Behavioural Brain Research 216 (2011) 424–432

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

The effects of VTA NMDA receptor antagonism on reward-related learning and associated c-fos expression in forebrain

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ARTICLE INFO

Article history:
Received 11 May 2010
Received in revised form 17 August 2010
Accepted 23 August 2010
Available online 27 August 2010

Key words:
Reward
Glutamate
AP-5
Motivation
Food
Conditioning
Nucleus accumbens
Prefrontal cortex
Caudate
VTA

ABSTRACT

The mechanisms whereby reward-associated stimuli come to function as conditioned stimuli and acquire the capacity to activate the same neural regions activated by primary rewards (i.e., dopamine terminal regions) is not fully understood. We hypothesized that NMDA receptor stimulation in the VTA is necessary for the acquisition by a CS to both produce conditioned approach and activate dopamine terminal regions. Rats were tested in a conditioned approach protocol that consisted of light stimulus-food conditioning sessions (30 randomly presented light stimulus-food pellet pairings), a session with no stimuli or food and 1 session with only light stimulus (CS-only) presentations. Food trough head entries during the CS and just prior to the CS were recorded and a CS/pre-CS ratio indicating the conditioned approach response was calculated. Brain tissue was harvested after the CS-only session and processed for c-fos expression in prefrontal cortex area 2, nucleus accumbens core and shell and medial and lateral caudate. When bilateral intra-VTA microinjections of AP-5 (0, 0.25 or 0.5 μg) were made prior to each of the conditioning sessions the 0.5 μg AP-5 dose prevented the acquisition of conditioned approach; when 0.5 μg AP-5 injections were made prior to the CS-only test they failed to affect expression of the response. Also, 0.5 μg AP-5 prior to conditioning significantly reduced c-fos expression in response to the CS in nucleus accumbens core. These results suggest that VTA NMDA receptor stimulation is necessary for both the acquisition of reward-related learning and acquisition by the CS to activate dopamine terminal regions.

Understanding the neural mechanisms of reward-related learning is central to understanding behavior in general and crucial to understanding psychopathologies like addiction, pathological impulsivity and depression. While the mechanisms underlying reward-related learning are not fully understood, it is likely that neural plasticity in brain regions mediating reward-related behavior is involved. The brain's reward system includes the dopamine (DA) neurons originating in the midbrain and projecting to forebrain regions such as the prefrontal cortex, nucleus accumbens (NAcc) and caudate. Consumption of natural [1–3] or drug [1,4,5] reward is associated with increases in extracellular levels of DA in these regions. Impairments in DA neurotransmission in NAcc, amygdala, prefrontal cortex and caudate diminishes the rewarding effects of natural [6,7] and drug [8–11] rewards, suggesting that these reward-associated DA signals are important for mediating the rewarding effects of these unconditioned stimuli. However, in addition to unconditioned stimuli, DA elevations are also observed during presentation of reward-associated stimuli or conditioned stimuli (CSs) [12–16]. Thus, it appears that a component of reward-related learning consists of the acquisition by CSs of the ability to produce activity that leads to DA release and its post-synaptic consequences in forebrain regions innervated by DA afferents.

An important question is, what processes underlie the changes necessary to enable the presentation of a CS to result in DA release? We have hypothesized that the ventral tegmental area (VTA), the site of origin of the mesocorticolimbic DA system, is a critical site for synaptic plasticity underlying food reward-related learning [17]. This site receives glutamate afferents from prefrontal cortex [18,19], amygdala and bed nucleus of the stria terminalis [20,21] and pedunculopontine nuclei [22], which could carry information about CSs. Also, long-term potentiation (LTP), a neurophysiological mechanism long believed to be important in learning, has been demonstrated in VTA [23]. We have suggested [17,24,25] that LTP in the VTA allows for previously neutral environmental signals, which stimulate the VTA in close temporal proximity to signals representing the receipt of primary rewards, to come to activate VTA DA cells in themselves. Generally, the development of LTP appears to be NMDA receptor dependent but the expression of LTP is not [26]. This would suggest that if NMDA receptor stimulation in the VTA is involved in reward-related learning, because of the LTP it produces,

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doi:10.1016/j.bbr.2010.08.026

then its role is limited to the acquisition of the learning, but not its expression. Another role for VTA NMDA receptor stimulation in reward-related learning may be to allow synaptic plasticity to occur in regions downstream of the VTA involved in reward-related learning. Stimulation of NMDA receptors in VTA plays a role in VTA DA cell burst firing [27], leading to phasic DA increases in terminal regions that could facilitate plasticity in those regions [28–30] and feed back an amplified CS signal to the VTA, resulting in a now strengthened CS signal capable of activating VTA DA cells. Thus, a role for VTA NMDA receptor stimulation in the acquisition of reward-related learning might be the mediation of LTP-dependent VTA plasticity, DA-mediated LTP in DA terminal regions, or both.

In previous work we tested the hypothesis that NMDA receptor stimulation in the VTA is necessary for the acquisition, but not expression, of food-reinforced instrumental responding. When animals were treated with intra-VTA AP-5, a selective NMDA receptor antagonist, prior to the initial sessions of an operant conditioning paradigm, they failed to demonstrate learning of the response, but when the treatment was made after acquisition it had no effect on the learned response [24]. The aims of the present studies were to further test the role of VTA NMDA receptor stimulation in reward-related learning and to investigate the forebrain regions downstream of the DA systems that might be associated with this NMDA-mediated plasticity.

Specifically, the aims of the present studies were to investigate (1) if the necessary role of VTA NMDA receptor stimulation in instrumental conditioning also applies to classical conditioning and (2) to test the hypothesis that a reward-associated stimulus becomes a CS because of VTA NMDA receptor mediated plasticity that allows it to activate DA terminal regions.

The first aim involved investigating the effects of intra-VTA injections of AP-5 on the acquisition and expression of conditioned approach. Given that a number of factors are involved in instrumental learning, including stimulus-response associations and other motor learning that are most likely mediated by regions other than the VTA [28–30], we thought it prudent to replicate the findings of our previous study by testing the role of NMDA receptors in the VTA in a reward-learning task that does not require the animal to acquire any new behaviors, but simply to form an association between a stimulus and a reward in a classical conditioning paradigm. If NMDA receptor antagonism prevented a CS from influencing behavior in this protocol, this would provide further support for our hypothesis regarding synaptic plasticity in the VTA underlying reward-related learning. Specifically, we hypothesized that VTA NMDA receptor antagonism would impair the acquisition of the conditioned approach response but not its expression. During the course of these experiments a report [31] has been published that investigated the same question and supports our hypothesis.

The second aim was to investigate the activation patterns, through the expression of c-fos protein, in reward-learning-implicated forebrain regions in association with conditioned approach. This approach is predicated on the presumption that CSs function as such because they acquire the capacity to appropriately activate the region or regions involved in reward-related responding. Further, we [25] and others [31,32] have hypothesized that one possible role of VTA NMDA receptor stimulation in reward-related learning is to initiate the synaptic plasticity whereby reward-related stimuli can come to activate these brain regions and cause conditioned reward approach. If this is the case then blockade of NMDA receptors in the VTA should both prevent the acquisition of conditioned approach, on the behavioral level, as well as the CS activation of the relevant brain regions, indicated by c-fos expression. Thus, in this second aim, we tested the hypothesis that intra-VTA AP-5 injections would impair the acquisition of conditioned approach and reduce the expression of c-fos in response to the CS in one or more of the following regions: prefrontal cortex area 2 (PFC2), NAcc core, NAcc shell, medial caudate and lateral caudate.

1. Methods

The protocols used in the present experiments were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Queens College Institutional Animal Care and Use Committee.

1.1. Subjects

Subjects consisted of 65 male Long Evans rats, facility-bred from males and females obtained from Charles River Laboratories (Raleigh, NC), with initial free-feeding weights between 350 and 375 g at the time of surgery. All rats were individually housed and maintained on a 12 h light:12 h dark cycle (lights off at 6 AM). All experimental sessions were conducted during the dark phase in order to test the rats during their active periods. All animals had unlimited access to food (Purina rat chow) until experimental sessions began, at which time access was restricted to daily rations that maintained their weights at 85% of their free-feeding values.

1.2. Surgery

All animals received an intraperitoneal (IP) injection of atropine sulfate (0.1 ml) and were anesthetized by sodium pentobarbital (65 mg/kg). Stainless steel guide cannulae (0.635 mm outer diameter, 0.3302 mm inner diameter) were bilaterally implanted to a depth that allowed for microinjections into the ventral tegmental area (VTA) using the following coordinates: −5.6 mm caudal to bregma, ±2.0 mm from the midline at a 10° angle toward the midline and −8.3 mm below the surface of the skull [33]. The cannulae were fixed in dental acrylic anchored to the skull by four stainless steel screws. Obturators (0.3048 mm diameter), extending 1 mm beyond the tip of the cannulae, were inserted at all times except during microinjections.

1.3. Apparatus

All behavioral testing was conducted in eight conditioning chambers each measuring 30 cm × 21 cm × 18 cm ($l \times w \times h$). One wall was equipped with a food trough and two white stimulus lights, each situated 2.54 cm above and 2.54 cm to the right or left of the food trough. Each chamber was housed in a ventilated, sound-attenuating box. The chambers were controlled by a PC through a MED Associates interface.

1.4. Conditioning experiments

Four to seven days after surgery animals began the food restriction diet to reduce their weights to 85% of their free-feeding values where they were maintained for the duration of the experiments. At least one day after food restriction began all rats were given 20 food pellets (45 mg, Purified Formula, Bioserv, Frenchtown, NJ) in their home cages on each of three days. All animals were then tested in one of two versions of the conditioned approach paradigm. One version, the acquisition test, was used to investigate the effects of treatment on the acquisition of conditioned approach and the other version, the expression test, was used to investigate the effects of treatment on the expression of a learned conditioned approach.

1.5. Conditioning procedure for acquisition test

Subjects were given one 20-min magazine training session in the conditioning chambers in which 20 food pellets were delivered on a random time schedule, to allow rats to become acquainted with magazine delivery of food pellets. Rats were then randomly assigned to groups receiving bilateral intra-VTA microinjections of vehicle (physiological saline) or the selective NMDA receptor antagonist AP-5 (0.25 and 0.5 μg) immediately before each of three 60-min conditioning sessions administered on consecutive days. During conditioning sessions, 30 food pellets were delivered on a random time 120-s schedule (range of 15–245 s). Each pellet delivery was preceded by a 3-s presentation of a light on the left side of the trough. In a random-control group the light presentations and food deliveries were not correlated to each other. After the 3 conditioning sessions with intra-VTA treatment, all rats received one 30-min session with no treatment during which no light or food presentations were programmed. This was followed by a CS-only test session in which rats were presented with light presentations under the same random time schedule as in conditioning but with no further consequences (no food). All rats received intra-VTA vehicle injections prior to the CS-only test session. For all rats the number of head entries during each session was counted and analyzed (see Section 1.10 below for details). After the last session the animals were killed and their brains were extracted and prepared for histological cannula placement verification and immunohistochemical procedures (details below). Only two rats were excluded from the data analysis due to misplaced cannulae.

1.6. Conditioning procedure for expression test

This procedure was similar to the one described in the acquisition test but with the following differences: the number of conditioning sessions was 7 (to ensure a learned response), no microinjections were made prior to the conditioning sessions, and microinjections of vehicle or 0.5 μg AP-5 were made prior to the CS-only test.

1.7. Microinjection procedure

Immediately prior to the appropriate sessions the obturator was removed from one of the guide cannulae and a stainless steel injector tube was inserted to extend 1 mm beyond the end of the guide cannula. The injector was connected by polyethylene tubing to a 10 μl Hamilton syringe (Reno, NV) preloaded with vehicle or AP-5. The compound was delivered manually over a 30-s period and the injector was kept in place for an additional 60 s before being removed and the obturator replaced. This procedure was repeated on the contralateral side, after which the animal was placed in the conditioning chamber and the session started.

1.8. Drugs

AP-5 (Sigma–Aldrich, St. Louis, MO) was dissolved in 0.9% saline before the start of the experiments. Each microinjection was delivered in a volume of 0.5 μl. The doses of AP-5 used were 0.25 and 0.5 μg and were chosen based on our previous experiments showing that these doses produced a dose-related significant attenuation of the acquisition of food-reinforced operant responding without affecting food motivation or food reward value [24].

1.9. Immunohistochemistry

Two additional groups were tested under the acquisition test protocol with either vehicle ($n = 7$) or 0.5 μg AP-5 ($n = 6$), and their brains were prepared for identification of the immediate early gene, c-fos. Seventy-five minutes after the end of the last session rats were anesthetized with sodium pentobarbital in preparation for perfusion. While under deep anesthesia the animals were perfused through the heart first with 0.9% saline followed by a phosphate-buffered (0.1 M) fixative containing 4% paraformaldehyde. Brains were removed from the skull and fixed with 4% paraformaldehyde overnight at 4 °C and sectioned through the NAcc/caudate/PFC2 in the coronal plane on a vibratome. Free-floating sections (40 μm) were collected into different wells for immunocytochemistry. Sections were first washed with phosphate-buffered saline (PBS) and then blocked in 5% NGS and 0.2% Triton X-100 for 1 h. Sections were then incubated with primary antibodies (rabbit anti-CFOS 1:5000, Calbiochem) in 0.1% Triton X-100, 2.5% NGS, and PBS at 4 °C overnight. Sections were rinsed several times with 2.5% NGS in PBS and then incubated in biotinylated secondary antibodies (biotinylated goat anti-rabbit; 1:200, Vector Labs, Burlingame, California) for 2 h at room temperature. Sections were rinsed several times with PBS and incubated for 1 h in an avidin-horseradish peroxidase mixture (Vector Labs, Burlingame, California). Sections were rinsed in PBS and then reacted with 0.05% diaminobenzidine in the presence of 0.0015% H_2O_2. Sections were collected onto gelatin-coated slides, dried for several hours, and coverslipped with Cytoseal.

1.10. Data analysis

For all rats in the acquisition and expression versions of the conditioned approach paradigm the data consisted of the number of food trough head entries made during (1) a 6-s period immediately preceding the onset of the CS (pre-CS period), (2) the 3-s period during the CS, (3) a 3-s period immediately following the offset of the CS and (4) at all other times (non-CS period). For analyses the 3-s periods during and immediately following the CS were combined for a 6-s total period and referred to as the CS period. For each session the total number of head entries during the CS periods and the total number of head entries during the pre-CS periods were used to calculate the CS/pre-CS ratio. This ratio indicates the magnitude of the conditioned approach response (i.e., the degree to which food trough head entries were elicited by the CS).

Separate two-way, mixed-design ANOVAs, with group (dose of AP-5) as a between groups factor and session as a repeated measures factor were conducted on the CS/pre-CS ratio data from sessions 1 to 3 (acquisition) or 1 to 7 (expression) for the acquisition and expression tests, respectively. Significant interactions were followed by tests of simple effect of session in each group (dose). Separate one-way ANOVAs were conducted on the CS/pre-CS ratio data from the CS-only test session for each conditioned approach procedure version (acquisition or expression). Separate one-way ANOVAs were conducted on the total head entries from the CS-only test session for each conditioned approach procedure. Post hoc tests consisted of Dunnett's tests.

To compare the effects of intra-VTA vehicle and AP-5 on c-fos expression in forebrain we identified a brain slice that contained NAcc core, shell, PFC2 and caudate at a rostral-caudal plane that was common for all rats; this was the slice that matched the 0.7 mm from bregma slice (Plate 15) in Paxinos and Watson [33]. c-fos-labeled nuclei were counted on an Olympus (Tokyo, Japan) BX51W microscope with a motorized stage. Stereo Investigator software (MicroBrightField, Williston, VT) was used to outline the NAcc core, NAcc shell, PFC2, medial caudate and lateral caudate. Planned independent sample *t*-tests with Bonferroni adjusted *P* values comparing c-fos counts in the vehicle to the AP-5 groups were conducted for each brain region.

2. Results

2.1. Cannula verification

Only the data of rats with verified VTA placements were included in these results. The VTA microinjection sites spanned much of the rostral-caudal length of the VTA but most were localized in the caudal portion of the VTA (−5.8 to −6.04 mm posterior to bregma) with some injections occurring in the central portion (−5.2 to −5.6 mm posterior to bregma) (see Fig. 1).

2.2. Effects of intra-VTA AP-5 on acquisition of conditioned approach

Rats receiving AP-5 prior to conditioning sessions made the greatest number of food trough head entries during sessions 1–3 while rats receiving vehicle made the fewest [Fig. 2A; dose effect: $F(3,32) = 7.625$, $P < .001$]. Across sessions 1–3, the total number of head entries did not change for the vehicle group while it declined for the 0.25 μg AP-5 and random-control groups and increased in the 0.5 μg AP-5 group [session × dose: $F(6,64) = 4.601$, $P < .001$]. In the CS-only test session, when all rats were treated with vehicle injections, the total number of head entries was similar for all groups (see Fig. 2A).

The left panel in Fig. 2B shows the ratio of CS/pre-CS food trough head entries in the three conditioning sessions. The pattern of change across sessions for this ratio was different among the groups [a two-way ANOVA revealed a significant session x dose interaction, $F(6,64) = 3.747$, $P < .005$]. The vehicle and 0.25 μg AP-5 groups both showed progressively larger CS/pre-CS ratios across sessions while for the 0.5 μg AP-5 and random control groups this ratio did not change (test of simple effect of session at each dose was <.01 for vehicle and <.001 for 0.25 μg AP-5). The right panel in Fig. 2B shows the CS/pre-CS ratio in the CS-only test session, when all groups were treated with vehicle. The ratios for the vehicle and 0.25 μg AP-5 groups were higher than for the 0.5 μg AP-5 and random-control groups (a one-way ANOVA revealed a significant group effect; $F(3,32) = 3.15$, $P < .05$). The ratios for the vehicle and 0.25 μg AP-5 groups were similar and the ratios for the 0.5 μg AP-5 and random-control groups were similar (Dunnett's tests showed that the vehicle group differed significantly from the 0.5 μg AP-5 and the random-control groups, all *P*s < .05).

The left panel of Fig. 3 shows the head entries emitted during the pre-CS and CS periods for these groups during the third conditioning session. The AP-5 groups emitted more head entries than the vehicle group, confirming the apparent stimulant effect observed in Fig. 1. In the case of the 0.25 μg group, responding was higher during the CS period than the pre-CS period, a response pattern that resembled that of the vehicle group, with the AP-5 stimulant effect affecting responding primarily during the CS period. In contrast, in the 0.5 μg AP-5 group, responding was similar during the pre-CS and CS periods, a response pattern that resembled that of the random-control group, with the AP-5 stimulant effect affecting responding during both the pre-CS and CS periods. A two-way ANOVA (CS as a repeated measures factor with pre-CS and CS as levels) revealed a significant group x CS effect [$F(3,32) = 7.589$, $P < .001$]. Tests of simple effects of CS at each level of group indicated significant CS effects in the vehicle and 0.25 μg AP-5 groups (*P*s < .05). The right panel of Fig. 3 shows the head entries emitted during the pre-CS and CS periods for these groups during the CS-only test session. Responding during the pre-CS period was similar

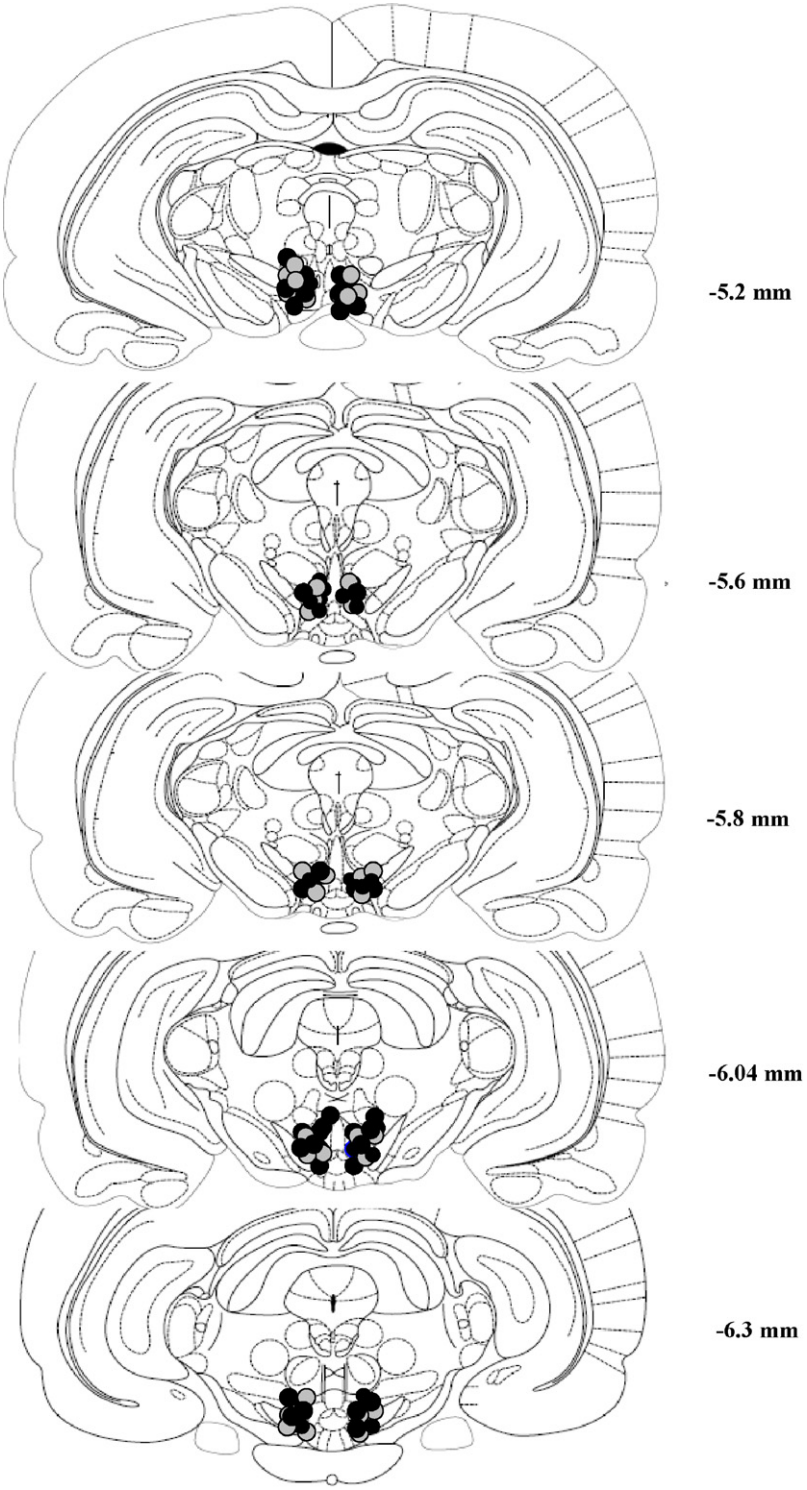

Fig. 1. Histological reconstruction of injection sites adapted from Paxinos and Watson [33]. Grey circles are groups treated with vehicle; black circles represent groups treated with AP-5. The numbers to the right of each section indicate the distance posterior to bregma.

in all groups. In the vehicle and 0.25 μg AP-5 groups responding during the CS period was much greater than during the pre-CS period. However, in the 0.5 μg AP-5 and random-control groups responding during the CS period was similar to responding during the pre-CS period. A two-way ANOVA revealed a significant group x CS effect [$F(3,32) = 4.534$, $P < .01$]. Tests of simple effects of CS at each level of group revealed significant CS effects in the vehicle and 0.25 μg AP-5 groups (Ps < .001).

2.3. Effects of intra-VTA AP-5 on expression of conditioned approach

Rats receiving vehicle or 0.5 μg AP-5 prior to the CS-only test session made similar amounts of food trough head entries during sessions 1–7 (with no treatment) that declined across these sessions [see Fig. 4; analyses revealed a significant session effect, $F(6,78) = 2.219$, $P < .05$, that did not significantly interact with the

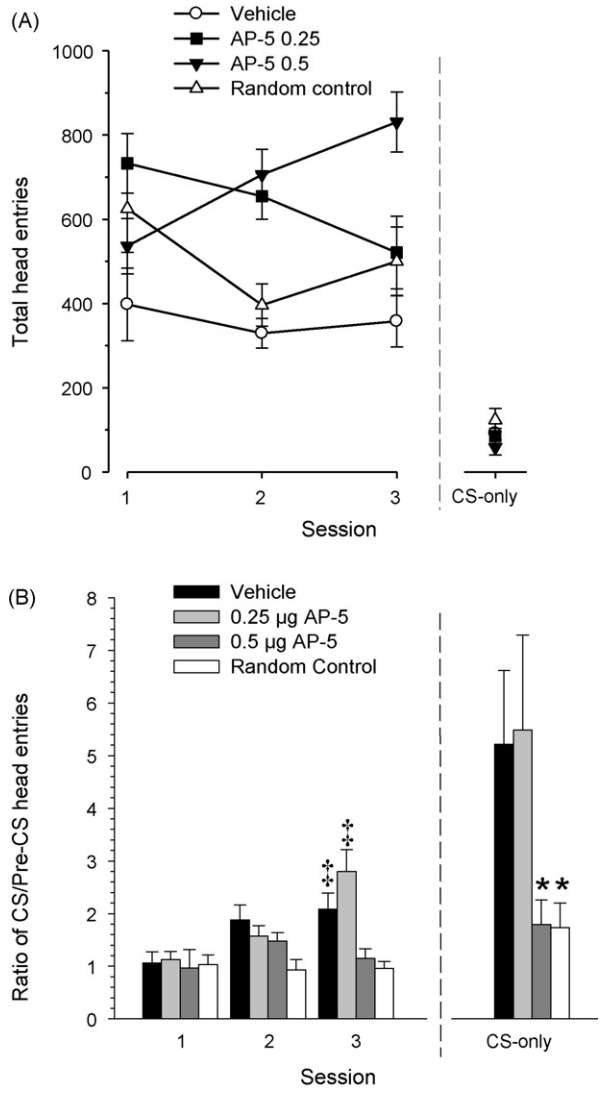

Fig. 2. Head entry data for rats receiving conditioning sessions in which light stimulus and food presentations were explicitly paired (vehicle, 0.25 and 0.5 μg AP-5 groups), or presented on independent, variable schedules (random-control group). (A) Mean (±SEM) total number of food trough head entries emitted during the three conditioning and the CS-only sessions for groups treated with AP-5 or vehicle prior to each conditioning session, and vehicle prior to the CS-only session. (B) Mean (±SEM) CS/pre-CS ratios for groups receiving AP-5 or vehicle prior to each conditioning session and vehicle prior to the CS-only session. ‡ represents a significant increase across the three conditioning sessions; * indicates a significant difference from the vehicle group.

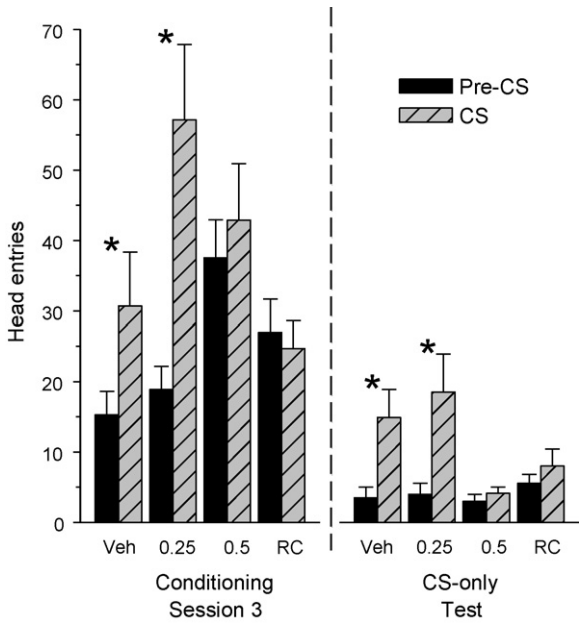

Fig. 3. Mean (±SEM) number of food trough head entries emitted during the pre-CS and CS periods in the third conditioning and the CS-only test sessions for groups treated with AP-5 or vehicle prior to each conditioning session, and vehicle prior to the CS-only test session. * indicates a significant CS effect in that group.

group factor]. In the CS-only test session, the group receiving 0.5 μg AP-5 showed a somewhat greater number of overall head entries than the group receiving vehicle but this difference was not significant.

The left panel in Fig. 4B shows the ratio of CS/pre-CS food trough head entries in the seven conditioning sessions. Although both groups received no treatments prior to sessions 1 to 7—that is, they were identical—the data are depicted separately according to the treatment that they would receive in the CS-only test session. The pattern of change in the CS/pre-CS ratio was similar for both groups across the conditioning sessions; both showed increasing ratios across the sessions and appeared to level off in the last few sessions (a two-way ANOVA revealed a significant session effect; $F(6,78) = 4.438$, $P < .005$). The right panel in Fig. 4B shows the ratio of CS/pre-CS food trough head entries in the CS-only test session after receiving vehicle or 0.5 μg AP-5. There was no significant difference between the ratios of each group.

2.4. Effects of intra-VTA AP-5 on acquisition of conditioned approach and c-fos expression in terminal areas of DA systems

To test the hypothesis that intra-VTA AP-5 reduction in CS/pre-CS ratios is associated with reduced activity in DA terminal regions we tested two additional groups, one with vehicle and the other with 0.5 μg AP-5 given prior to conditioning sessions and stained for c-fos after the CS-only test. Similar to the previous groups tested under the same conditions, the AP-5 group showed significantly smaller CS/pre-CS ratios than vehicle group during the CS-only test [see Fig. 5A; total head entries and CS/pre-CS ratios across the three conditioning sessions (data not shown) were similar to the corresponding groups in the first acquisition experiment shown in Fig. 2]. C-fos expression in all regions tested was lower in the AP-5 group than in the vehicle group (see Fig. 5B). Our planned comparisons revealed that in the NAcc core the amount of c-fos in the AP-5 group was significantly lower than in the vehicle group [$t(11) = 2.038$, $P < .05$); t-tests for the NAcc shell, medial caudate and PFC2 approached significance, Ps = .1 for each]. Fig. 6 consists of representative brain sections, one from a vehicle (left) and the other from an AP-5 (right) treated animal, showing c-fos in the NAcc core at the level of magnification used during counting.

3. Discussion

Our findings demonstrate that NMDA receptor stimulation in the VTA is necessary for the acquisition, but not expression, of conditioned approach behavior and for a CS to significantly activate the NAcc core. Rats that received intra-VTA injections of the 0.5 μg dose of AP-5 prior to conditioning sessions showed no evidence of acquiring conditioned approach cued by a food-associated light stimulus. This was indicated first by a lack of increase in the ratio of CS/pre-CS head entries across three conditioning sessions by the 0.5 μg AP-5 treated rats, which was similar to the random-control group, while the conditioned approach ratio did

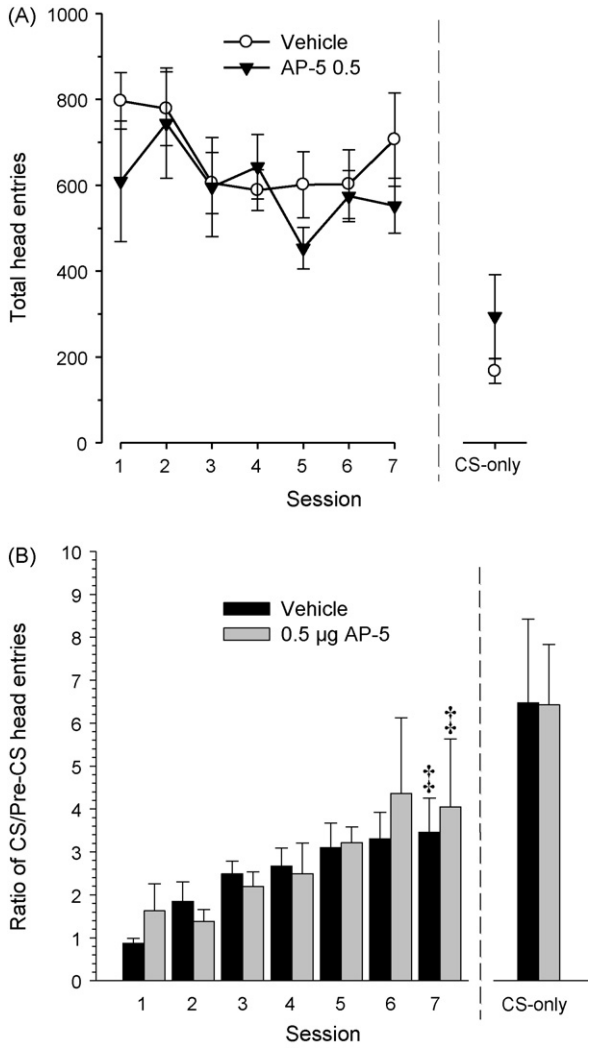

Fig. 4. (A) Mean (±SEM) total number of food trough head entries emitted during the seven conditioning and the CS-only sessions for groups receiving no treatment prior to each conditioning session and vehicle or 0.5 μg AP-5 prior to the CS-only session. (B) Mean (±SEM) CS/pre-CS ratios for groups receiving no treatment prior to each conditioning session and vehicle or 0.5 μg AP-5 prior to the CS-only session. ‡ represents significant increase across the seven conditioning sessions.

increase across those sessions for the vehicle and 0.25 μg AP-5 groups. More importantly, during the CS-only test session, which directly followed vehicle injections for all groups, the conditioned approach ratio associated with the presentation of the CS was significantly higher in the vehicle and 0.25 μg AP-5 groups than the 0.5 μg AP-5 group, which in turn was not significantly different from the random-control group. The absolute head entry data indicated that the significantly reduced CS/pre-CS ratio in the 0.5 μg AP-5 group did not result from increased pre-CS responding but rather from decreased CS responding relative to the vehicle group.

It is unclear why total head entries for the 0.5 μg AP-5 group increased across the three conditioning sessions, whereas rats receiving 0.25 μg AP-5 decreased their total head entries. Our most parsimonious explanation for this effect is that all animals treated with AP-5 had an overall increase in their activity. However, because animals receiving the 0.5 μg dose did not learn to treat the light presentation as a CS, overall head entries continued to increase as animals were stimulated by the receipt of the primary reward, whereas animals receiving 0.25 μg AP-5 learned to enter the food trough selectively in response to the CS, thereby overriding any possible locomotor effects. Although we did not quantify activity in this set of studies apart from head entries, increased activity with intra-VTA AP-5 is consistent with our previous findings of increased stereotypy with intra-VTA AP-5 [24] as well as a previous report of increased forward locomotion [34]. The increased locomotor activity induced by intra-VTA AP-5 is not due to alterations in accumbens DA transmission [35]. Although the mechanisms of NMDA receptor antagonist-induced increased locomotor activity are not yet understood, reduced activity of GABA neurons may play a role, as selective lesions of VTA GABA neurons also results in increased activity [36]. While we did not quantify the behavior in this experiment, animals receiving AP-5 appeared to be somewhat disinhibited and looser in their movements, but not uncoordinated or impaired in any way.

In contrast to our findings when NMDA receptors were blocked prior to conditioning sessions, rats that received no treatment prior to the conditioning sessions and 0.5 μg AP-5 prior to the CS-only test session demonstrated a conditioned approach ratio similar to the group that received vehicle prior to the CS-only test. Thus, in animals where the conditioned approach response was already acquired, intra-VTA AP-5, at a dose that blocked acquisition of conditioned approach, failed to block the expression of the learned response.

In addition, our findings demonstrate that NMDA receptor stimulation in the VTA is necessary for CS-associated activity in DA terminal regions. Rats treated with intra-VTA injections of a 0.5 μg AP-5 dose prior to conditioning sessions not only failed to acquire the conditioned approach response, but also showed significantly less c-fos expression in the NAcc core in response to the food-associated CS during the CS-only test session than animals treated with vehicle. These results suggest that (1) expression of CS-controlled responding involves CS-induced activation of cells in NAcc core and (2) the neural plasticity that allows a CS to acquire the ability to control responding and CS-induced NAcc core activity both require NMDA receptor stimulation in VTA during acquisition. To our knowledge, this is the first study to demonstrate that antagonism of VTA NMDA receptor stimulation leads to both a significant impairment in the acquisition of reward-related learning and a significant decrease in CS-induced neural activity. Therefore, this is the first demonstration of a causal link between VTA NMDA receptor stimulation and both acquisition of reward-related learning and acquisition of CS-induced neural activity.

Altogether, the present results strongly support the hypothesis that NMDA receptor stimulation in the VTA is necessary for the acquisition, but not the expression, of reward-related learning. In this experiment, NMDA receptor blockade, while leading to an overall increase in activity, prevented an increase in head entries relative to the CS, both during training following treatment with drug, and during a CS-only test session with no treatment. Rats treated with the high dose of AP-5 behaved as if the CS had acquired no special significance, just as it appeared to remain meaningless to the random-control rats. The present results also support the hypothesis that the neural plasticity underlying reward-related learning involves CS-induced activation of DA terminal regions and requires NMDA receptor stimulation in the VTA. We should note, however, that these experiments do not rule out the unlikely possibility that VTA treatment with AP-5 itself later reduces the capacity of DA terminal regions to express c-fos.

The findings of this study are consistent with previous studies showing a role for NMDA receptors in reward-related learning. As noted earlier, others [31] have investigated the role of NMDA receptors in conditioned approach using a paradigm similar to the one used presently and found that intra-VTA injections of AP-5 prevented the acquisition of conditioned approach but not the expression of this learning. We have previously reported that intra-VTA AP-5 blocked the acquisition of food-reinforced instrumental responding but not its expression [24]. Using drugs of abuse as

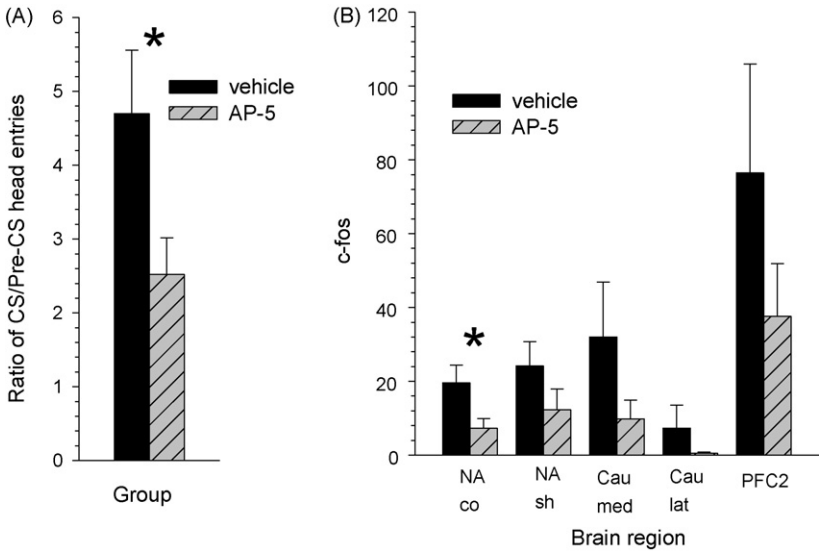

Fig. 5. (A) Mean (±SEM) CS/pre-CS ratios during the CS-only test for the two groups whose brains were analyzed for c-fos immediately after the CS-only session. * represents a significant group difference. (B) Mean (±SEM) c-fos counts in nucleus accumbens core and shell, medial and lateral caudate and prefrontal cortex area 2 in rats treated with vehicle or 0.5 μg AP-5 prior to each of three conditioning sessions and vehicle prior to the CS-only session. For all rats tissue was harvested 75 min after the end of the CS-only test and processed for c-fos. * represents a significant group difference at that site.

unconditioned stimuli Aston-Jones' group found that intra-VTA treatment with an NMDA antagonist impaired the establishment of morphine conditioned place preference [37]. There is an accumulation of evidence that NMDA receptor stimulation in the VTA plays a necessary role in reward-related learning for both natural (e.g., food) and drug reward. The neural plasticity involved in reward-related learning and in which VTA NMDA receptor stimulation plays a role remains to be elucidated.

We have proposed a model of reward-related learning [17,24,25] predicated on the assumption that this learning occurs when the CS acquires the ability to activate the same neural system that produces unconditioned approach [38]. This is a Hebbian model that proposes that the VTA is a site where signals from reward-associated and unconditioned stimuli (USs; i.e., primary rewards) converge onto DA cells and, through NMDA-dependent LTP, CS signals acquire the capacity to activate VTA DA cells by themselves. In the VTA, muscarinic ACh receptor stimulation has been shown repeatedly to mediate the rewarding effects of USs like food [17,39], brain stimulation reward [39–42] and cocaine [43] and therefore could serve as the US signal in this model. Also in the VTA, glutamate is released from afferents of neurons originating in prefrontal cortex, pedunculopontine nuclei and other brain regions that are involved in processing information about environmental stimuli. Thus, the VTA is a site where convergence of US and CS signals is possible. Support for this model comes from previous studies showing that blockade of mACh receptor stimulation in the VTA blocks the acquisition, but not the expression, of food-reinforced instrumental responding [17] and other food-related learning [44]. Support for the role of NMDA receptor stimulation in this model comes from the present and other [31] findings that blockade of NMDA receptors impairs the acquisition of the conditioned approach or instrumental [24] response but not the expression of this learning.

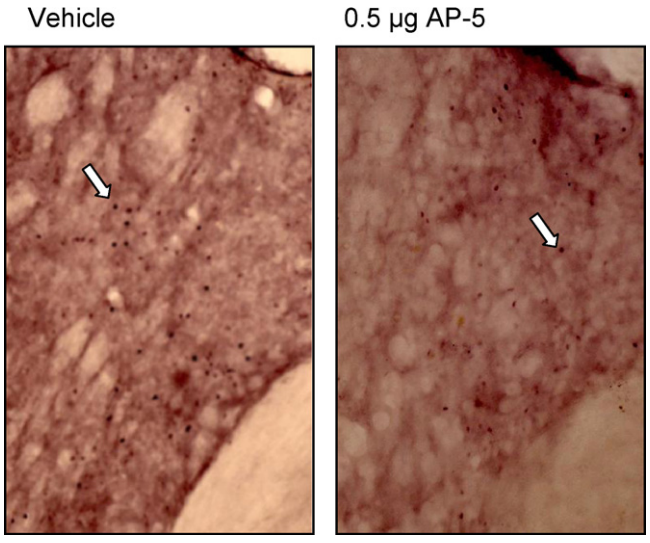

Fig. 6. Two representative sections indicating c-fos protein in identical portions of the nucleus accumbens core in a rat treated with 0.5 μg AP-5 and one with vehicle prior to conditioning sessions and vehicle prior to the CS-only session (same animals as in Fig. 5). Each photograph is taken with the microscope set to the same level of magnification used during counting. The white arrow indicates c-fos.

Additional evidence supporting our model is that the VTA and its terminal regions show progressive changes in activity as neutral stimuli are paired with rewards, indicating that associative processes are taking place. Midbrain DA neurons respond to both primary rewards and CSs (see Ref. [45] for review), but responding to CSs develops over time. Generally, midbrain DA neurons fire in response to reward receipt until animals are well trained at which time responding of DA cells comes primarily under the control of CSs [46–48]. The present findings that intra-VTA AP-5, in addition to impairing the acquisition of conditioned approach also resulted in significantly less c-fos in NAcc core and a trend toward less c-fos in other DA terminal regions in response to the CS, is consistent with the hypothesis that the reward-associated stimulus failed to control behavior because it failed to acquire the capacity to activate DA cells, resulting in less cellular activation in DA terminal regions. However, some findings do not support this model, at least not in the case when food is the US. Stuber et al. [31] have found that although conditioned approach was associated with synaptic strengthening onto VTA DA cells to the CS, this LTP-like enhanced response was temporary; it developed during acquisition of conditioned approach but dissipated after the behavioral response stabilized.

Another possibility is that NMDA receptor stimulation in the VTA is needed to produce a DA signal downstream that is itself essential for synaptic plasticity. Phasic, or *burst*, firing of VTA DA cells is at least partly dependent on NMDA receptor stimulation [27,49], and this NMDA effect appears dependent on intact signaling from afferents originating in the laterodorsal tegmentum [50]. It has long been hypothesized that a DA signal in terminal regions, time-locked to reward events, serves as a necessary input for the synaptic plasticity in these regions that leads to reward-related learning [28–30]. In this model, stimuli associated with reward provide a glutamatergic signal to output neurons in DA terminal regions while primary rewards provide a DA signal to the same. This convergent stimulation would produce synaptic plasticity in these output neurons allowing reward-related stimuli to function as CSs, activating these output neurons to produce conditioned responding. Kelley and co-workers have conducted several studies demonstrating the necessity of both DA and NMDA receptor stimulation in NAcc [51], prefrontal cortex [52] and amygdala [53] for the acquisition, but not performance, of instrumental responding. But in addition to the stimulus-response type of learning that might be represented by the Kelley and co-workers' studies it is also possible that DA signals in terminal regions mediate the synaptic plasticity that underlies stimulus-reward associations; such plasticity may then result in amplified CS-related glutamate signals back to the VTA [54] causing the CS-induced activation of DA cells [46–48] and CS-induced DA release [12–16] that is observed in reward-related learning. Thus in both of these cases—where phasic DA activity is necessary for stimulus-response or stimulus-reward associative learning—if phasic DA activity is dependent on NMDA receptor stimulation in the VTA [27,50], then antagonism of this neurochemical pathway should impair acquisition of conditioned approach learning. In the case where phasic DA activity is necessary for synaptic plasticity leading to enhanced CS-related glutamate signals to VTA, blockade of VTA NMDA receptors during learning should also result in the diminished capacity of CSs to activate VTA DA cells leading to reduced cellular activity in DA terminal regions in response to CSs, a finding observed in the present study.

In summary, we tested the hypothesis that NMDA receptor stimulation in VTA is necessary for both the acquisition of conditioned approach and CS-induced cellular activity in DA terminal regions. We found that intra-VTA microinjections of AP-5 prior to conditioning sessions blocked the acquisition of conditioned approach but injections made after acquisition had no effect on the learned response. We also found that VTA AP-5, in addition to blocking reward-related learning, significantly reduced the expression of c-fos in response to the CS in the NAcc core (as well as producing trends to less c-fos in NAcc shell, PFC2 and medial caudate). These results support our hypothesis and suggest that NMDA receptor stimulation in the VTA is necessary for the neural plasticity underlying reward-related learning.

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