C, in wildtype mice, the reinstatement magnitude of morphine-induced CPP was significantly lower than that of fentanyl-induced CPP, while in PKC ϵ knockout mice, the reinstatement magnitude was significantly increased. Thus, we may conclude that

morphine and fentanyl control drug-induced contextual memory in an agonist-selective manner, and that PKC ϵ plays an essential role in modulating the extinction and reinstatement, but not acquisition, of morphine-induced CPP response.

3.4 Morphine modulates the extinction of spatial reference memory via a PKC ϵ -dependent pathway.

We then used MWM task to determine the effect of morphine and fentanyl, as well as the role of PKC ϵ in regulating overall contextual memory. Wildtype and PKC ϵ knockout mice were subcutaneously injected daily with morphine, fentanyl or an equal volume of saline, for 5 consecutive days, and trained in the water maze apparatus on days 1-5 (Fig. 4A). Three-way ANOVAs on swimming latency and distance to the hidden platform during the training session revealed no significant treatment $\times$ genotype $\times$ day interactions ($F_{8, 210}=0.21$, $p>0.05$; $F_{8, 210}=0.13$, $p>0.05$), and no significant effect of genotype ($F_{1, 210}=0.57$, $p>0.05$; $F_{1, 210}=0.99$, $p>0.05$) or treatment ($F_{2, 210}=1.23$, $p>0.05$; $F_{2, 210}=0.90$, $p>0.05$) (Fig. 4, B and C). The extinction of spatial reference memory was tested until the conditioned response was totally diminished (no significant difference between the target and other quadrants). As shown in Fig. 4, D and E, in wildtype mice, morphine treatment resulted in a significantly higher rate of memory extinction compared to saline and fentanyl (time: $F_{2, 84}=89.88$, $p<0.001$; distance: $F_{2, 84}=76.05$, $p<0.001$). In PKC ϵ knockout mice, however, the spatial reference memory extinction phase was dramatically prolonged (time: $F_{1, 56}=142.00$, $p<0.001$; distance: $F_{3, 56}=123.60$, $p<0.001$). There was no significant difference on memory extinction between saline-, morphine- and fentanyl-treated mice (time: $F_{2, 147}=0.80$, $p>0.05$; distance: $F_{2, 147}=0.70$, $p>0.05$) (Fig. 4, D and E). Fentanyl had no significant effect on the rate of memory extinction in mice of either genotype (time: $F_{3, 196}=1.09$, $p>0.05$; distance: $F_{3, 196}=0.04$, $p>0.05$). These results suggest that morphine, but not fentanyl, affects the retention but not acquisition of spatial reference memory via a PKC ϵ -dependent pathway, which agrees with the conclusion from CPP trials.

3.5 PKC ϵ /Prox1 mediates morphine-induced in vivo lineage-preferential differentiation of mouse hippocampal NSPCs.

Our previous in vitro studies have demonstrated that Prox1 is a major regulator downstream of PKC ϵ , which is down-regulated upon morphine-induced activation of OPRM1 (Xu et al., 2014; Xu et al., 2015). Therefore, we then examined the effect of Prox1 on lineages of NSPCs in mouse DG. In order to exclusively increase the expression of Prox1 in Nestin-expressing hippocampal NSPCs, the lentiviruses that express either the negative control oligonucleotide (control-vir) or *Nestin*-promoter-controlled *Prox1* cDNA (Nestin-Prox1-vir) was stereotaxically injected into hippocampi of both wildtype and PKC ϵ knockout mice. The position of the stereotaxical injection was tested (Fig. S2A) and the expression of Prox1-EGFP delivered by the lentivirus was found in approximately 68% of all Nestin-expressing cells in the hippocampus (Fig. S2B). After the surgery, all mice experienced a resting period of 7 days in their home cages before receiving BrdU injection (Fig. 5A). As shown in Fig. 5B and C, morphine treatment resulted in a significant decrease in BrdU $^{+}$ DCX $^{+}$ cell population and an increase in astrocyte (BrdU $^{+}$ GFAP $^{+}$) population in all wild type mice injected with either control-vir or Nestin-Prox1-vir, compared to saline-treated mice, but had no effect on PKC ϵ knockout mice. On the other hand, Nestin-Prox1-vir facilitated

neuronal differentiation of NSPCs by dramatically increasing immature neuron population and decreasing astrocyte population in mice with both genotypes and treatments (three-way ANOVA, effect of virus: $F_{1, 56}=72.49$, $p<0.001$; $F_{1, 56}=99.45$, $p<0.001$; effect of genotype: $F_{1, 56}=15.17$, $p<0.001$; $F_{1, 56}=21.96$, $p<0.001$; effect of treatment: $F_{1, 56}=28.25$, $p<0.001$; $F_{1, 56}=25.65$, $p<0.001$). Thus, probably, morphine facilitates in vivo astrocyte-preferential differentiation of hippocampal NSPCs via the PKC ϵ /Prox1 pathway.

3.6 PKC ϵ /Prox1 regulates the extinction and reinstatement of morphine-induced contextual memory of adult mice.

Due to the role of Prox1 in mediating adult NSPC differentiation in mouse hippocampus, we further examined its effect on morphine-induced contextual memory. After the stereotaxical injection of control-vir or Nestin-Prox1-vir, all mice were housed in their home cages for 7 days during the recovery period before the CPP paradigm (Fig. 6A). No significant difference was found between various groups in CPP acquisition (two-way ANOVA, effect of virus: $F_{2, 42}=0.80$, $p>0.05$; effect of genotype: $F_{1, 42}=0.09$, $p>0.05$), whereas the injection of Nestin-Prox1-vir dramatically decreased CPP extinction rates of both wildtype ($F_{2, 84}=29.25$, $p<0.001$) and PKC ϵ knockout mice ($F_{2, 189}=17.04$, $p<0.001$) (Fig. 6B). As shown in Fig. 6C, PKC ϵ knockout was able to dramatically increase the magnitude of CPP reinstatement, and the injection of Nestin-Prox1-vir enhanced the reinstatement of both wildtype and PKC ϵ knockout mice (two-way ANOVA, effect of virus: $F_{2, 42}=8.29$, $p<0.001$; effect of genotype: $F_{1, 42}=19.54$, $p<0.001$). Together, these results indicate that the PKC ϵ /Prox1 pathway is crucial to both extinction and reinstatement of morphine-induced contextual memory.

3.7 PKC ϵ /Prox1 regulates morphine-modulated extinction of spatial reference memory in adult mice.

We next examined the role of PKC ϵ /Prox1 pathway in spatial reference memory by using the MWM paradigm. The control-vir and Nestin-Prox1-vir were injected into the hippocampi of all mice (day -13), followed by a 7-day resting period (Fig. 7A). No significant difference in swimming latency was found during the training session (four-way ANOVA, effect of genotype: $F_{1, 280}=0.10$, $p>0.05$; effect of virus: $F_{1, 280}=0.01$, $p>0.05$; effect of treatment: $F_{1, 280}=1.03$, $p>0.05$), indicating no effect of genotype or virus injection on the acquisition of spatial memory (Fig. 7B). During the extinction session, however, both PKC ϵ knockout and Nestin-Prox1-vir injection gave rise to significantly lower rate and prolonged period of spatial memory extinction, either in saline- or morphine-treated mice (four-way ANOVA, effect of genotype: $F_{1, 224}=136.47$, $p<0.001$; $F_{1, 224}=63.51$, $p<0.001$; effect of virus: $F_{1, 224}=314.59$, $p<0.001$; $F_{1, 224}=151.20$, $p<0.001$; effect of treatment: $F_{1, 224}=106.26$, $p<0.001$; $F_{1, 224}=47.04$, $p<0.001$). Moreover, in PKC ϵ knockout mice injected with Nestin-Prox1-vir, an even lower extinction rate compared to the control group (wildtype mice injected with control-vir and treated with saline) was observed (Fig. 7, C and D), suggesting a cooperating mechanism between PKC ϵ knockout and Prox1 overexpression, which was confirmed by the significant genotype $\times$ virus interaction (time: $F_{1, 224}=4.94$, $p<0.05$; distance: $F_{1, 224}=4.77$, $p<0.05$). Data of all groups are shown as track plots during the third probe (day 14), when the discrepancy between groups were most

significant (Fig. 7E). These results indicate an essential role of the PKC ϵ /Prox1 pathway as a major regulator of spatial reference memory extinction in adult mice.

4. Discussion

In this study, we examined the in vivo effects of morphine on adult hippocampal neurogenesis and contextual memory tasks. In combination with our earlier works (Xu et al., 2014; Xu et al., 2015), we have demonstrated in molecular, cellular and behavioral levels that morphine regulates the contextual memory of adult mice by modulating the lineage of NSPC differentiation in the DG, which is dependent on PKC ϵ /Prox1 activity. NSPC differentiation in adult DG mainly contributes to adult neurogenesis, a process during which newborn neurons are generated and incorporate into the existing neural circuitry, and recognized as a robust form of neural plasticity (Aimone et al., 2014; Goncalves et al., 2016; Spalding et al., 2013). During the past decade, it has been demonstrated that adult neurogenesis contributes to learning and memory, and is associated to a number of neurological and psychiatric diseases (Aimone et al., 2014; Goncalves et al., 2016). Thus, it is reasonable to hypothesize that PKC ϵ /Prox1 regulates drug-related contextual memory by mediating adult neurogenesis and the plasticity of neural circuitry in adult DG. In recently reported studies, it was reported that adult neurogenesis is essential to the cocaine addiction circuit (Castilla-Ortega et al., 2016b), thus contributing to cocaine-related maladaptive cognition (Castilla-Ortega et al., 2017) and modifies cocaine-conditioned CPP (Castilla-Ortega et al., 2016a; Noonan et al., 2010). In our current study focusing on opioid receptor agonists, we found that morphine activates PKC ϵ and thereby suppresses Prox1 expression, thus resulting in decreases adult neurogenesis, which in turn gives rise to impaired retention and reinstatement of drug-associated memory. By manipulating PKC ϵ /Prox1 activity via drug administration or lentivirus injection, we were able to modify the retention of contextual memory and relapse of addictive drugs. As adult neurogenesis and drug-associated contextual memory is crucial to psychological dependence in drug addiction (Canales, 2007), our current findings may contribute to novel treatments for drug abuse in the future.

The conclusion of our current study that morphine regulates drug memory retention by inhibiting adult neurogenesis is consistent with previous studies showing that morphine attenuates drug memory retention and reinstatement by inhibiting the differentiation of neural progenitor cells in adult DG (Zheng et al., 2013). This effect of morphine was demonstrated to be a result of its incapability of activating NeuroD1 via regulation of miR-190 (Zheng et al., 2010c; Zheng et al., 2013). In combination with our earlier studies, it is clear that the agonist-selective manner of OPRM1 agonists in regulating adult neurogenesis, and thereby the contextual memory, is mediated by dual mechanisms. On the one hand, morphine regulates cell lineages during NSPC differentiation by activating the PKC ϵ -dependent miR-181a-5p/Prox1/Notch1 pathway via a post-transcriptional mechanism that modulates the processing and maturation of miR-181a-5p. On the other hand, fentanyl activates ERK a β -arrestin-dependent manner, which accounts for its incapability of modulating Notch1, and in turn, the lineage of NSPCs (Xu et al., 2014; Xu et al., 2015). However, the β -arrestin-dependent ERK phosphorylation induced by fentanyl might lead to the activation miR-190/NeuroD1 pathway, by facilitating the phosphorylation of Yin Yang 1

(YY1), which may also contribute to alterations of adult neurogenesis (Zheng et al., 2010a; Zheng et al., 2008; Zheng et al., 2013). Our current study also confirms the putative common GPCR/miRNA modulating pathway that we proposed earlier, which is a crucial mechanism of NSPC regulation by addictive drugs (Xu et al., 2016). Furthermore, due to the revealed association between NeuroD1 and Notch1 in controlling neurogenesis (Seo et al., 2007), it is reasonable to infer the crosstalk between, or even a complicated network that encompass both pathways. Since a number of other molecules, such as paired-box 6 (Pax6) (Maekawa et al., 2005), Neurogenin 2 (Ngn2) (Galichet et al., 2008), or other microRNAs (Chandrasekar and Dreyer, 2009), were demonstrated to be related to the two pathways, this potential neurogenesis-regulating network might expand with further research on adult neurogenesis.

By using the *Nestin* promoter, we were able to limit the overexpression of *Prox1* in early-stage NSPCs in the adult DG. Although the maturation of NSPCs is reported to be a crucial step in the formation of new neural circuitry (Bortolotto and Grilli, 2017), our findings nevertheless establish the essentiality of early-stage NSPCs in determining cell lineages, adult neurogenesis, and finally the behavior of adult animals. This is consistent with a previous study demonstrating that NeuroD1 regulates opioid tolerance via a time-dependent pathway through contextual learning, which was carried out by overexpressing *Neurod1* and miR-190 driven by the same *Nestin* promoter (Li et al., 2014). Thus, the early-stage NSPC in the adult DG may play roles in multiple functions, from anti-nociceptive tolerance to drug memory retention, and is therefore an intriguing potential target for the treatment of neurogenesis-related neural malfunctions.

Although we have demonstrated the effect of Prox1 on adult neurogenesis and memory of the drug experiences during CPP and MWM paradigms, it is still uncertain which sessions of the behavioral tasks are crucial for the modulation of drug-associated contextual memory. Also, since the total time courses of both paradigms (>35 days) are even longer than the entire process of adult neurogenesis (Kempermann et al., 2015), it is difficult to rule out the probability that the behavioral changes observed might be a result of natural neurogenesis during such a long period, rather than an effect of Prox1 overexpression. Therefore, it is necessary to examine the effect of Prox1 during acquisition, extinction and retrieval of the CPP paradigm, along with pre-training, training and probe-tests of the MWM paradigm, in order to delineate the temporal effects of Prox1 during the entire process of the contextual memory tasks. This goal should be accomplished by using the inducible expression system in the future, so that a more detailed mechanism on drug-mediated contextual memory would be revealed.

It is worth noting that all data in the present study were obtained in mice. However, the significance of the study could be undermined by the potential discrepancies in the mechanisms between rodents and human, and moreover, by the current controversy over the persistence of human hippocampal neurogenesis (Boldrini et al., 2018; Sorrells et al., 2018). Sorrells et al. discovered an early decline of hippocampal neurogenesis throughout human childhood, which finally reaches an undetectable level after adolescence, thus raising questions about the different functions of DG between humans and other species, and even the existence of adult neurogenesis in human brain (Sorrells et al., 2018). However, Bodrini

et al. showed the contrary by analyzing postmortem brains, as adult neurogenesis persists in healthy adult humans of 14-79 years old (Boldrini et al., 2018). This controversy may stem from a series of issues, especially the technical issues. First, the postmortem delay of as long as 48 h in the study of Sorrells et al. may give rise to significant inaccuracy of marker studies, whereas the results of Bodrini et al. are more accurate (< 26 h). Second, Sorrells et al. used tissue from epilepsy surgery, which may have severe damage to the neurogenic niche. Third, the sparse DCX-positive cell may not represent all NSPCs in DG, thus resulting in underestimated quantification of neurogenesis (Kempermann et al., 2018). Finally, even in the cases of early decline of neurogenesis, there is still possibility that such function might recover due to activities and administration of certain neurogenic drugs (van Praag et al., 1999; Zhang et al., 2017). Therefore, more parameters other than histological studies, such as behavioral studies of humans, are needed for increasing the accuracy of human neurogenesis quantification in the future.

Apart from their differences in extinction of drug-associated memory and spatial memory, the two opioids, morphine and fentanyl, were found to perform differentially in the context of clinical practice, especially in the treatment of chronic pain of humans. For example, despite the similar analgesic efficacies of both drugs, fentanyl has 80-100 times more potency than morphine, along with its faster onset and shorter duration of analgesic effect (Vallejo et al., 2011). The two opioids also differ in immune opioid actions, as the immunoregulatory actions of morphine is not shared with fentanyl, which does not influence nitric oxide release or cell adhesion (Bilfinger et al., 1998). We may infer that these clinical differences might attribute to their preferred signaling pathways that lead to differential effects on adult neurogenesis, but this relevance needs to be confirmed by further investigation.

In conclusion, our current work demonstrated that one could modulate the extinction and reinstatement of morphine-induced contextual memory, indicating a potential target for drug addiction treatment. It is our future aim to elucidate a more detailed network that control adult neurogenesis and drug memory, in order that more practical treatments of drug abuse could be developed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. George L. Wilcox (Department of Neuroscience, University of Minnesota) for providing PKC ϵ -knockout mice. We thank Dr. Hui Zheng (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences) for providing the pLenti-Nestin vector. We thank Dr. Kyu Young Song for his help in plasmid construction.

Funding

This work was supported by NIDA Grant DA031442-05, the National Natural Science Foundation of China (81701313), the National Science & Technology Major Project (No. 2017ZX09101003-003-007) and Program for Changjiang Scholars and Innovative Research Team in University (IRT1193).

References

- Abrous DN, Koehl M, Le Moal M, 2005 Adult neurogenesis: from precursors to network and physiology. *Physiol Rev* 85, 523–569. [PubMed: 15788705]
- Aimone JB, Li Y, Lee SW, Clemenson GD, Deng W, Gage FH, 2014 Regulation and function of adult neurogenesis: from genes to cognition. *Physiol Rev* 94, 991–1026. [PubMed: 25287858]
- Apple DM, Fonseca RS, Kokovay E, 2017 The role of adult neurogenesis in psychiatric and cognitive disorders. *Brain Research* 1655, 270–276. [PubMed: 26801829]
- Bilfinger TV, Fimiani C, Stefano GB, 1998 Morphine's immunoregulatory actions are not shared by fentanyl. *Int J Cardiol* 64 Suppl 1, S61–66. [PubMed: 9687094]
- Boldrini M, Fulmore CA, Tartt AN, Simeon LR, Pavlova I, Poposka V, Rosoklija GB, Stankov A, Arango V, Dwork AJ, Hen R, Mann JJ, 2018 Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell* 22, 589–599 e585. [PubMed: 29625071]
- Bortolotto V, Cuccurazzu B, Canonico PL, Grilli M, 2014 NF-kappaB mediated regulation of adult hippocampal neurogenesis: relevance to mood disorders and antidepressant activity. *Biomed Res Int* 2014, 612798. [PubMed: 24678511]
- Bortolotto V, Grilli M, 2017 Opiate Analgesics as Negative Modulators of Adult Hippocampal Neurogenesis: Potential Implications in Clinical Practice. *Front Pharmacol* 8, 254. [PubMed: 28536527]
- Canales JJ, 2007 Adult neurogenesis and the memories of drug addiction. *Eur Arch Psychiatry Clin Neurosci* 257, 261–270. [PubMed: 17401727]
- Castilla-Ortega E, Blanco E, Serrano A, Ladron de Guevara-Miranda D, Pedraz M, Estivill-Torrus G, Pavon FJ, Rodriguez de Fonseca F, Santin LJ, 2016a Pharmacological reduction of adult hippocampal neurogenesis modifies functional brain circuits in mice exposed to a cocaine conditioned place preference paradigm. *Addict Biol* 21, 575–588. [PubMed: 25870909]
- Castilla-Ortega E, Ladron de Guevara-Miranda D, Serrano A, Pavon FJ, Suarez J, Rodriguez de Fonseca F, Santin LJ, 2017 The impact of cocaine on adult hippocampal neurogenesis: Potential neurobiological mechanisms and contributions to maladaptive cognition in cocaine addiction disorder. *Biochem Pharmacol*.
- Castilla-Ortega E, Serrano A, Blanco E, Araos P, Suarez J, Pavon FJ, Rodriguez de Fonseca F, Santin LJ, 2016b A place for the hippocampus in the cocaine addiction circuit: Potential roles for adult hippocampal neurogenesis. *Neurosci Biobehav Rev* 66, 15–32. [PubMed: 27118134]
- Chandrasekar V, Dreyer JL, 2009 microRNAs miR-124, let-7d and miR-181a regulate cocaine-induced plasticity. *Mol Cell Neurosci* 42, 350–362. [PubMed: 19703567]
- Cunningham CL, Patel P, Milner L, 2006 Spatial location is critical for conditioning place preference with visual but not tactile stimuli. *Behav Neurosci* 120, 1115–1132. [PubMed: 17014262]
- Galichet C, Guillemot F, Parras CM, 2008 Neurogenin 2 has an essential role in development of the dentate gyrus. *Development* 135, 2031–2041. [PubMed: 18448566]
- Goncalves JT, Schafer ST, Gage FH, 2016 Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. *Cell* 167, 897–914. [PubMed: 27814520]
- Goodman J, Packard MG, 2016 Memory Systems and the Addicted Brain. *Frontiers in Psychiatry* 7.
- Guo SJ, Cui Y, Huang ZZ, Liu H, Zhang XQ, Jiang JX, Xin WJ, 2016 Orexin A-mediated AKT signaling in the dentate gyrus contributes to the acquisition, expression and reinstatement of morphine-induced conditioned place preference. *Addict Biol* 21, 547–559. [PubMed: 25757577]
- Kempermann G, Gage FH, Aigner L, Song H, Curtis MA, Thuret S, Kuhn HG, Jessberger S, Frankland PW, Cameron HA, Gould E, Hen R, Abrous DN, Toni N, Schinder AF, Zhao X, Lucassen PJ, Frisen J, 2018 Human Adult Neurogenesis: Evidence and Remaining Questions. *Cell Stem Cell*.
- Kempermann G, Song HJ, Gage FH, 2015 Neurogenesis in the Adult Hippocampus. *Cold Spring Harbor Perspectives in Biology* 7.
- Khasar SG, Lin YH, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO, 1999 A novel nociceptor signaling pathway revealed in protein kinase C epsilon mutant mice. *Neuron* 24, 253–260. [PubMed: 10677042]

- Kibaly C, Kam AYP, Loh HH, Law PY, 2016 Naltrexone Facilitates Learning and Delays Extinction by Increasing AMPA Receptor Phosphorylation and Membrane Insertion. *Biological Psychiatry* 79, 906–916. [PubMed: 26049209]
- Kramar CP, Chefer VI, Wise RA, Medina JH, Barbano MF, 2014 Dopamine in the dorsal hippocampus impairs the late consolidation of cocaine-associated memory. *Neuropsychopharmacology* 39, 1645–1653. [PubMed: 24442095]
- Kutlu MG, Gould TJ, 2016 Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: contributions to development and maintenance of addiction. *Learn Mem* 23, 515–533. [PubMed: 27634143]
- Lavado A, Lagutin OV, Chow LM, Baker SJ, Oliver G, 2010 Prox1 is required for granule cell maturation and intermediate progenitor maintenance during brain neurogenesis. *PLoS Biol* 8.
- Li W, He S, Zhou Y, Li Y, Hao J, Zhou X, Wang F, Zhang Y, Huang Z, Li Z, Loh HH, Law PY, Zheng H, 2014 Neurod1 modulates opioid antinociceptive tolerance via two distinct mechanisms. *Biol Psychiatry* 76, 775–784. [PubMed: 24993058]
- Maekawa M, Takashima N, Arai Y, Nomura T, Inokuchi K, Yuasa S, Osumi N, 2005 Pax6 is required for production and maintenance of progenitor cells in postnatal hippocampal neurogenesis. *Genes Cells* 10, 1001–1014. [PubMed: 16164600]
- Marchese NA, Artur de la Villarmois E, Basmadjian OM, Perez MF, Baiardi G, Bregonzio C, 2016 Brain Angiotensin II AT1 receptors are involved in the acute and long-term amphetamine-induced neurocognitive alterations. *Psychopharmacology (Berl)* 233, 795–807. [PubMed: 26613735]
- Mendez IA, Montgomery KS, LaSarge CL, Simon NW, Bizon JL, Setlow B, 2008 Long-term effects of prior cocaine exposure on Morris water maze performance. *Neurobiology of Learning and Memory* 89, 185–191. [PubMed: 17904876]
- Nader MA, 2016 Animal models for addiction medicine: From vulnerable phenotypes to addicted individuals. *Prog Brain Res* 224, 3–24. [PubMed: 26822351]
- Noonan MA, Bulin SE, Fuller DC, Eisch AJ, 2010 Reduction of adult hippocampal neurogenesis confers vulnerability in an animal model of cocaine addiction. *J Neurosci* 30, 304–315. [PubMed: 20053911]
- Schuster DJ, Kitto KF, Overland AC, Messing RO, Stone LS, Fairbanks CA, Wilcox GL, 2013 Protein kinase Cepsilon is required for spinal analgesic synergy between delta opioid and alpha-2A adrenergic receptor agonist pairs. *J Neurosci* 33, 13538–13546. [PubMed: 23946412]
- Seo S, Lim JW, Yellajoshiyula D, Chang LW, Kroll KL, 2007 Neurogenin and NeuroD direct transcriptional targets and their regulatory enhancers. *EMBO J* 26, 5093–5108. [PubMed: 18007592]
- Shalev U, Grimm JW, Shaham Y, 2002 Neurobiology of relapse to heroin and cocaine seeking: a review. *Pharmacol Rev* 54, 1–42. [PubMed: 11870259]
- Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, James D, Mayer S, Chang J, Auguste KI, Chang EF, Gutierrez AJ, Kriegstein AR, Mathern GW, Oldham MC, Huang EJ, Garcia-Verdugo JM, Yang Z, Alvarez-Buylla A, 2018 Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555, 377–381. [PubMed: 29513649]
- Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, Huttner HB, Bostrom E, Westerlund I, Vial C, Buchholz BA, Possnert G, Mash DC, Druid H, Frisen J, 2013 Dynamics of hippocampal neurogenesis in adult humans. *Cell* 153, 1219–1227. [PubMed: 23746839]
- Steinmetz AB, Freeman JH, 2016 Cannabinoid modulation of memory consolidation within the cerebellum. *Neurobiology of Learning and Memory* 136, 228–235. [PubMed: 27818269]
- Steriopoulou A, Elkouris M, Politis PK, 2014 Prospero-related homeobox 1 (Prox1) at the crossroads of diverse pathways during adult neural fate specification. *Front Cell Neurosci* 8, 454. [PubMed: 25674048]
- Tramullas M, Martinez-Cue C, Hurler MA, 2008 Chronic administration of heroin to mice produces up-regulation of brain apoptosis-related proteins and impairs spatial learning and memory. *Neuropharmacology* 54, 640–652. [PubMed: 18201731]
- Trezza V, Baarendse PJ, Vanderschuren LJ, 2010 The pleasures of play: pharmacological insights into social reward mechanisms. *Trends Pharmacol Sci* 31, 463–469. [PubMed: 20684996]

- Vallejo R, Barkin RL, Wang VC, 2011 Pharmacology of opioids in the treatment of chronic pain syndromes. *Pain Physician* 14, E343–360. [PubMed: 21785485]
- van Praag H, Kempermann G, Gage FH, 1999 Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* 2, 266–270. [PubMed: 10195220]
- Vorhees CV, Williams MT, 2006 Morris water maze: procedures for assessing spatial and related forms of learning and memory. *Nat Protoc* 1, 848–858. [PubMed: 17406317]
- Wang J, Gallagher D, DeVito LM, Cancino GI, Tsui D, He L, Keller GM, Frankland PW, Kaplan DR, Miller FD, 2012 Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation. *Cell Stem Cell* 11, 23–35. [PubMed: 22770240]
- Watanabe S, 2011 Drug-social interactions in the reinforcing property of methamphetamine in mice. *Behav Pharmacol* 22, 203–206. [PubMed: 21422916]
- Xu C, Loh HH, Law PY, 2016 Effects of addictive drugs on adult neural stem/progenitor cells. *Cell Mol Life Sci* 73, 327–348. [PubMed: 26468052]
- Xu C, Zhang Y, Zheng H, Loh HH, Law PY, 2014 Morphine modulates mouse hippocampal progenitor cell lineages by upregulating miR-181a level. *Stem Cells* 32, 2961–2972. [PubMed: 24964978]
- Xu C, Zheng H, Loh HH, Law PY, 2015 Morphine Promotes Astrocyte-Preferential Differentiation of Mouse Hippocampal Progenitor Cells via PKCepsilon-Dependent ERK Activation and TRBP Phosphorylation. *Stem Cells* 33, 2762–2772. [PubMed: 26012717]
- Yonelinas AP, 2013 The hippocampus supports high-resolution binding in the service of perception, working memory and long-term memory. *Behavioural Brain Research* 254, 34–44. [PubMed: 23721964]
- Zhang Y, Kibaly C, Xu C, Loh HH, Law PY, 2017 Temporal effect of manipulating NeuroD1 expression with the synthetic small molecule KHS101 on morphine contextual memory. *Neuropharmacology* 126, 58–69. [PubMed: 28842345]
- Zhang Y, Xu C, Zheng H, Loh HH, Law PY, 2016 Morphine Modulates Adult Neurogenesis and Contextual Memory by Impeding the Maturation of Neural Progenitors. *PLoS One* 11, e0153628. [PubMed: 27078155]
- Zheng H, Chu J, Zeng Y, Loh HH, Law PY, 2010a Yin Yang 1 phosphorylation contributes to the differential effects of mu-opioid receptor agonists on microRNA-190 expression. *J Biol Chem* 285, 21994–22002. [PubMed: 20457614]
- Zheng H, Loh HH, Law PY, 2008 Beta-arrestin-dependent mu-opioid receptor-activated extracellular signal-regulated kinases (ERKs) Translocate to Nucleus in Contrast to G protein-dependent ERK activation. *Mol Pharmacol* 73, 178–190. [PubMed: 17947509]
- Zheng H, Zeng Y, Chu J, Kam AY, Loh HH, Law PY, 2010b Modulations of NeuroD activity contribute to the differential effects of morphine and fentanyl on dendritic spine stability. *J Neurosci* 30, 8102–8110. [PubMed: 20554861]
- Zheng H, Zeng Y, Zhang X, Chu J, Loh HH, Law PY, 2010c mu-Opioid receptor agonists differentially regulate the expression of miR-190 and NeuroD. *Mol Pharmacol* 77, 102–109. [PubMed: 19854889]
- Zheng H, Zhang Y, Li W, Loh HH, Law PY, 2013 NeuroD modulates opioid agonist-selective regulation of adult neurogenesis and contextual memory extinction. *Neuropsychopharmacology* 38, 770–777. [PubMed: 23303051]

Highlights

- Morphine regulates NSPC lineage determination via a PKC ϵ -mediated pathway
- PKC ϵ inhibits neuronal differentiation of NSPCs by inhibiting Prox1 expression
- PKC ϵ /Prox1 regulates extinction phase and reinstatement of morphine-induced CPP
- PKC ϵ /Prox1 regulates morphine-modulated extinction of spatial reference memory

Figure 1. In vivo expression of crucial molecules in NSPC lineage determination after OPRM1 agonist administration in wildtype and PKCε KO mice.

(A-E) The mRNA levels of *DCX* (A), *GFAP* (B), *Prox1* (C), *Notch1* (D) and the expression of miR-181a-5p in the hippocampi of both wildtype and PKCε KO mice were determined by qRT-PCR after the administration of 5 mg/kg morphine or 50 μg/kg fentanyl for 4 consecutive days. The results were normalized against the level of GAPDH, and further normalized against the result obtained from the saline wildtype group (set as 100%). All data represent the mean ± SEM of four independent experiments. Significant differences among groups were determined using one-way analyses of variance, followed by Newman-Keuls

post hoc comparisons. *, $p < 0.05$ compared to the saline wildtype group; #, $p < 0.05$ compared to the morphine PKC ϵ KO group.

(F) The protein levels of DCX, GFAP, Prox1 and Notch1 in the hippocampi of both wildtype and PKC ϵ KO mice were determined by western-blot. The results were normalized against the level of β -actin, and further normalized against the result obtained from the saline wildtype group.

(G-J) Quantification of DCX (G), GFAP (H), Prox1 (I) and Notch1 (J) protein levels. All data represent the mean $\pm$ SEM of four independent experiments. Significant differences among groups were determined using one-way analyses of variance, followed by Newman-Keuls post hoc comparisons. *, $p < 0.05$ compared to the saline wildtype group; #, $p < 0.05$ compared to the morphine PKC ϵ KO group. WT, wildtype; KO, knockout.

Figure 2. The lineages of adult hippocampal NSPC differentiation after OPRM1 agonist administration in wildtype and PKC ϵ KO mice.

(A) Timeline of the experiment. Animals received BrdU injection (200 mg/kg, i.p.) on days 1-3 and a following CPP training with 5 mg/kg morphine, 50 μ g/kg fentanyl or an equal volume of saline on days 4-7. The animals were sacrificed on day 8 and analyzed by immunohistochemistry.

(B) Fluorescent images showing the co-localization of BrdU and the immature neuron marker DCX or astrocyte marker GFAP, in the DG of wildtype and PKC ϵ KO mice treated

with morphine or fentanyl. Red: BrdU; Green: DCX; Purple: GFAP; Blue: DAPI. Images represent eight individual animals with similar results. Scale bar: 50 μ m.

(C) Quantification of the co-localization between BrdU and DCX or GFAP. Total numbers of BrdU-labeled cells co-stained with DCX (BrdU⁺DCX⁺) or GFAP (BrdU⁺GFAP⁺) antibodies in adult mouse hippocampus sections were counted and compared. Error bars represent SEM (N=8). Significant differences among groups were determined using one-way analyses of variance, followed by Newman-Keuls post hoc comparisons. *, $p < 0.05$ compared to the saline wildtype group; #, $p < 0.05$ compared to the morphine wildtype group.

Figure 3. PKCε knockout results in prolonged extinction phase and enhanced reinstatement of morphine- but not fentanyl-induced CPP.

(A) Timeline of the CPP paradigm. After 3 days of habituation and preconditioning (days 1-3), wildtype and PKCε knockout mice were trained for 4 consecutive days (days 4-7), during which they received injection of either morphine (5 mg/kg, s.c.) or fentanyl (50 μg/kg, s.c.), and an equal volume of saline. All animals were then tested every 7 days from day 8 in the CPP apparatus until the place preference extinguished. CPP reinstatement was tested after total extinction.

(B) The CPP scores of wildtype and PKCε knockout mice treated with morphine or fentanyl during the acquisition and extinction sessions. Error bars represent SEM (N=8). Significant differences among groups were determined using two-way analyses of variance, followed by Newman-Keuls post hoc comparisons.

(C) The CPP scores of wildtype and PKCε knockout mice treated with morphine or fentanyl at reinstatement. Error bars represent SEM. N=8. Significant differences among groups were determined using Student's *t* tests. *, *p*<0.05 compared to the morphine wildtype group.

Figure 4. Morphine modulates the extinction of spatial reference memory via a PKC ϵ -dependent pathway.

(A) Timeline of the MWM paradigm. Wildtype and PKC ϵ knockout mice were subcutaneously injected daily with 5 mg/kg morphine, 50 μ g/kg fentanyl or an equal volume of saline, for 5 consecutive days (days -5 to -1). After being habituated for 1 day (day 0), all mice were trained in the water maze apparatus for 5 consecutive days (days 1-5). The probe-test was repeated every 4 days until the conditioned response was totally diminished. (B-C) The time spent (B) and distance covered (C) before finding the hidden platform during the training session of the MWM paradigm. Error bars represent SEM. N=8 for each group. Significant differences among groups were determined using three-way analyses of variance, followed by Newman-Keuls post hoc comparisons.

(D-E) The time spent (D) and distance covered (E) in the target quadrant during the probe tests in the extinction session. Error bars represent SEM. N=8 for each group. Significant differences among groups were determined using three-way analyses of variance, followed

by Newman-Keuls post hoc comparisons. **, $p < 0.01$; ***, $p < 0.001$ compared to all other groups on the same day.

Figure 5. PKCε/Prox1 mediates morphine-induced in vivo lineage-preferential differentiation of mouse hippocampal NSPCs.

(A) Timeline of the BrdU-labeling experiment with lentivirus injection. The lentivirus was stereotactically injected into hippocampi of both wildtype and PKCε knockout mice 7 days before the experiment started (day -7).

(B) Fluorescent images showing the co-localization of BrdU and the immature neuron marker DCX or astrocyte marker GFAP, in the DG of wildtype and PKCε KO mice treated with morphine or fentanyl, and injected with lentiviruses that express either the negative control oligonucleotide (control-vir) or *Nestin*-promoter-controlled *Prox1* cDNA (Nestin-

Prox1-vir). Red: BrdU; Green: DCX; Purple: GFAP; Blue: DAPI. Images represent eight individual animals with similar results. Scale bar: 50 μ m.

(C) Quantification of the co-localization between BrdU and DCX or GFAP. Total numbers of BrdU-labeled cells co-stained with DCX (BrdU⁺DCX⁺) or GFAP (BrdU⁺GFAP⁺) antibodies in adult mouse hippocampus sections were counted and compared. Error bars represent SEM (N=8). Significant differences among groups were determined using three-way analyses of variance, followed by Newman-Keuls post hoc comparisons. *, $p < 0.05$ compared to the saline-treated wildtype mice injected with the same virus; #, $p < 0.05$ compared to the morphine-treated wildtype mice injected with control virus; &, $p < 0.05$ compared to the morphine-treated mice injected with control virus, and with the same genotype.

Figure 6. PKC ϵ /Prox1 regulates the extinction and reinstatement of morphine-induced CPP of adult mice.

(A) Timeline of the CPP paradigm with lentivirus injection. The lentivirus was stereotactically injected into hippocampi of both wildtype and PKC ϵ knockout mice 7 days before the pre-conditioning session (day -7).

(B) The CPP scores of wildtype and PKC ϵ knockout mice injected with saline (no virus), control virus (con-vir) or *Nestin*-promoter-controlled *Prox1* cDNA (*Nestin-Prox1-vir*) and treated with morphine during the acquisition and extinction sessions. Error bars represent SEM (N=8). Significant differences among groups were determined using two-way analyses of variance, followed by Newman-Keuls post hoc comparisons.

(C) The CPP scores of wildtype and PKC ϵ knockout mice injected with saline, con-vir or *Nestin-Prox1-vir*, and treated with morphine at reinstatement. Error bars represent SEM.

Significant differences among groups were determined using two-way analyses of variance, followed by Newman-Keuls post hoc comparisons. N=8; *, $p<0.05$ compared to groups of the same genotype injected with saline or control-vir. #, $p<0.05$ compared to wildtype mice injected with the same virus.

Figure 7. PKC ϵ /Prox1 regulates morphine-modulated extinction of spatial reference memory in adult mice.

(A) Timeline of the MWM paradigm with lentivirus injection. The lentivirus was stereotactically injected into hippocampi of both wildtype and PKC ϵ knockout mice 8 days before agonist administration (day -13).

(B) The time spent before finding the hidden platform during the training session of the MWM paradigm. Error bars represent SEM. N=8 for each group. Significant differences among groups were determined using four-way analyses of variance, followed by Newman-

Keuls post hoc comparisons. WT: wildtype; Mor: morphine; Sal: saline; Con: control; KO: knockout.

(C-D) The time spent (C) and distance covered (D) in the target quadrant during the probe tests in the extinction session. Error bars represent SEM. N=8 for each group. Significant differences among groups were determined using four-way analyses of variance, followed by Newman-Keuls post hoc comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. The asterisks designate the significant differences of the “WT Mor Con-vir”, “WT Sal Nestin-Prox1-vir”, “PKC ϵ KO Sal Nestin-Prox1-vir” and “PKC ϵ KO Mor Nestin-Prox1-vir” groups compared to all other groups.

(E) Track plots of wildtype and PKC ϵ KO mice injected with saline, con-vir or Nestin-Prox1-vir, and treated with morphine during the third probe test (day 14). The southwest (third) quadrant is the target quadrant where the platform was previously located during training.