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Retrieval of morphine-associated context induces cFos in dentate gyrus neurons

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Abstract

Addiction has been proposed to emerge from associations between the drug and the reward-associated contexts. This associative learning has a cellular correlate, as there are more cFos+ neurons in the hippocampal dentate gyrus (DG) after psychostimulant conditioned place preference (CPP) vs. saline controls. However, it is unknown whether morphine CPP leads to a similar DG activation, or whether DG activation is due to locomotion, handling, pharmacological effects, or – as data from contextual fear learning suggests – exposure to the drug-associated context. To explore this, we employed an unbiased, counterbalanced, and shortened CPP design that led to place preference, more DG cFos+ cells, and yet decreased locomotion. Next, mice underwent morphine CPP but were then sequestered into the morphine-paired (conditioned stimulus+ [CS+]) or saline-paired (CS–) context on test day. Morphine-paired mice sequestered to CS+ had ~30% more DG cFos+ cells than saline-paired mice. Furthermore, Bregma analysis revealed morphine-paired mice had more cFos+ cells in CS+ compared to CS– controls. Notably, there was no significant difference in DG cFos+ cell number after handling alone or after receiving morphine in home cage. Thus, retrieval of morphine-associated context is accompanied by activation of hippocampal DG granule cell neurons.

Keywords

addiction; conditioned place preference (CPP); hippocampus; Immediate Early Gene (IEG); re-exposure

Drug addiction is a brain disease of compulsive drug taking, seeking, and use with many negative health and societal consequences. The focus of most addiction research is the canonical reward pathway, which includes the nucleus accumbens, ventral tegmental area, and prefrontal cortex (Koob and Volkow, 2010; Nestler, 2005). However, the hippocampus and its dentate gyrus (DG) subregion have also been implicated in addiction (Noonan et al., 2010). For instance, cocaine-dependent humans show cue-induced hippocampal dopamine release (Fotros et al., 2013). Furthermore, conditioned place preference (CPP) in rodents, which involves context-dependent learning associations with drugs (Bardo et al., 1995; Tzschentke, 2007), relies on an intact DG (Hernandez-Rabaza et al., 2008). CPP has also been used to assess hippocampal neuroadaptations that correlate and contribute to drug-

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reward memories (Hernandez-Rabaza et al., 2008; Koob and Volkow, 2010; Meyers et al., 2006), and are thought to negatively impact cognition and mood (NIDA; Russo and Nestler, 2013). Thus, understanding how the formation of drug-reward memories alters the neurobiology of the hippocampal DG may shed light on the later and more persistent aspects of addiction.

In learning and memory research, DG expression of the immediate early gene (IEG) cFos, correlates with (Kubik et al., 2007; Lopez et al., 2012) and causes retrieval of memory (Liu et al., 2012; Liu et al., 2014). Correlative studies in addiction research also show increased DG expression of cFos after retrieval of psychostimulant CPP (Chauvet et al., 2011; Rademacher et al., 2006), suggesting DG cFos involvement in context-reward memory. In order to determine whether DG cFos expression plays a causative role in addiction related behaviors, more fundamental information is needed about the involvement of DG cFos in context-reward memory. For example, the hippocampus and DG have been proposed to play a role in determining the salience, or importance, of contexts (Penner and Mizumori, 2012), which influences choice behavior for a rewarding context (Kennedy and Shapiro, 2009). However, whether DG cFos is involved in defining the salience of a context has yet to be determined. As such, CPP is well poised to examine DG cFos after choice behavior (i.e. free access to CPP chambers on test day)(Tzschentke, 2007) and elimination of choice by sequestration (i.e. confinement to the previously-paired saline or morphine context on test day). Therefore, the involvement of DG cFos was examined in mice tested in both choice behavior and sequestration CPP paradigms. The correlative data presented below support the role of DG cFos in reward and memory, and strengthen the rationale for future examination of DG cFos contribution to retrieval of reward memory, which can ultimately lead to better treatments for addicted humans.

Based on the role of DG cFos in contextual fear learning (Liu et al., 2014), we hypothesized that DG cellular activation (cFos+ cells) after CPP was due to retrieval of the drug-associated context reward memory. To examine the retrieval of a drug-associated context reward memory, male C57BL/6J mice underwent morphine CPP (5 days, n=6–13, Fig. 1A, see Detailed Methods). Mice paired with morphine (Sal/Mor, days 2–4, s.c.15 mg/kg, n=6; Fig. 1A) and tested on day 5 had a positive CPP score compared to saline controls (Sal/Sal) on test day (n=13, one-way ANOVA, $P<0.001$, Fig. 1B), therefore validating the use of this shorter morphine CPP paradigm (Ribeiro Do Couto et al., 2003; Smith and Aston-Jones, 2014; Zarrindast et al., 2002). Mice were then killed 90 min post-test, a time point at which spatial/retrieval stimulus-induced cFos protein levels are high in many brain areas (Clark et al., 2010; Kee et al., 2007).

Due to functional and structural heterogeneity of the DG (Snyder et al., 2009), cFos+ nuclei (Fig. 1C inset) were quantified in DG granule cell layer (GCL) and GCL subregions. In agreement with previous reports with psychostimulants (Chauvet et al., 2011; Rademacher et al., 2006), mice (Sal/Mor) previously paired with saline in one context (CS–) and morphine in another context (CS+) had ~30% more cFos+ cells in the DG GCL than mice that received saline in both contexts (n=5/group, Student's *t*-test, $^{\#}P<0.05$, Fig. 1D). Analysis between Sal/Sal and Sal/Mor groups for DG GCL cFos+ cells across the coronal septotemporal axis of the hippocampus revealed a main effect treatment ($F_{(1,154)}=25.87$,

$P < 0.001$) and distance ($F_{(13,154)} = 28.28$, $P < 0.001$), but had no significant interaction (data not shown). It is unlikely that increased locomotion contributed to the increased DG GCL cFos+ cells during test day, as no difference in total beam breaks during test day was observed between Sal/Mor and Sal/Sal groups (Fig 1E).

Subregional GCL analysis revealed Sal/Mor mice had more cFos+ cells in the suprapyramidal (supra) blade as well as the inner (iGCL) and outer (oGCL) compared to Sal/Sal controls ($n = 5/\text{group}/\text{region}$, Student's t -test, supra, $^{\#}P < 0.05$; iGCL, $P < 0.01$; oGCL, $P < 0.05$; Supporting Information Fig. 1B–D). No significant difference in infrapyramidal (infra) blade cFos+ cell number was found in Sal/Mor compared to Sal/Sal (Supporting Information Fig. 1E). Taken together, these data show that re-exposure to the CPP box after morphine pairing can generally activate DG GCL neurons relative to saline-paired controls, suggesting that drug experience and the context (CS+) can activate the DG.

Previous studies have shown that drug-induced locomotion (Zhang et al., 2006), restraint (Hoffman et al., 2013), and spatial contexts (Lopez et al., 2012) can induce DG cFos expression. Therefore, we also explored whether the increase in cFos+ cells in the DG GCL (Fig. 1D) was due to these alternative explanations, or even to prior morphine exposure. In regards to locomotion, the total number of beam breaks between Sal/Sal and Sal/Mor mice on pretest and test day was not significantly different, suggesting that locomotor activity did not contribute to the increase in DG GCL cFos+ cells in Sal/Mor mice during test day (Fig. 1E). In addition, no difference in DG GCL volume was observed between Sal/Sal and Sal/Mor after morphine CPP (Supporting Information Fig. 2). In regards to prior morphine exposure, naïve vs. handled ($n = 9\text{--}12$, Fig. 2A) and homecage Sal/Sal vs. Sal/Mor groups ($n = 5\text{--}6$, Fig. 2C) showed no significant difference in DG GCL cFos+ cells (Fig. 2B, Naïve vs. Handled, $P = 0.613$; Fig. 2D, Sal/Sal vs. Sal/Mor, $P = 0.592$). Taken together, these data show the increase of DG GCL cFos+ cells initially observed after choice behavior (Fig. 1D) was not due to locomotion or other variables that coincided with performing the CPP paradigm, such as handling or effects of prior morphine exposure.

One question that remained was whether the increase in DG GCL cFos+ cells (Fig. 1D) was due to choice behavior and/or retrieval of drug-associated context reward memory. Optogenetically-induced cFos activation in the DG GCL is sufficient to recall fear memory (Liu et al., 2012). However, in regards to CPP, it is unclear if the retrieval of a rewarding memory vs. the morphine CPP paradigm itself led to more DG GCL cFos+ cells (Laakso et al., 2002). We considered choice behavior as a possible variable leading to more DG GCL cFos+ cells, and developed a paradigm to sequester mice into their saline-paired (CS–) or morphine-paired (CS+) contexts. Surprisingly, Sal/Sal and Sal/Mor mice sequestered on test day to CS+ or CS– (Fig. 3A) showed a main effect of treatment ($n = 7\text{--}9/\text{group}/\text{treatment}$, Fig. 3B, $F_{(3,28)} = 3.311$, $P = 0.034$). Posthoc analysis revealed Sal/Mor CS+ mice had more DG GCL cFos+ cells than Sal/Sal CS+ controls [Fig. 3B, $M = -1749$, 95% CI $(-3417, -79.90)$, $P < 0.05$]. Analysis between Sal/Sal and Sal/Mor CS– groups for DG GCL cFos+ cells across the coronal septotemporal axis of the hippocampus revealed no significant difference (Fig. 3C). However, analysis between Sal/Sal and Sal/Mor CS+ groups for DG GCL cFos+ cells across the coronal septotemporal axis of the hippocampus showed a significant main effect of treatment ($F_{(1,156)} = 22.92$, $P < 0.001$) and distance

($F_{(12,156)}=37.03$, $P<0.001$), but no significant interaction ($F_{(12,156)}=1.658$, $P=0.081$). Posthoc analysis of the CS+ group revealed significantly more DG GCL cFos+ cells in Sal/Mor at Bregma points -2.32 [$M=-33.57$, 95% CI($-66.51,4.084$), $P<0.05$] and -2.92 mm [$M=-35.57$, 95% CI($-68.51,-2.631$), $P<0.05$], compared to Sal/Sal controls (Fig. 3D). To determine if the context was important in establishing reward-associated context memory, analysis between CS- and CS+ in both Sal/Sal (Fig. 3E) and Sal/Mor (Fig. 3F) groups was performed. While both Sal/Sal and Sal/Mor groups showed a main effect of distance or interaction across the coronal septotemporal axis of the hippocampus (Sal/Sal, $F_{(12,182)}=43.09$, $P=0.001$; Sal/Mor, $F_{(13,196)}=60.58$, $P=0.001$), only the Sal/Mor had a main effect of context (Fig. 3F; $F_{(1,196)}=7.45$, $P=0.01$).

Subregional analysis by one-way ANOVA showed a significant interaction for the infra ($F_{(3,28)}=3.852$, $P=0.02$) and oGCL ($F_{(3,28)}=3.464$, $P=0.0294$) regions (Supporting Information Fig. 3B,D). Posthoc analysis revealed Sal/Mor CS+ mice had more cFos+ cells than Sal/Sal CS+ for both infra [$M=-690.4$, 95% CI($-1285,-95.55$), $P<0.05$, Supporting Information Fig. 3B] and oGCL regions [$M=-1237$, 95% CI($-2425,-48.74$), $P<0.05$, Supporting Information Fig. 3D]. Notably, on test day there was no difference in total number of beam breaks between all groups (data not shown). Subregional analysis also found significant differences in Sal/Mor CS+ versus Sal/Sal CS+ for supra ($P<0.0218$, Supporting Information Fig. 3A) and iGCL ($P<0.0477$, Supporting Information Fig. 3C) regions. A significant difference in Sal/Mor CS+ versus Sal/Mor CS- was also observed (Student's *t*-test, $^{\#}P<0.05$) in the oGCL region. Therefore, our data suggest that after sequestration to a previously-learned drug context, more DG GCL cFos+ cells are observed after retrieval of a drug-associated context reward memory.

In sum, using a brief morphine CPP paradigm (3 pairing days, 15 mg/kg morphine) and free access to the CPP chambers on test day, we found a correlation between morphine-paired mice in the morphine-paired context (CS+) and more DG GCL cFos+ cells compared to saline-paired mice. Control experiments showed the increase in DG GCL cFos+ cells was not due to handling or pharmacological effects of morphine. However, additional morphine CPP experiments revealed that, as in the choice behavior experiment, morphine-paired mice sequestered to CS+ on test day had more DG GCL cFos+ cells compared to saline-paired mice. Taken together, these data correlatively suggest that DG cellular activation after morphine CPP appears to be dependent on the drug-associated context. For example, on test day when choice for a salient context was removed and only the drug-associated context was presented, a significant difference in DG GCL cFos+ cells in Sal/Sal and Sal/Mor mice was observed in the CS+ group, but not the CS- group (Fig. 3). However, we cannot rule out that changes in the contextual environment on test day contribute to DG cFos activation.

It is useful to consider the present results in the context of the literature relevant to context dependent-reward and -fear learning and memory. Thus far, it was presumed that CPP led to rodents making an association between a drug and a context (Bardo and Bevins, 2000). The closest experiment to our shortened sequestration morphine CPP paradigm examined retrieval of drug/texture association using cocaine CPP (Johnson et al., 2010; Zombeck et al., 2008). In both of these studies, mice were paired to different textures (CS+ cocaine-paired texture; CS- saline-paired texture) with black walls throughout, and not given a

choice between textures on test day. In conflict with our results where more DG cFos+ cells were observed after retrieval of a drug-associated context (sequestration to CS+), these prior experiments found either fewer DG cFos+ cells in CS+ (Johnson et al., 2010) or no significant difference (Zombeck et al., 2008) compared to CS- controls. The discrepancy with our results may be due to differences in spatial vs. textural contexts (Lopez et al., 2012), number of pairing days (Guzowski et al., 2001; Tzschentke, 2007), and drug type. Fear (contextual fear conditioning) and reward (psychostimulant CPP) learning and memory experiments have also shown that retrieval is sufficient to increase cFos in the DG (Beck and Fibiger, 1995; Rademacher et al., 2006) and septal DG (Chauvet et al., 2011). In addition, region specific activity in the hippocampus is necessary for the acquisition and expression of cocaine CPP (Meyers et al., 2006). Our data agree with the concept of region specific activity within the hippocampus after retrieval since we observed an increase in cFos in the intermediate region of the DG (Fig. 3D) after retrieval of a morphine-associated context. However, more work is necessary to determine the function(s) of each region and how they may interact with one another during retrieval of a drug-reward memory. Taken together, our data provide an interesting parallel between the formation of reward vs. fear memory, which may be useful in developing treatments for addicted humans similar to those treatment that have been developed for the extinction of fear memory.

Our work lays the foundation for future studies that can test the hypothesis that DG cellular activation is necessary and sufficient to establish drug-associated context. While data is not available on DG activation during retrieval of morphine-paired CPP, recently published electrophysiology data from CA1 show retrieval of morphine-paired CPP correlates with increased basal synaptic transmission, impaired hippocampal LTP, and increased synaptic expression of the NR1 NMDAR subunit in the CA1 region (Portugal et al., 2014). It remains to be tested whether these changes in CA1 are causative, whether they are due to re-exposure-induced activation or modulation of the DG, and to what extent other inputs to the hippocampus or networks are involved in these changes (Otis et al., 2013; Tang and Dani, 2009). In addicted humans, drug-associated contextual memories are strong and long lasting, making it difficult not to relapse when exposed to familiar associations (Koob and Volkow, 2010). Given the cognitive flexibility and enhanced contextual discrimination afforded by DG neurogenesis (Burghardt et al., 2012; Garthe et al., 2009; Kheirbek et al., 2012), one possible treatment avenue for addiction consists of increasing new DG neuron number or survival in the DG (Clelland et al., 2009). This would be predicted to allow better sparse encoding in the DG (Deng et al., 2010; Leutgeb et al., 2007), which may enhance extinction, or the learning that a previous drug-associated context is no longer rewarding. However, it is also possible that aside from DG neurogenesis, DG interneurons (Ikrar et al., 2013) and DG granule-like cells (Williams et al., 2007) may play a role in modulating DG output during retrieval of a drug/associated context reward memory. All of these studies would be made more feasible by a better understanding of the neuroplastic changes specific to the hippocampal DG during CPP retrieval.

DETAILED METHODS (See also Supplemental Information)

Conditioned Place Preference (CPP)

Mice were trained in a 3-compartment apparatus (Olson et al., 2005; Taniguchi et al., 2012). The CPP apparatus consisted of 2 large pairing and one smaller middle compartment, each with three distinct environments (Grey compartment [G], grey walls with course mesh flooring and dim lights; Middle compartment [M], white walls with bar flooring and brighter lights; Striped compartment [S], black and white striped walls with fine mesh flooring and dim lights). No differences in time in middle compartment were observed for all groups tested (data not shown). An unbiased conditioning apparatus and counterbalanced compartment assignment CPP design was used (Tzschentke, 2007). Mice were trained on CPP with a 5-day paradigm inspired by previous studies with rat psychostimulant CPP (Smith and Aston-Jones, 2014; Zarrindast et al., 2002).

Briefly, on day 1 (pretest) mice were placed in the middle compartment (~11-1 P.M.) and had full access to the CPP box for 30 min. Time spent in each compartment was recorded by 8 photo beams per large compartment (i.e. the larger grey and striped compartments; Med Associates Inc.). For each mouse the time in the grey compartment was subtracted from the time in the striped compartment (i.e. difference score). The difference score was then used to assign a mouse to either a grey or striped CS+ compartment, respectively, such that the final pretest CPP score (i.e. difference score) within a cage was close to zero [modified from (Russo et al., 2007)]. All mice within a cage received either saline/saline (Sal/Sal) or saline/morphine (Sal/Mor), which is why we balanced preferences within each cage. An equal number of mice were conditioned in the grey and striped compartments. Mice that spent more than 20% (>6 min) of their CPP score in any compartment were excluded from future analysis to ensure subjects did not have a bias in the conditioning apparatus (Fig.1, Sal/Sal, n=3; Sal/Mor, n=5; Fig. 3, Sal/Sal, n=3; Sal/Mor, n=8).

During pairing days (D 2–4), the CPP box was divided into the 3 compartments (G, M, S). All mice received saline (subcutaneous administration [s.c.], sterile bacteriostatic 0.9% saline [Hospira]) in the morning (~9–11 A.M.) and were paired to a context (conditioned stimulus, CS–) for 20 min and the number of photo beam breaks was recorded (Bardo et al., 1995). This was repeated in the alternate context (CS+) following saline or morphine (s.c., 15 mg/kg morphine in 0.9% saline; morphine sulfate powder, NIDA) in the afternoon (2–4 P.M.). On day 5 (test day), mice were again allowed access to all 3 compartments of the CPP box for 30 min and time spent in each compartment was recorded. In order to examine the rewarding effects of morphine, any Sal/Mor mouse that had a negative CPP test score was excluded from future analysis (Fig. 1, n=5).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Morphine CPP produces place preference and results in more DG GCL cFos+ cells
 (A) Time line of morphine CPP experiment and schematic of experimental groups. Using a condensed 5-day morphine CPP paradigm Sal/Sal and Sal/Mor mice are placed into the CPP chamber on day 1 (pretest) and allowed to freely move throughout the CPP chamber. On days 2–4, Sal/Sal and Sal/Mor groups are administered saline (S) in the morning and saline or morphine (M) in the afternoon, respectively. On the fifth day, mice are tested and allowed to freely move throughout the CPP chamber. (B) CPP score of Sal/Mor compared to Sal/Sal. One-way ANOVA followed by Sidak post hoc comparisons, *** $P=0.001$; $n=6-13$ /group.

(C) Photomicrograph of cFos+ cells in the DG GCL. Inset: cFos+ nuclei. Scale bar: (C) 100 μm ; (inset) 10 μm . (D) Stereological quantification of cFos+ cells in the DG GCL. Student's *t*-test, $^{\#}P=0.05$; $n=5/\text{group}$. (E) Analysis of total beam breaks on pretest and test day. $n=6-13$ (mean $\pm$ SEM). S=saline, M=morphine, GCL=granule cell layer, H=hilus.

Figure 2. Home cage control experiments result in no change in DG GCL cFos+ cells
 (A) Time line of naïve vs. handled home cage experiment. Briefly, all mice were kept in home cage for 5 days. On the fifth day, mice were either handled or remained in home cage.
 (B) Stereological quantification of cFos+ cells in the DG GCL. n=9–12. (C) Time line of Sal/Sal vs. Sal/Mor home cage experiment. Briefly, all mice followed a similar paradigm as in Figure 1A, but in home cage. (D) Stereological quantification of cFos+ cells in the DG GCL. n=5–6 (mean $\pm$ SEM).

Figure 3. Sequestration to a previously-paired morphine context (CS+) results in more DG GCL cFos+ cells

(A) Time line of morphine CPP sequestration experiment. Briefly, a same paradigm in Figure 1A was used; however on the fifth day mice were sequestered into their previously saline- (CS-) or morphine-paired (CS+) context. (B) Stereological quantification of cFos+ cells in the DG GCL comparing Sal/Mor and Sal/Sal in CS- and CS+ groups, respectively. (C–F) Bregma analysis comparing cFos+ cells per section in CS- (C), CS+ (D), Sal/Sal (E), and Sal/Mor (F) groups. One- and Two-way ANOVA followed by Sidak post hoc comparisons, * $P=0.05$; $n=7-9$ /group (mean $\pm$ SEM).