Discrimination between cocaine-associated context and cue in a modified conditioned place preference paradigm: role of the nNOS gene in cue conditioning

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Abstract

The conditioned place preference (CPP) paradigm entails appetitive learning and is utilized to investigate the motivational effects of drug and natural reward in rodents. However, a typical CPP design does not allow dissociation between cue- and context-dependent appetitive learning. In humans, context and cues that had been associated with drug reward can elicit conditioned response and drug craving. Therefore, we investigated (\(a\)) methods by which to discriminate between cue- and context-dependent appetitive learning, and (\(b\)) the role of the neuronal nitric oxide synthase (nNOS) gene in appetitive learning. Wild-type (WT) and nNOS knockout (KO) mice were trained by cocaine (20 mg/kg) in a discrete context paired with a light cue (a compound context-cue stimulus). In test 1, approach behaviour to either the training context or to the cue in a novel context was determined. WT mice showed robust preference for both cocaine-associated context and cue. nNOS KO mice acquired approach behaviour for the cocaine-associated context but not cue. This finding suggests that the nNOS gene is required for cue-dependent appetitive learning. On the following day (test 2), mice were tested for approach behaviour to the compound context-cue stimulus. Context but not cue exposure in test 1 reduced approach behaviour to the compound context-cue stimulus in test 2, suggesting that repeated context but not cue exposures diminished the conditioned response. Hence, this modified CPP paradigm is useful for the investigation of approach behaviour for both drug-associated context and cue, and allows further investigation of mechanisms underlying cue- and context-dependent appetitive learning.

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Key words: Appetitive learning, approach behaviour, cocaine, compound context-cue stimulus, neuronal nitric oxide synthase (nNOS).

Introduction

In classical Pavlovian conditioning, pairing of an unconditioned stimulus (US) with a neutral context and cues, such as light or sound, confers conditioned stimulus (CS) properties to these entities. Depending on the nature of the US, re-exposure to the CS elicits either approach or avoidance behaviour, i.e. conditioned response (CR). When a specific context is paired with reinforcing stimuli, the CR is approach (e.g. conditioned place preference; CPP). The acquisition of place conditioning requires first, an US that changes the affective state of the organism, and second, learning and memory processes (White & Carr, 1985). The CPP paradigm has been used to investigate the motivational effects of drug and natural reward (Liu \textit{et al.} 2008). Most reinforcing drugs (e.g. cocaine) that promote self-administration produce CPP in rodents (Bardo & Bevins, 2000).
In a typical place-conditioning apparatus, cues such as floor texture and wall colour or pattern are embedded in the context, making it difficult to distinguish between cues and context. Thus the reinforcer-paired ‘environment’ acquires properties of a CS. Subsequent exposure to the CS elicits approach behaviour to the drug-paired environment. This type of learning is also viewed as ‘habit learning,’ which has a major role in the development of drug addiction (Atallah et al. 2007; Everitt & Robbins, 2005). While the context of drug exposure may elicit drug craving, presentation of cocaine- and alcohol-related cues to cocaine and alcohol abusers elicits limbic activation, craving and physiological responses similar to the drugs’ effects, suggesting the emergence of a CR to drug-associated cues (Childress et al. 1999; Newlin, 1992; Robbins et al. 1999).

Given the pivotal role of cue-dependent appetitive learning in the development and expression of maladaptive behaviours associated with repeated drug use, the aim of the present study was 2-fold. Using the cocaine CPP paradigm in mice, we investigated (a) possible means of dissociating between approach behaviour to discrete context and cue which were paired with cocaine administration, and (b) the role of the neuronal nitric oxide synthase (nNOS) gene in the acquisition of cue- and context-dependent approach behaviour.

The nitric oxide (NO) signalling pathway has a role in hippocampus (Arancio et al. 2001; Puzzo et al. 2006) and amygdala (Chien et al. 2003; Schafe et al. 2005) long-term potentiation (LTP). Evidence from behavioural studies in invertebrates suggest that NO has a major role in consolidation of long-term memory (Kemenes et al. 2002; Lewin & Walters, 1999; Matsumoto et al. 2006; Muller, 2000). More recent studies have suggested the role of NO signalling in contextual (Resstel et al. 2008) and cued (Schafe et al. 2005) fear learning. Our recent studies have shown that adult male mice with a targeted mutation of the nNOS gene (knockout; KO) had a major deficit in contextual fear learning and a mild deficit in cued fear learning (Kelley et al. 2009). However, nNOS KO mice have acquired ‘normal’ cocaine-induced CPP, but expressed only a transitory place preference compared to their wild-type (WT) counterparts (Balda et al. 2006). Together these findings suggest the role of the nNOS gene in associative learning.

In the present study, we investigated the development of approach behaviour to discrete contexts and cues associated with cocaine administration, using a modified CPP paradigm in mice. In addition, the role of the nNOS gene in the development of conditioned behaviour to drug-associated contextual and cued stimuli was investigated.

Materials and methods

Animals

Mice purchased from Jackson Laboratories (USA) were bred in our facilities at the University of Miami, Miller School of Medicine, Miami, FL, as described previously (Balda et al. 2006; Itzhak & Anderson, 2008). Both genotypes, WT and nNOS KO, were generated on a mixed B6;129S genetic background (Huang et al. 1993). For the experiments described herein, adult (aged 7–8 wk) WT and nNOS KO males were used. Animals were housed in a temperature-(22 ± 0.5 °C) and humidity-(50%) controlled room and maintained on a 12-h light/dark schedule (lights on 07:00 hours) with free access to food and water. Animal care was in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, 1996) and approved by the University of Miami Animal Care and Use Committee.

Drugs

Cocaine HCl (Sigma, USA) was dissolved in saline (0.9% NaCl). Drug and saline vehicle were administered intraperitoneally in a volume of 0.1 ml/10 g. Mice were conditioned by 20 mg/kg cocaine, a dose we found optimal in WT and nNOS KO mice for the acquisition of cocaine CPP (Balda et al. 2006).

Training and testing apparatus

Custom-designed Plexiglas cages (42 l x 20 w x 20 h cm; Opto-Max Activity Meter v. 2.16; Columbus Instruments, USA) were used. The training context consisted of two compartments, separated by a removable guillotine door, one comprising four black walls with a smooth black floor and the other four white walls and a floor covered with sandpaper (fine grit 150C, Norton) (Fig. 1a). Each compartment was covered by a transparent ceiling, perforated with an array of 16 small holes (1-cm diameter, in an array of four rows with four holes each) to allow ventilation (not shown in Fig. 1). We introduced four flashing lights (mini bulbs; 2.5 V each) through the ceiling perforations (Fig. 1a) as a cue. A compound test cue stimulus was always paired with cocaine administration. Because experiments were carried out in an unbiased design (half of the subjects were conditioned by cocaine in the black compartment and the other half...
in the white compartment) the light cue was present either in the black or in the white compartment of the training apparatus. Upon completion of the training (see below), approach behaviour towards cocaine-associated context and cue was tested as follows, one group was tested in the training context in the absence of the light cue and a second group was tested in a novel context (Fig. 1b) in the presence of the light cue. Drug-associated cue preference was tested in the novel context (b). One compartment was covered with white stars (2.5 cm in diameter) on a black background, and the second compartment was covered with black and white stripes (2 cm wide × 20 cm high). These removable covers were washed with diluted laboratory-grade soap (Alconox) followed by a water rinse after each test. Each cage was equipped with 2 horizontal sensors mounted alongside opposing lengths. The two compartments (21 × 20 × 20 cm) were each scanned by seven infrared beams at a rate of 10 Hz (2.54-cm intervals). A null zone 8-cm wide was assigned at the interface of the two compartments to ensure that only full entry into each compartment was registered as ‘real’ time spent in each zone.

Training procedure

Training and testing were carried out in dimmed lighting (30 W; a reading lamp with two 18-in. F15T8 white fluorescent bulbs, 15 W each, faced a wall) in a test room separate from the housing room. On the first day, between 12:00 and 14:00 hours, mice were habituated (20 min) to the training context (Fig. 1a) in the absence of the light cue; time spent in each compartment was recorded to determine preconditioning compartment-preference/aversion. To ensure a strictly unbiased training design, mice that showed initial preconditioning preference of >10–12% of the total time (20 min) for either compartment were discarded. As we have previously described (Itzhak et al. 2009), routinely 20–23% of the mice of each genotype had initial biased preference either to the black or to the white compartment of the cage and were subsequently omitted from the experiment. This bias was random towards either compartment, and no genotype-dependent bias was observed. For the next 4 d (days 2–5) WT and nNOS KO mice (n = 32–34 per group) were trained by a morning (10:00–12:00 hours) saline session and an afternoon (14:00–16:00 hours) cocaine (20 mg/kg) session, each lasting 30 min. Successful acquisition of cocaine CPP in nNOS KO mice required daily training by cocaine (Balda et al. 2006) instead of alternate-day training (Itzhak et al. 1998). For the unbiased design, training was counterbalanced, half of the subjects were trained with drug in the black compartment and the other half in the white compartment. The order of injections (saline-drug) was not counterbalanced because drug administration in the morning session may have a remaining effect into the afternoon session. Cocaine was administered immediately before the animal was placed into the appropriate compartment, and the light cue was turned on 10 min later. Animals thus experienced the presence of flashing lights for the final 20 min of the drug-training session. This period corresponds to the peak effect of cocaine (Itzhak, 1997). Control groups of both genotypes (n = 18–20 per group) received saline instead of cocaine in the afternoon session. Following each training session, the sandpaper was removed, the cage
Table 1. Experimental design

<table>
<thead>
<tr>
<th>Experimental timeline (days)</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7</td>
<td>Context/Cue</td>
<td>Context/Cue</td>
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<table>
<thead>
<tr>
<th>Group I</th>
<th>Habituation Training</th>
<th>Context</th>
<th>Context/Cue</th>
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<tr>
<td>WT/KO</td>
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<table>
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<tr>
<th>Group II</th>
<th>Habituation Training</th>
<th>Cue</th>
<th>Context/Cue</th>
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<td>WT/KO</td>
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On day 1, wild-type (WT) and neuronal nitric oxide synthase knockout (KO) mice were habituated to the training context (Fig. 1a) in the absence of the light cue. On days 2–5 mice were trained by a morning saline session and afternoon cocaine session, in the presence of the light cue, as described in Materials and methods section. On day 6 test 1 was performed. Mice of both genotypes were assigned to two groups; group I was tested for place preference in the training context (Fig. 1a) in the absence of the light cue, and group II was tested for cue preference in the novel context (Fig. 1b). On day 7, test 2 was performed in the training context in presence of the light cue (compound context-cue stimulus).

Testing procedure

All tests were carried out between 12:00 and 14:00 hours, i.e. during the same time period in which the pretraining habituation had been recorded. Each test was performed in a drug-free state and it lasted for only 10 min to minimize extinction learning. Group I of each genotype was first tested for context preference (in test 1), while group II of each genotype was first tested for cue preference (in test 1). See Table 1 for the experimental timeline and group assignments.

Test 1

The day following the completion of training, group I of each genotype (n=16–18) was tested for context preference. Animals were placed in the training context in the absence of the light cue (Fig. 1a), and time spent in each compartment was recorded for 10 min. Group II of each genotype (n=16–18) was tested for cue preference in the novel context (Fig. 1b). The light cue was activated and flashing prior to entry of the mice into the testing room. Mice were then brought into the room and placed in the center of the cages (Fig. 1b). Preference for the cue was recorded for 10 min. Half of the mice of group II (n=8–9) were tested with the light cue in the same side of the cage in which the cue had been present during training. To avoid influence of spatial learning of the location of the light cue, the remaining mice from group II (n=8–9) were tested with the light cue located in the part of the cage corresponding to the spatial location opposite to that present during training. The outcome of these two tests was remarkably similar, suggesting that animals responded to the drug-associated cue and not to the spatial location of the light; hence, the results of the two tests were combined.

Test 2

Twenty-four hours later, both groups I (context-tested) and II (cue-tested) of each genotype were retested for approach behaviour to the compound context-cue stimulus. The light cue was placed in the training context, on the side in which drug had been administered, and preference for each side of the apparatus was registered for 10 min. Table 1 summarizes the experimental design of training and testing.

Statistical analysis

The raw values (time spent in saline- and drug-associated context/cue) of each test were analysed by Wilcoxon signed-rank test (Fig. 2). The Wilcoxon signed-rank test is a non-parametric procedure, which does not require assuming normality or equal variance. This test is used when: (a) the effect of a single treatment on the same individual is tested, and (b) when it is unknown if the treatment effect is normally distributed with the same variances. The parametric paired t test is used when one knows that the effects are normally distributed. Results showed that when a significant p value was observed in the Wilcoxon test, a significant p value was also observed in the paired t test, suggesting that the effect was normally distributed. Subsequently, the magnitude of preference for cocaine-associated context and cue in test 1 across the genotypes was analysed by two-way ANOVA: genotype x group (group I context vs group II cue) (Fig. 3a). Test 2 investigated approach behaviour to the compound context-cue stimulus. To determine if results of test 2 were influenced by test 1, i.e. if the first exposure to either the context or cue had an effect on approach behaviour to the compound stimulus, two-way ANOVA was performed. The variables were genotype and groups (group I context-tested vs group II cue-tested) (Fig. 3b). Specific differences between groups were analysed by post-hoc Newman–Keuls test. A p value <0.05 was considered significant.
**Results**

*Response to context and cue following training of WT mice by cocaine*

WT mice that had been conditioned by cocaine and were tested in the training context (group I, test 1)
showed significant preference for the drug-paired compartment over the saline-paired compartment $(\Delta = 196 \pm 28 \text{ s}, \text{z} = 3.89, \text{p} < 0.001; \text{Fig. 2a})$, suggesting the development of CPP. The following day, retesting of group I in the training context in the presence of the cue (a compound context-cue stimulus, test 2) resulted in significant preference for the compound stimulus compared to the saline-paired context $(\Delta = 125 \pm 17 \text{ s}, \text{z} = 3.15, \text{p} < 0.01; \text{Fig. 2a}, \text{test 2})$. However, the magnitude of approach behaviour in test 2 $(125 \pm 7 \text{ s}, \text{Fig. 3b})$ was significantly lower than in test 1 $(196 \pm 28 \text{ s}, \text{z} = 2.24, \text{p} < 0.05; \text{Fig. 3a})$.

WT mice that had been conditioned by cocaine, and were first tested for cue in the novel context (group II, test 1) showed significant preference for the light cue associated with cocaine administration over the opposite compartment $(\Delta = 156 \pm 25 \text{ s}, \text{z} = 4.06, \text{p} < 0.001; \text{Fig. 2b})$. This finding suggests that the light cue associated with cocaine administration was sufficient to evoke approach behaviour. The following day, results of test 2 showed that group II had high preference for the compound context-cue stimulus over the saline-paired context $(\Delta = 254 \pm 30 \text{ s}, \text{z} = 4.62, \text{p} < 0.001; \text{Fig. 2b})$.

Response to context and cue following training of nNOS KO mice by cocaine

nNOS KO mice that had been conditioned by cocaine and were first tested for cue in the novel context (group II, test 1) showed significant preference for the drug-paired compartment vs. the saline-paired compartment $(\Delta = 195 \pm 37 \text{ s}, \text{z} = 3.73, \text{p} < 0.001; \text{Fig. 2c})$, suggesting the development of CPP. On the following day, results of test 2 showed that group I of nNOS KO mice had no significant preference for the compound context-cue stimulus (Fig. 2c), suggesting complete loss of place preference.

nNOS KO mice that had been conditioned by cocaine and were first tested for cue preference in the novel context (group II, test 1) showed no significant preference for the light cue $(\Delta = 40 \pm 33 \text{ s}, \text{Fig. 2d})$. Hence, in contrast to WT mice, nNOS KO mice did not acquire preference for the light cue that had been associated with cocaine administration. The finding that nNOS KO mice had no significant preference to either compartment of the novel context suggests that the novel context was indeed different from the training context. The following day, results of test 2 showed that group II had high preference for the compound context-cue stimulus over the saline-paired context $(\Delta = 214 \pm 36 \text{ s}, \text{z} = 4.69, \text{p} < 0.001; \text{Fig. 2d})$. Notably, exposure of nNOS KO mice to the compound context-cue stimulus, in test 2, elicited approach behaviour similar in magnitude to the group I that was first tested for context-dependent approach behaviour $(\Delta = 195 \pm 37 \text{ s}, \text{Fig. 2c})$.

Comparison between results from WT and nNOS KO mice

Further analysis of the results from WT and nNOS KO mice was assessed by comparing the magnitudes of conditioned context and cue preference between the genotypes. Results in Fig. 3 represent the difference between the times spent in drug- and saline-associated context/cue as a measure of ‘magnitude’. In test 1, the magnitude of preference for cocaine-associated context and cue across the genotypes was analysed by two-way ANOVA: genotype x group (context vs. cue). There was a significant genotype effect $(F_{1,64} = 10.98, \text{p} < 0.01)$, a significant group effect $(F_{1,64} = 20.55, \text{p} < 0.001)$ and a significant interaction $(F_{1,64} = 9.37, \text{p} < 0.01; \text{Fig. 3a})$. Newman–Keuls post hoc analysis resulted in a significant genotype effect within the cue groups $(q = 6.28, \text{p} < 0.001)$, and a significant group effect (context vs. cue) within nNOS KO mice $(q = 7.49, \text{p} < 0.001)$. No significant group effect was observed in WT mice $(q = 1.49, \text{p} > 0.05; \text{Fig. 3a})$, suggesting that the magnitude of approach behaviour of WT mice for the context and cue was similar (Fig. 3a).

To determine if results of test 2 were influenced by test 1, i.e. if the first exposure to either the context or cue had an effect on the expression of approach behaviour in test 2, a two-way ANOVA was performed for results of test 2. The variables were genotype and groups (group I context-tested vs. group II cue-tested). There was a significant genotype effect $(F_{1,64} = 5.35, \text{p} < 0.05)$ and a significant group effect $(F_{1,64} = 29.33, \text{p} < 0.000)$. Newman–Keuls post hoc analysis revealed the following significant differences, (1) WT vs. KO mice in test 2, when test 1 had been carried out in the training context $(q = 3.29, \text{p} < 0.05)$; (2) between WT mice that were exposed to context or cue in test 1 $(q = 4.53, \text{p} < 0.01)$; (3) between KO mice that were exposed to context or cue in test 1 $(q = 6.277, \text{p} < 0.001)$ (Fig. 3b). No significant differences between WT and KO mice were observed in test 2 if the genotypes had been exposed to the cue in test 1 (Fig. 3b).

Response to context and cue following training of WT and nNOS KO mice by saline

This experiment determined if (a) the training context, (b) the light cue and (c) the novel context (stars and stripes) have affective properties that might have influenced approach and avoidance behaviour in WT
neither approach nor avoidance behaviour in either session lasted 10 min. The environments tested produced (stars and stripes) in the presence of the light cue. Each test presence of the light cue, and in a novel testing apparatus (black and white) in the presence of the light cue had no ad-

tion of the training apparatus to drug-associated context and cue, and (b) the nNOS gene has a more prominent role in the acquisition of CR to drug-associated cue than to drug-associated context.

The traditional CPP paradigm has been used to investigate several elements of addictive behaviour such as habit formation and reactivity to drug-associated environment, as well as extinction and reinstatement of conditioned behaviour (Itzhak & Martin, 2002; Sanchis-Segura & Spanagel, 2006; Tzschentke, 2007). However, it has been difficult to dissociate between CR to drug-associated context and cue in the traditional CPP paradigm because cues such as wall colour or pattern and floor texture are embedded in the context of the conditioning apparatus. Given the important role of drug-associated cues in the precipitation of relapse (Childress et al. 1999; Newlin, 1992; Robbins et al. 1999), the present study has attempted to discriminate between the development of CR to drug-associated context and cue by modifying the traditional CPP paradigm. To this end we introduced a flashing light cue during the conditioning phase (Fig. 1a). Separate groups of WT mice that had been conditioned by cocaine were tested for preference to cocaine-associated context and for preference to cocaine-associated cue in a novel environment. Results show that both WT groups developed similar magnitude of preference for cocaine-associated context and cue (Fig. 3a). In contrast, nNOS KO mice expressed preference for cocaine-associated context but not cue. The finding that mice of both genotypes that had been conditioned by saline only, had neither preference nor aversion to the training and novel contexts and cue, suggests that contextual and cued stimuli that we used had no influence on the results of conditioning by cocaine. These findings suggest that WT mice acquired both context- and cue-dependent appetitive learning while nNOS KO mice had deficits in cue-dependent appetitive learning.

These results are different from our recent studies on fear conditioning where we found that nNOS KO mice had a greater deficit in context-dependent fear learning (Kelley et al. 2009). Notably however, multiple fear conditioning sessions (four) significantly improved context-dependent fear learning (Kelley et al. 2009). Given that in the present study mice had not only one, but four cocaine training sessions, it is unclear why the deficit in cue-dependent cocaine conditioning was persistent. Although appetitive and aversive learning may recruit similar substrate, such as the amygdala (cue-dependent learning) and hippocampus (spatial learning), the NO signalling pathway may have a differential role in

Table 2. Time (s) spent in each compartment of the conditioning apparatus after training by saline (controls)

<table>
<thead>
<tr>
<th></th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
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<tbody>
<tr>
<td>WT mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Context</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>Cue</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Time (s)</td>
<td>271 ± 23</td>
<td>258 ± 31</td>
</tr>
<tr>
<td>Context</td>
<td>Stars</td>
<td>Black</td>
</tr>
<tr>
<td>Cue</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Time (s)</td>
<td>237 ± 29</td>
<td>266 ± 31</td>
</tr>
<tr>
<td>nNOS KO mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Context</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>Cue</td>
<td>No</td>
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<tr>
<td>Time (s)</td>
<td>261 ± 31</td>
<td>241 ± 25</td>
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<td>Stars</td>
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</tr>
<tr>
<td>Cue</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Time (s)</td>
<td>254 ± 29</td>
<td>247 ± 27</td>
</tr>
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</table>

WT, Wild type; nNOS, neuronal nitric oxide synthase knockout.
Mice were trained daily by two saline sessions for 4 d. Results represent the mean ± S.E.M. time spent in each compartment of the training apparatus (black and white) in the absence and presence of the light cue, and in a novel testing apparatus (stars and stripes) in the presence of the light cue. Each test session lasted 10 min. The environments tested produced neither approach nor avoidance behaviour in either genotype.

and nNOS KO mice. Both genotypes were treated in the same manner as the experimental group, except that the cocaine injection of the afternoon session was replaced by saline. Results are summarized in Table 2. Both WT and nNOS KO mice spent about equal time in the black and white compartments of the conditioning apparatus (Table 2, test 1). Addition of the light cue into the training context did not change the preference for either side of the cage (test 2). Likewise, time spent in the two compartments of the novel context (stars and stripes) in the presence of the light cue was similar, and retesting in the training context (black and white) in the presence of the light cue had no additional effect on time spent in each compartment. The results suggest that the light cue and the novel context were neutral, and had no influence on approach/avoidance behaviour in control WT and nNOS KO mice.

Discussion
The two major findings of the present study are, (a) a modified CPP paradigm can be used to investigate CR to drug-associated context and cue, and (b) the nNOS gene has a more prominent role in the acquisition of CR to drug-associated cue than to drug-associated context.

The traditional CPP paradigm has been used to investigate several elements of addictive behaviour such as habit formation and reactivity to drug-associated environment, as well as extinction and reinstatement of conditioned behaviour (Itzhak & Martin, 2002; Sanchis-Segura & Spanagel, 2006; Tzschentke, 2007). However, it has been difficult to dissociate between CR to drug-associated context and cue in the traditional CPP paradigm because cues such as wall colour or pattern and floor texture are embedded in the context of the conditioning apparatus. Given the important role of drug-associated cues in the precipitation of relapse (Childress et al. 1999; Newlin, 1992; Robbins et al. 1999), the present study has attempted to discriminate between the development of CR to drug-associated context and cue by modifying the traditional CPP paradigm. To this end we introduced a flashing light cue during the conditioning phase (Fig. 1a). Separate groups of WT mice that had been conditioned by cocaine were tested for preference to cocaine-associated context and for preference to cocaine-associated cue in a novel environment. Results show that both WT groups developed similar magnitude of preference for cocaine-associated context and cue (Fig. 3a). In contrast, nNOS KO mice expressed preference for cocaine-associated context but not cue. The finding that mice of both genotypes that had been conditioned by cocaine only, had neither preference nor aversion to the training and novel contexts and cue, suggests that contextual and cued stimuli that we used had no influence on the results of conditioning by cocaine. These findings suggest that WT mice acquired both context- and cue-dependent appetitive learning while nNOS KO mice had deficits in cue-dependent appetitive learning.

These results are different from our recent studies on fear conditioning where we found that nNOS KO mice had a greater deficit in context-dependent fear learning (Kelley et al. 2009). Notably however, multiple fear conditioning sessions (four) significantly improved context-dependent fear learning (Kelley et al. 2009). Given that in the present study mice had not only one, but four cocaine training sessions, it is unclear why the deficit in cue-dependent cocaine conditioning was persistent. Although appetitive and aversive learning may recruit similar substrate, such as the amygdala (cue-dependent learning) and hippocampus (spatial learning), the NO signalling pathway may have a differential role in
appetitive (cocaine) and aversive (fear) learning. That is, the NO signalling may be required for cue-dependent appetitive learning more than for cue-dependent aversive learning. Additionally, evidence suggests a major role for the dorsal striatum in appetitive learning and habit formation (Belin et al. 2009). In our previous studies, we found selective up-regulation of nNOS immunoreactive neurons in the dorsal striatum following repeated cocaine administration to WT mice (Balda et al. 2008). Hence the critical role of dorsal striatum nNOS-containing neurons in cocaine effects we described, may partially explain the persistent deficit in cocaine-associated cue-dependent learning (e.g. habit formation) observed in nNOS KO mice in the present study. Further, it should be noted that in aversive conditioning (fear) the cue was auditory while in the appetitive conditioning (cocaine) the cue was visual (flashing light). It is thought that rapid auditory cue learning is mediated by direct projections from the auditory thalamus to the lateral amygdala while slow visual cue learning is mediated by indirect projections from the visual thalamus to the lateral amygdala (Newton et al. 2004). These differences may also partially explain the differential response of nNOS KO mice to the visual cue in the present study and the auditory cue in the fear-conditioning study (Kelley et al. 2009).

We further investigated animals’ response to the compound context-cue stimulus. We anticipated that approach behaviour for the compound stimulus would be higher than the response to context or cue alone. However, WT and nNOS KO mice that were tested for context preference in test 1 showed reduction (WT mice) or absence (nNOS KO mice) of approach behaviour in test 2 (Fig. 3b). We hypothesize that the diminished approach behaviour in WT mice was due to initiation of extinction learning. Because WT mice were exposed to the training context in the absence of drug (US) for two consecutive days (tests 1 and 2), the reduced approach behaviour in test 2 was probably due to the acquisition of extinction learning. Repeated exposure to the CS in the absence of the US elicits extinction learning (Bouton, 2004).

However, we hypothesize that the loss of approach behaviour in nNOS KO mice following exposure to the context in test 1 (Fig. 3b) was due to disruption of memory reconsolidation rather than accelerated extinction learning compared to WT mice. This hypothesis is supported by our previous studies. nNOS KO males acquired cocaine place preference as had WT mice, but upon a second test, place preference was no longer apparent, and subsequent cocaine priming did not reinstate place preference in nNOS KO mice as it did in their WT counterparts (Balda et al. 2006). This finding suggests that in the absence of NO signalling, cocaine place preference was acquired but did not persist as it did in WT mice. To investigate whether the limited expression of cocaine place preference in nNOS KO mice was due to impairment of memory reconsolidation in the absence of NO signalling, two experiments were performed. In the first, WT mice that have been conditioned by cocaine received systemic administration of the nNOS inhibitor 7-nitroindazole or saline (controls) upon the first retrieval of cocaine place preference. In subsequent tests, place preference was apparent in the control group but not in subjects that received the nNOS inhibitor (Itzhak & Anderson, 2007). This finding suggests that administration of the nNOS inhibitor upon memory retrieval of cocaine-associated context disrupted reconsolidation and thus subsequent place preference was no longer apparent. In a second experiment, we investigated if systemic administration of the NO donor molsidomine to nNOS KO mice upon retrieval of cocaine place preference prolongs the expression of place preference. Indeed, the NO donor afforded a transient prolongation of place preference compared to nNOS KO mice that received saline. This finding suggests that NO signalling is required for stabilization of reconsolidation of place preference (Itzhak & Anderson, 2007). These and our previous studies suggest that, in the absence of NO signalling pathway, the acquisition of context-dependent appetitive learning is not impaired. However, upon memory retrieval the reconsolidation process may be impaired and consequently long-term contextual memory is disrupted. Several studies have suggested that disruption of memory reconsolidation by drugs that block, (a) extracellular signal-regulated kinase (ERK) pathway (Miller & Marshall, 2005; Valjent et al. 2006), (b) protein synthesis (Milekic et al. 2006), (c) β-adrenergic (Bernardi et al. 2006), (d) muscarinic cholinergic, and (e) N-methyl-D-aspartate (NMDA) (Kelley et al. 2007) receptors abolished further expression of cocaine or morphine CPP.

Interestingly, when WT and nNOS KO mice were first tested for cue reactivity (test 1) they subsequently showed robust approach behaviour for the compound context-cue stimulus (test 2, Fig. 3b). These findings suggest that exposure to the cue in the novel context (test 1) had no influence on subsequent re-exposure to the cue in the training context (test 2). That is, exposure of WT mice to the cue in the novel context did not elicit extinction learning as had the exposure to the training context in test 1. The finding that WT mice pre-exposed to the cue (test 1) showed significantly
higher approach behaviour in test 2 compared to WT mice that had been pre-exposed to the training context (test 1) (Fig. 3b) further supports the concept that context but not cue exposure elicited extinction learning. This observation is in accordance with the premise that the training context has a major role in extinction learning (Bouton, 2004).

nNOS KO mice did not acquire cue-dependent appetitive learning (test 1). Exposure of nNOS KO mice to the compound context-cue stimulus (test 2) elicited approach behaviour similar in magnitude (Fig. 3b) to the group that was first tested for context-dependent approach behaviour (test 1) (Fig. 3a). Hence, the light cue had no significance either in the acquisition or in the expression of cocaine-induced approach behaviour in nNOS KO mice.

In summary, the present results demonstrate the utilization of a modified place-conditioning paradigm to investigate both context- and cue-dependent appetitive learning. This paradigm allows dissociation between approach behaviour to discrete contextual and cued stimuli associated with the motivational effect of cocaine. Moreover, we found that the nNOS gene has a more prominent role in the acquisition of approach behaviour to cocaine-associated cue than cocaine-associated context.

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Statement of Interest

None.

References


