

# Role of a Hippocampal Src-Family Kinase-Mediated Glutamatergic Mechanism in Drug Context-Induced Cocaine Seeking

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Glutamatergic neurotransmission in the dorsal hippocampus (DH) is necessary for drug context-induced reinstatement of cocaine-seeking behavior in an animal model of drug relapse. Furthermore, *in vitro* studies suggest that the Src family of tyrosine kinases critically regulates glutamatergic cellular functions within the DH. Thus, Src-family kinases in the DH may similarly control contextual cocaine-seeking behavior. To test this hypothesis, rats were trained to lever press for un-sigaled cocaine infusions in a distinct context followed by extinction training in a different context. Cocaine-seeking behavior (non-reinforced active lever pressing) was then assessed in the previously cocaine-paired and extinction contexts after AP5 (*N*-methyl-D-aspartate glutamate (NMDA) receptor (NMDAR) antagonist; 0.25 or 2.5 µg/0.5 µl/hemisphere), PP2 (Src-family kinase inhibitor; 6.25 or 62.5 ng/0.5 µl/hemisphere), Ro25-6981 (NR2B subunit-containing NMDAR antagonist; 0.2 or 2 µg/0.5 µl/hemisphere), or vehicle administration into the DH. Administration of AP5, PP2, or Ro25-6981 into the DH dose-dependently impaired drug context-induced reinstatement of cocaine-seeking behavior relative to vehicle, without altering instrumental behavior in the extinction context or food-reinforced instrumental responding and general motor activity in control experiments. Cocaine-seeking behavior during the first 20 min of the test session in the cocaine-paired context was associated with an increase in NR2B subunit activation, and intra-DH PP2 pretreatment disrupted this relationship. Together, these findings suggest that Src-family kinase activation, NMDAR stimulation, and likely Src-family kinase-mediated NR2B subunit-containing NMDAR activation in the DH are necessary for incentive motivational and/or memory processes that promote contextual cocaine-seeking behavior.

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## INTRODUCTION

Exposure to drug-associated environmental stimuli provokes relapse to drug use in cocaine users (Childress *et al*, 1988; Grant *et al*, 1996; Childress *et al*, 1999). Paralleling these clinical findings, exposure to a previously cocaine-paired context induces cocaine-seeking behavior in the extinction-reinstatement animal model of drug relapse (Crombag *et al*, 2008; Fuchs *et al*, 2008a). Furthermore, drug context-induced reinstatement of cocaine-seeking behavior depends on glutamatergic neurotransmission, in part via the stimulation of metabotropic glutamate receptor subtype 1 (mGluR1), within the dorsal hippocampus (DH) (Xie *et al*, 2010).

Similar to mGluR1s, *N*-methyl-D-aspartate glutamate (NMDA) receptors (NMDARs) critically regulate environmentally induced drug-seeking behaviors. Systemic admin-

istration of NMDAR antagonists inhibits the expression of cocaine-conditioned place preference and explicit cue-induced instrumental cocaine- or ethanol-seeking behaviors (Bachteler *et al*, 2005; Backstrom and Hyytia, 2006; Maldonado *et al*, 2007). These effects are mediated by NMDAR populations in multiple brain regions. Consistent with this, NMDAR antagonism in the central amygdala or the nucleus accumbens core attenuates the expression of morphine-conditioned place preference (Rezayof *et al*, 2007) and explicit cue-induced instrumental cocaine-seeking behavior (Backstrom and Hyytia, 2007), respectively. NMDAR antagonism in the DH also disrupts the expression of amphetamine-induced conditioned place preference (Sakurai *et al*, 2007); however, it has yet to be investigated whether the stimulation of NMDAR populations in the DH is necessary for drug context-induced reinstatement of instrumental drug-seeking behavior.

NMDAR function is critically regulated by the Src family of tyrosine kinases (Grovesman *et al*, 2012; Salter and Kalia, 2004). Five members of the family have been identified in the mammalian brain, including Src, Fyn, Lyn, Yes, and Lck (Salter and Kalia, 2004). In cultured hippocampal neurons or brain slices, activation of Src-family kinases increases the phosphorylation of NR2B NMDAR subunits (Lu *et al*, 1999a;

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Stramiello and Wagner, 2008), which is required for NR2B subunit surface expression and NMDAR function (Ali and Salter, 2001; Groveman *et al*, 2012; Lu *et al*, 1999a; Salter and Kalia, 2004; Stramiello and Wagner, 2008). Conversely, pharmacological inhibition of Src-family kinases in the dorsomedial striatum decreases the phosphorylation of NR2B subunits and the expression of drug-primed alcohol-seeking behavior (Wang *et al*, 2010). However, the putative contributions of Src-family kinases in the DH to context-induced motivated behaviors, and specifically to drug context-induced cocaine-seeking behavior, have not been explored.

The present study evaluated the hypothesis that NMDARs and Src-family kinases within the DH critically regulate drug context-induced cocaine-seeking behavior. In experiments 1 and 2, we investigated the dose-dependent effects of the NMDAR antagonist, AP5, and the Src-family kinase inhibitor, PP2, in the DH on drug context-induced cocaine-seeking behavior and the possible rate-altering effects of these manipulations on food-reinforced instrumental responding and general locomotor activity. In experiment 3, we examined the effects of the behaviorally effective dose of PP2 on NR2B subunit activation in the DH in relation to drug context-induced cocaine-seeking behavior. Finally, in experiment 4, we examined the dose-dependent effects of intra-DH administration of the selective NR2B subunit-containing NMDAR antagonist, Ro25-6981, on drug context-induced cocaine-seeking behavior, food-reinforced instrumental responding, and locomotor activity.

## MATERIALS AND METHODS

### Animals

Male Sprague–Dawley rats (Charles-River,  $N=64$ ; 250–275 g) were individually housed in a temperature- and humidity-controlled vivarium on a reversed light/dark cycle. Rats were maintained on 20–25 g of rat chow per day and *ad libitum* water. The housing and treatment of the rats followed the guidelines of the ‘Guide for the Care and Use of Laboratory Rats’ (Institute of Laboratory Animal Resources, Commission on Life Sciences 2011) and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

### Food Training and Surgery

To expedite cocaine self-administration training, rats were first trained to lever press under a fixed-ratio 1 (FR-1) schedule of food reinforcement overnight. Forty-eight hours later, they were surgically implanted with intravenous jugular catheters and 26-Ga stainless steel guide cannulae (Plastics One), aimed bilaterally at the DH (angled laterally by 15°; AP  $-3.4$  mm, ML  $+/-3.1$  mm, DV  $-2.15$  mm). The food training, surgery, and post-operative care procedures have been described previously (Fuchs *et al*, 2007; Fuchs *et al*, 2008b; Xie *et al*, 2010).

### Cocaine Self-Administration and Extinction Training

After surgical recovery, daily 2-h self-administration training sessions were conducted during the rats’ dark cycle.

The operant conditioning chambers were configured to one of two distinctly different contexts (Contexts 1 and 2, described in Supplementary Materials and Methods). Active lever presses resulted in cocaine reinforcement (cocaine hydrochloride; 0.15 mg/0.05 ml per infusion, *i.v.*; NIDA, Research Triangle Park, NC) under a FR-1/20 s-time-out schedule (Fuchs *et al*, 2007). Responses on the other (inactive) lever were recorded but had no scheduled consequences. Rats that did not reach a minimum of 10 sessions with  $\geq 10$  cocaine infusions/session were eliminated from the experiment. The remaining rats received daily 2-h extinction-training sessions in an alternate context (1 or 2). During extinction training, lever responses were recorded but had no scheduled consequences. Before the fourth extinction-training session, rats were adapted to the intracranial microinfusion procedure, as described previously (Fuchs *et al*, 2007). As active lever responses during cocaine self-administration training averaged between 50 and 75/session (see Supplementary Table S1), extinction training continued until a criterion of  $\leq 25$  active lever responses/session on at least two consecutive days was reached. Rats that reached this criterion were used for subsequent reinstatement testing.

### Reinstatement Testing

Cocaine-seeking behavior was assessed in the cocaine-paired and extinction contexts during four test sessions, using a counterbalanced experimental design, as described previously (Xie *et al*, 2010) and in the Supplementary Materials and Methods. Rats received bilateral vehicle or AP5 (0.25 or 2.5  $\mu\text{g}/0.5$   $\mu\text{l}/\text{hemisphere}$ ;  $N=7$ , experiment 1), PP2 (6.25 or 62.5 ng/0.5  $\mu\text{l}/\text{hemisphere}$ ;  $N=8$ , experiment 2), or Ro25-6981 (0.2 or 2  $\mu\text{g}/0.5$   $\mu\text{l}/\text{hemisphere}$ ;  $N=8$ , experiment 4) microinfusions into the DH immediately before testing. In experiment 3, rats received bilateral vehicle or PP2 (62.5 ng/0.5  $\mu\text{l}/\text{hemisphere}$ ;  $N=6-9$ ) microinfusions into the DH and either a 1-h test session in the cocaine-paired context or exposure to the home cage, followed by euthanasia.

### Locomotor Activity and Food-Reinforced Instrumental Behavior

Pharmacological manipulations may produce motor side effects that alter the expression of motivated behavior. Hence, in experiments 1, 2, and 4, at least 72 h after the last reinstatement test session, we examined the effects of AP5, PP2, and Ro25-6981 on general locomotor activity and on food-reinforced instrumental motor performance in different subsets of rats ( $N=7-8/\text{group}$ ), as described previously by Xie *et al* (2010) and in Supplementary Materials and Methods.

### Histology and Western Blotting

In experiments 1, 2, and 4, rats were overdosed using ketamine and xylazine (66.6 and 1.3 mg/kg intravenous or 133.2 and 2.6 mg/kg intraperitoneal, depending on catheter patency). The brains were dissected out, post-fixed with 10% formaldehyde, sectioned at 75  $\mu\text{m}$ , and stained with cresyl violet (Kodak, Rochester, NY). The most ventral

point of each cannula track was mapped onto schematics from the rat brain atlas (Paxinos and Watson 1997).

In experiment 3, rats were euthanized via rapid decapitation immediately after the 1-h reinstatement test session or home cage exposure. Brains were dissected out, flash frozen in isopentane at  $-20^{\circ}\text{C}$ , and stored at  $-80^{\circ}\text{C}$ . DH punches were taken with 19-Ga neuropunches (Fine Science Tools) and 40- $\mu\text{m}$  coronal tissue sections were also collected to verify cannula placement. Punched tissue was stored at  $-80^{\circ}\text{C}$  in lysis buffer, containing 10 mM HEPES, 1% SDS, 11% sucrose, and 1x protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St Louis, MO). Tissue samples were homogenized manually and boiled in a dry heat block at  $100^{\circ}\text{C}$  for 10 min. The protein concentration of samples was determined using the Biorad DC Protein Assay kit. Proteins were separated on 7.5% Tris-HCl polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) for 1 h at 100 V. Membranes were then blocked in 5% milk for 1 h, and incubated with rabbit anti-phospho-NMDA NR2B polyclonal antibody (pTyr1472, 1:1000, Sigma-Aldrich) overnight (16–20 h) at  $4^{\circ}\text{C}$ . Membranes were incubated in donkey anti-rabbit polyclonal secondary antibody (1:10 000, GE Healthcare, Piscataway, NJ) conjugated to horseradish peroxidase (HRP) for 1 h followed by development with an enhanced chemiluminescence system (Pierce Biotech, Rockford, IL). Membranes were washed with stripping buffer (62.5 mM Tris-HCl at pH 6.7, 2% SDS, 100 mM beta-mercaptoethanol) to permit re-probing with antibodies to total NR2B (1:1000, Millipore) and actin-HRP (ie, loading control; 1:50 000, Santa Cruz Biotechnology, Santa Cruz, CA). pNR2B, total NR2B, and actin protein levels were quantified by densitometry, using NIH Image J software. pNR2B levels were normalized to total NR2B levels, and no difference was observed in total NR2B levels, when normalized to actin. Subsequent discussion of NR2B activation refers to normalized pNR2B/total NR2B levels.

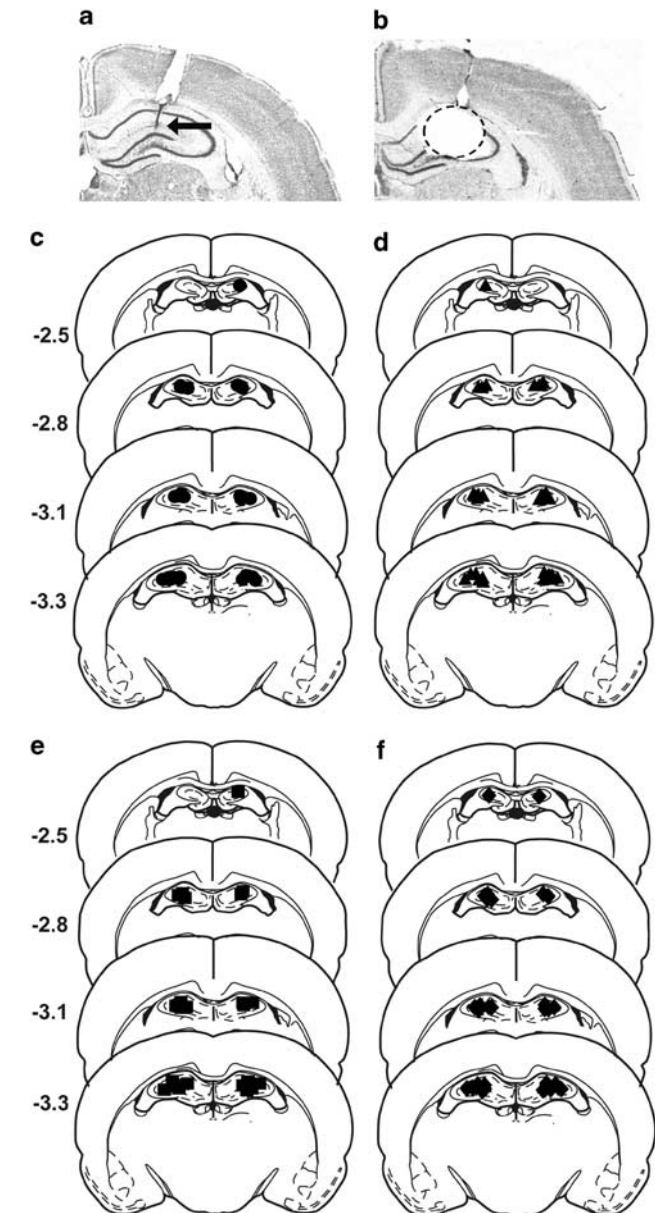
## Data Analysis

Data were analyzed with univariate correlation and mixed-factorial analyses of variance (ANOVAs), where appropriate, as described in Supplementary Material and Methods. Significant ANOVA main and interaction effects were further investigated using Tukey *post hoc* tests, when appropriate. Alpha ( $\alpha$ ) was set at 0.05.

## RESULTS

### Histology

Schematics illustrating cannula placement and photomicrographs showing a representative cannula tract and DH micropunch are included in Figure 1. The most ventral point of the infusion cannula tracks was located within the *Cornu Ammonis* 1/3 (CA1/CA3) subregions of the DH bilaterally in all rats in experiments 1 (Figure 1a), 2 (Figure 1b), and 4 (Figure 1d). Furthermore, high power microscopy did not reveal any evidence of abnormal tissue damage (ie, extensive cell loss or gliosis) at the infusion site. DH micropunches were collected bilaterally around the



**Figure 1** Schematic and photographic representation of injection cannula placements within the dorsal hippocampus (DH). On the photomicrographs of cresyl violet-stained sections, the arrow and segmented circle identify a representative cannula placement (a) and sampling area for western blot analysis (b), respectively. On the schematics adapted from Paxinos and Watson (1997), filled circles (c), filled triangles (d), filled squares (e), and filled diamonds (f) represent the most ventral point of the cannula tracks for rats in experiments 1, 2, 3, and 4, respectively. Numbers indicate distance from bregma in millimeters.

infusion site in all rats in experiment 3 (Figure 1c). The sample sizes are reported in the figure captions.

### Cocaine Self-Administration and Extinction

There were no pre-existing differences between the experimental groups in cocaine intake, active or inactive lever responding during cocaine self-administration training or extinction training, or in the number of days required to reach the extinction criterion. The corresponding

descriptive statistics are provided in Supplementary Results and Supplementary Table S1.

## Reinstatement

In each experiment, following vehicle administration into the DH, active and inactive lever responding was independent of testing context order, treatment order, and treatment on the other test day (ie, dose of AP5, PP2, or Ro25-6981). Therefore, data were collapsed across these variables to form a single-vehicle condition. Inactive lever responding was low in all experiments and was not altered by the pharmacological manipulations (all treatment main and interaction effects,  $F_s < 1.64$ ,  $p > 0.21$ ; Supplementary Figure S2).

## Effects of Intra-DH AP5 Treatment on Drug Context-Induced Cocaine-Seeking and Motor Behaviors

Intra-DH AP5 treatment attenuated active lever responding as a function of testing context and dose (Figure 2a). Following pretreatment with vehicle or the 0.25  $\mu\text{g}$  dose of AP5 into the DH, exposure to the cocaine-paired context increased active lever responding relative to responding in the extinction context (all treatment main and interaction effects,  $F_{(1-2, 25)} = 4.16-33.98$ ,  $p = 0.0001-0.03$ , Tukey test,  $p < 0.05$ ). The 0.25  $\mu\text{g}$  dose of AP5 failed to alter active lever responding in either context relative to vehicle. Conversely, the 2.5  $\mu\text{g}$  dose of AP5 decreased active lever responding in the cocaine-paired context (Tukey test,  $p < 0.05$ ), without altering responding in the extinction context, relative to vehicle.

During the locomotor activity test session, the number of photobeam breaks gradually decreased (time main effect,  $F_{(2, 12)} = 21.32$ ,  $p = 0.0001$ ; 20-min interval 1 > 2-3, Tukey test,  $p < 0.05$ ). Furthermore, the behaviorally effective, 2.5  $\mu\text{g}$  dose of AP5 did not alter the number of photobeam breaks relative to vehicle (treatment main and interaction effects,  $F_{(1-2, 6-12)} = 0.35-2.05$ ,  $p = 0.17-0.58$ ; Figure 2b).

Food reinforcement elicited greater responding on the active lever than on the inactive lever (lever main effect,  $F_{(1, 6)} = 31.76$ ,  $p = 0.001$ ). Furthermore, 2.5  $\mu\text{g}$  dose of AP5 did not alter food-reinforced active or inactive lever responding, relative to vehicle (all treatment main

and interaction effects,  $F_{(1, 6)} = 0.49-0.51$ ,  $p = 0.50-0.51$ ; Figure 2c).

## Effects of Intra-DH PP2 Treatment on Drug Context-Induced Cocaine-Seeking and Motor Behaviors

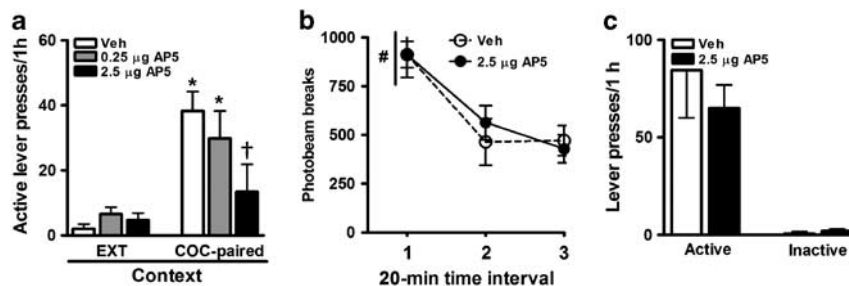
Intra-DH PP2 treatment attenuated active lever responding as a function of testing context and dose (Figure 3a). Following pretreatment with vehicle or the 6.25 ng dose of PP2, exposure to the cocaine-paired context increased active lever responding relative to responding in the extinction context (all treatment main and interaction effects,  $F_{(1-2, 29)} = 4.13-43.73$ ,  $p = 0.0001-0.03$ , Tukey test,  $p < 0.05$ ). The 6.25 ng dose of PP2 failed to alter active lever responding in either context relative to vehicle. In contrast, the 62.5 ng dose decreased active lever responding in the cocaine-paired context (Tukey test,  $p < 0.05$ ), without altering active lever responding in the extinction context, relative to vehicle.

During the locomotor activity test session, the number of photobeam breaks gradually decreased (time main effect,  $F_{(2, 14)} = 39.14$ ,  $p = 0.0001$ ; 20-min interval 1 > 2-3, Tukey test,  $p < 0.05$ ; Figure 3b). Furthermore, the behaviorally effective, 62.5 ng dose of PP2 did not alter the number of photobeam breaks relative to vehicle (treatment main and interaction effects,  $F_{(1-2, 7-14)} = 0.35-0.86$ ,  $p = 0.38-0.71$ ).

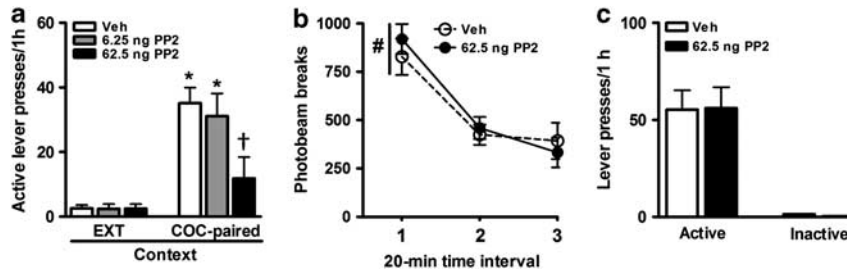
Food reinforcement elicited robust responding on the active lever (lever main effect,  $F_{(1, 7)} = 158.60$ ,  $p = 0.0001$ ; Figure 3c). However, the 62.5 ng dose of PP2 did not alter food-reinforced active or inactive lever responding, relative to vehicle (all treatment main and interaction effects,  $F_{(1, 7)} = 0.003-0.016$ ,  $p = 0.90-0.96$ ).

## Effects of Intra-DH PP2 Treatment on NR2B Activation and Drug Context-Induced Cocaine Seeking

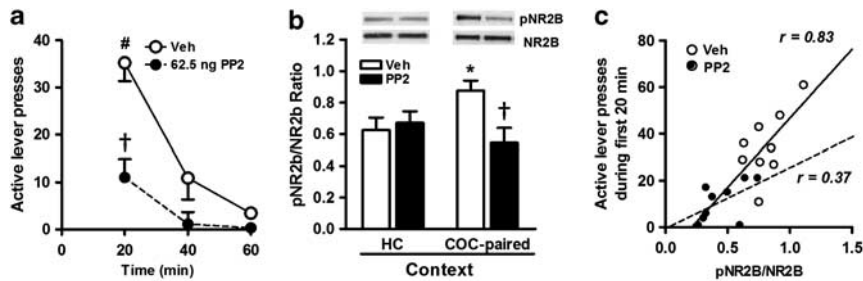
Replicating experiment 2, intra-DH administration of the 62.5 ng dose of PP2 altered active lever responding as a function of context and time (all treatment main and interaction effects,  $F_{(1, 16)} = 18.21-45.72$ ,  $p = 0.0001-0.001$ ; Supplementary Figure S2). Specifically, following bilateral administration of vehicle in the DH, robust active lever responding occurred during the first 20 min, and it declined during the test session in the cocaine-paired



**Figure 2** Effects of bilateral AP5 pretreatment in the dorsal hippocampus (DH) on non-reinforced active lever responses (a: mean/1 h + SEM;  $N = 7-14$ /dose) during testing in the extinction (EXT) and previously cocaine-paired contexts (COC), on locomotor activity in a novel context (b: mean photobeam breaks/1 h + SEM;  $N = 7$ /dose), and on food-reinforced instrumental behavior in a neutral context (c: mean active and inactive lever presses/1 h + SEM;  $N = 7$ /dose). Asterisks represent significant difference relative to responding in the extinction context (analysis of variance (ANOVA) context simple main effect, Tukey test,  $p < 0.05$ ). Dagger represents significant difference relative to vehicle treatment (ANOVA treatment simple main effect, Tukey test,  $p < 0.05$ ). Pound sign represents significant difference relative to all other time points (ANOVA time simple main effect, Tukey test,  $p < 0.05$ ).



**Figure 3** Effects of bilateral PP2 pretreatment in the dorsal hippocampus (DH) on non-reinforced active lever responses (a: mean/1 h + SEM;  $N = 8-16$ /dose) during testing in the extinction (EXT) and previously cocaine-paired contexts (COC), on locomotor activity in a novel context (b: mean photobeam breaks/1 h + SEM;  $N = 8$ /dose), and on food-reinforced instrumental behavior in a neutral context (c: mean active and inactive lever presses/1 h + SEM;  $N = 8$ /dose). Asterisks represent significant difference relative to responding in the extinction context (analysis of variance (ANOVA) context simple main effect, Tukey test,  $p < 0.05$ ). Dagger represents significant difference relative to vehicle treatment (ANOVA treatment simple main effect, Tukey test,  $p < 0.05$ ). Pound sign represents significant difference relative to all other time points (ANOVA time simple main effect, Tukey test,  $p < 0.05$ ).



**Figure 4** Effects of bilateral PP2 pretreatments in the dorsal hippocampus (DH) on non-reinforced active lever responses (a: mean/20 min + SEM;  $N = 9$ /dose) during a 60-min test in the previously cocaine-paired context (COC), on NR2B N-methyl-D-aspartate glutamate (NMDA) subunit activation in the DH following testing in the cocaine-paired context or home cage (HC) exposure (b: mean ratio + SEM;  $N = 9$ /dose), and on the relationship between the level of NR2B NMDA subunit activation and active lever responding during the first 20 min of testing in the cocaine-paired context (c:  $N = 9$ ). Pound sign represents significant difference relative to all other time points (analysis of variance (ANOVA) time simple main effect, Tukey test,  $p < 0.05$ ). Daggers represent significant difference relative to vehicle treatment (ANOVA treatment simple main effect, Tukey test,  $p < 0.05$ ). Asterisk represents significant difference relative to HC control (Tukey test,  $p < 0.05$ ). The  $r$  values refer to Pearson's correlational coefficients (vehicle,  $p = 0.005$ ; PP2,  $p = 0.32$ ).

context (Tukey test,  $p < 0.01$ , 20-min interval 1 > 2-3; Figure 4a). Bilateral administration of 62.5 ng dose of PP2 into the DH reduced active lever responding during the first 20 min in the cocaine-paired context, relative to vehicle (all treatment main and interaction effects,  $F_{(1-2, 16-32)} = 9.63-41.90$ ,  $p = 0.0001-0.001$ , Tukey test,  $p < 0.01$ ; Figure 4a).

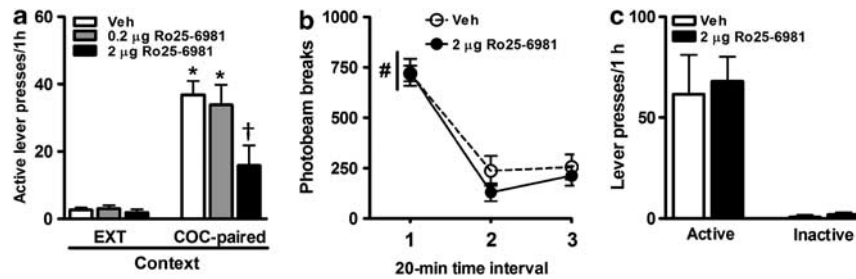
Western blot analyses of NR2B activation were conducted on tissues collected immediately after this 1-h test session or home cage exposure (ie, 60-65 min following PP2 administration). In the vehicle-treated rats, NR2B activation in the DH was greater following testing in the cocaine-paired context, relative to home cage exposure (treatment main and interaction effects,  $F_{(1, 31)} = 4.47-8.33$ ,  $p = 0.008-0.044$ , Tukey test,  $p < 0.05$ ; context main effect,  $F_{(1, 31)} = 5.05$ ,  $p = 0.033$ ; Figure 4b). Furthermore, PP2 pretreatment attenuated the increase in NR2B activation following testing in the cocaine-paired context (Tukey test,  $p < 0.05$ ), but did not alter NR2B activation following home cage exposure (Figure 4b). For vehicle-treated rats, there was a positive correlation between NR2B activation and active lever responding during the first 20 min the test session (Pearson's  $r = 0.83$ ,  $p = 0.005$ ; Figure 4c), and PP2 eliminated this correlation (Pearson's  $r = 0.37$ ,  $p = 0.32$ ; Figure 4c). Notably, correlations could not be detected between NR2B activation and (1) active lever responding during the second and third 20-min intervals of the test session, likely due to low variability in responding after behavioral extinction (Pearson's

$r = 0.30-0.49$ ,  $p = 0.09-0.79$ ; data not shown), and (2) cocaine history (ie, total drug intake during self-administration training, Pearson's  $r = 0.21$ ,  $p = 0.46$ ; data not shown).

### Effects of Intra-DH Ro 25-6981 Treatment on Drug Context-Induced Cocaine-Seeking and Motor Behaviors

Intra-DH Ro25-6981 treatment attenuated active lever responding as a function of testing context and dose (Figure 5a). Following pretreatment with vehicle or the 0.2  $\mu\text{g}$  dose of Ro25-6981, exposure to the cocaine-paired context increased active lever responding relative to responding in the extinction context (all treatment main and interaction effects,  $F_{(1-2, 29)} = 4.08-72.74$ ,  $p = 0.0001-0.02$ , Tukey test,  $p < 0.05$ ). The 0.2  $\mu\text{g}$  dose of Ro25-6981 failed to alter active lever responding in either context relative to vehicle. In contrast, the 2.0  $\mu\text{g}$  dose decreased active lever responding in the cocaine-paired context (Tukey test,  $p < 0.05$ ), without altering active lever responding in the extinction context, relative to vehicle.

During the locomotor activity test session, the number of photobeam breaks gradually decreased (time main effect,  $F_{(2, 14)} = 66.18$ ,  $p = 0.0001$ ; 20-min interval 1 > 2-3, Tukey test,  $p < 0.05$ ). Furthermore, the behaviorally effective, 2.0  $\mu\text{g}$  dose of Ro25-6981 did not alter the number of photobeam breaks relative to vehicle (treatment main and interaction effect,  $F_{(1-2, 7-14)} = 0.20-1.17$ ,  $p = 0.34-0.67$ ; Figure 5b).



**Figure 5** Effects of bilateral Ro25-6981 pretreatment in the dorsal hippocampus (DH) on non-reinforced active lever responses (a: mean/1 h + SEM;  $N = 8-16$ /dose) during testing in the extinction (EXT) and previously cocaine-paired contexts (COC), on locomotor activity in a novel context (b: mean photobeam breaks/1 h + SEM;  $N = 8$ /dose), and on food-reinforced instrumental behavior (c: mean active and inactive lever presses/1 h + SEM;  $N = 8$ /dose). Asterisks represent significant difference relative to responding in the extinction context (analysis of variance (ANOVA) context simple main effect, Tukey test,  $p < 0.05$ ). Dagger represents significant difference relative to vehicle treatment (ANOVA treatment simple main effect, Tukey test,  $p < 0.05$ ). Pound sign represents significant difference relative to all other time points (ANOVA time simple main effect, Tukey test,  $p < 0.05$ ).

Food reinforcement elicited greater responding on the active lever than on the inactive lever (lever main effect,  $F_{(1, 7)} = 42.11$ ,  $p = 0.0001$ ). Furthermore, the 2.0 μg dose of Ro25-6981 did not alter food-reinforced active or inactive lever responding, relative to vehicle (all treatment main and interaction effects,  $F_{(1, 7)} = 0.04-0.09$ ,  $p = 0.77-0.84$ ; Figure 5c).

## DISCUSSION

This study evaluated the contribution of Src-family kinases, NMDARs, and specifically NR2B subunit-containing NMDARs, in the expression of drug context-induced cocaine-seeking behavior. PP2-induced Src-family kinase inhibition or AP5-induced NMDAR antagonism in the DH dose-dependently attenuated this behavior relative to vehicle. NR2B NMDAR subunit activation in the DH increased following the test of cocaine-seeking behavior in the previously cocaine-paired context, relative to home cage exposure. The behaviorally effective dose of PP2 selectively decreased NR2B activation in the DH, which was associated with reduction in drug context-induced cocaine-seeking behavior. Similarly, Ro25-6981-induced selective antagonism of NR2B subunit-containing NMDARs in the DH dose-dependently attenuated drug context-induced cocaine-seeking behavior relative to vehicle. These manipulations did not produce non-specific performance deficits, as they did not alter instrumental performance in the extinction context, general locomotor activity, or food-reinforced instrumental behavior. Furthermore, the manipulations were probably selective to the anterior CA1/CA3 regions of the DH, as we have previously shown that global neuronal inactivation of the overlying cortex or of the adjacent dentate gyrus or posterior CA1/CA3 regions of the DH fails to alter drug context-induced cocaine seeking (Fuchs *et al*, 2005; Lasseter *et al*, 2010). Together, these findings suggest that activation of Src-family kinases, stimulation of NMDAR, and likely Src-family kinase-mediated NR2B-containing NMDAR activation in the DH are necessary for drug context-induced cocaine-seeking behavior.

The present study significantly extends upon our previous finding that mGluR1 stimulation in the DH is necessary for drug context-induced cocaine-seeking behavior (Xie *et al*, 2010) by demonstrating the critical

role of NMDAR in this phenomenon. Furthermore, in the present study, NMDAR antagonism and selective NR2B inhibition had similar behavioral effects, suggesting that NR2B subunit-containing NMDARs have a critical role in drug context-induced cocaine seeking. However, these pharmacological manipulations were not aimed at a particular subregion of the DH (eg, CA1-3, dentate gyrus). NMDARs are mostly postsynaptic in the hippocampus (Nyiri *et al*, 2003; Takumi *et al*, 1999), and have a greater expression of NR2B subunits than other NMDA subunits (ie, NR2A) in the CA1 subregion, but not in the CA3 subregion or the dentate gyrus (Coultrap *et al*, 2005; Pandis *et al*, 2006). Also, within the CA1 subregion, NMDAR subunit composition and density vary across cell types (Angulo *et al*, 2004; Nyiri *et al*, 2003; Serrano *et al*, 2006; Serrano *et al*, 2008; Siegel *et al*, 1994; Szabadits *et al*, 2011; Todd *et al*, 2006; Xue *et al*, 2011). Thus, given the functional heterogeneity of hippocampal NMDAR populations (Kesner, 2007; Lee *et al*, 2003), future studies will be necessary to dissect the role of distinct NR2B subunit-containing NMDAR subpopulations within the DH in drug context-induced cocaine-seeking behavior.

Although NMDAR stimulation or Src-family kinase activity in the DH was not necessary for food-reinforced lever pressing behavior in the present study, it is unclear whether NMDARs or Src-family kinases in the DH contribute to contextual natural reward-seeking behavior *per se*. When applied to a food reinforcer, our training protocol is not sufficient to elicit contextual food-seeking behavior (Xie *et al*, 2010). This may be due to overshadowing by highly predictive, food-related visual, olfactory, and gustatory stimuli during food self-administration training, which diminishes the salience and associative value of the food-predictive context (Xie *et al*, 2010). Given that food-related sensory stimuli could not be eliminated, a direct comparison of the role of NMDARs or Src-family kinases in contextual drug-seeking *vs* natural reward-seeking behaviors was not feasible in the present study.

NMDARs in the DH have been implicated in various memory processes. For instance, NMDAR stimulation is necessary for the retrieval or expression of spatial, inhibitory avoidance, and trace fear memory (Corcoran *et al*, 2011; Matus-Amat *et al*, 2007; Melik *et al*, 2006; Quinn *et al*, 2005; Rodrigues *et al*, 2001), but not contextual fear memory (Fellini *et al*, 2009; Jamali-Raeufy *et al*, 2011; Quinn *et al*, 2005;

Yoshihara and Ichitani, 2004). Adding to this literature, the present findings suggest that NMDARs, specifically NR2B subunit-containing NMDARs, in the DH are required for the recall or utilization of context-response-cocaine associative memories and/or for motivational processes that underlie contextual control over drug-seeking behavior (Fuchs *et al*, 2008a). Conversely, NMDAR stimulation in the DH is not critical for the retrieval or utilization of extinction memories as NMDAR antagonism did not increase lever responding in the extinction context.

Functional interactions between Src-family kinases and NMDARs are well documented. Src-family kinases can phosphorylate NR2B subunits at tyrosine residue 1472, which is critical for NR2B subunit-containing NMDAR surface expression and functional potentiation (Ali and Salter, 2001; Groveman *et al*, 2012; Lu *et al*, 1999a; Salter, 1998; Salter and Kalia, 2004; Sinai *et al*, 2010; Stramiello and Wagner, 2008). The present study revealed that NR2B phosphorylation at Y1472 in the DH increased following testing in the cocaine-paired context, relative to home cage exposure. Although this increase was independent of cocaine history, it was positively correlated with the magnitude of cocaine-seeking behavior during the first 20 min of the test session. PP2 treatment in the DH disrupted this relationship and reduced NR2B activation following testing in the cocaine-paired context to a level seen following home cage exposure, without altering NR2B activation following home cage exposure. These findings suggest that Src-family kinase-mediated NR2B activation is critical for drug context-induced cocaine-seeking behavior. However, future studies will need to explore whether DH NR2B activation is sufficient to increase drug context-induced cocaine-seeking behavior or goal-directed behavior in general.

PP2 strongly inhibits several members of the Src tyrosine kinase family, including Src, Lck, Fyn, and Hck *in vitro* ( $IC_{50}$  = 4–170 nM; Bain *et al*, 2003; Hanke *et al*, 1996; Lawrence and Niu, 1998). *In vivo* studies demonstrate that Fyn activation is necessary for a number of aversive conditioned behaviors as well as alcohol-conditioned place preference, alcohol self-administration, and drug-primed reinstatement of alcohol-seeking behavior, in a brain region-specific manner (Isosaka *et al*, 2008; Kojima *et al*, 2005; Schafe *et al*, 1996; Wang *et al*, 2007; Wang *et al*, 2010; but see Yaka *et al*, 2003). Within the DH, Fyn activation is required for contextual fear conditioning (Isosaka *et al*, 2008). Fyn knockout mice exhibit deficiency in hippocampal long-term potentiation (LTP) induction and spatial learning (Grant *et al*, 1992), while overexpression of constitutively active mutant Fyn results in enhanced excitatory postsynaptic potentials in response to afferent stimulation and reduced hippocampal LTP induction threshold (Lu *et al*, 1999b). Given that functional heterogeneity may exist across members of the Src tyrosine kinase family, future studies will be necessary in order to identify whether Fyn or other Src-family kinases exert dominant control over drug context-induced cocaine-seeking behavior.

Various neurochemical mechanisms, including glutamate or dopamine receptor stimulation, may trigger Src-family kinase-mediated NR2B activation in the DH. For example, mGluR1 stimulation activates Src-family kinases (Heuss

*et al*, 1999) and potentiates NMDAR current in hippocampal pyramidal cells (Benquet *et al*, 2002). Dopamine D1-like receptor stimulation in the hippocampus also enhances the Src-family kinase-mediated phosphorylation of NR2B subunits and potentiates D1-like receptor-mediated LTP (Stramiello and Wagner, 2008). Furthermore, mGluR1 or D1-like receptor stimulation in the DH is necessary for drug context-induced cocaine-seeking behavior (Xie *et al*, 2010; Xie and Fuchs, unpublished data). However, distinct pools of Src-family kinase activated by different intracellular signaling pathways may have different functions. Therefore, it will be important to identify the upstream activators of Src-family kinases in the DH that contribute to drug context-induced cocaine-seeking behavior *per se*.

Importantly, plasticity in Src-family kinase function may fundamentally contribute to the development of compulsive cocaine-seeking and -taking behaviors. Consistent with this, acute cocaine exposure increases Src-family kinase activation in the ventral tegmental area (Schumann *et al*, 2009). Furthermore, chronic alcohol exposure produces long-lasting Fyn activation in the dorsomedial striatum, which is critical for alcohol self-administration and drug-primed alcohol-seeking behavior (Wang *et al*, 2010). Similar plasticity in DH Src-family kinase physiology has yet to be demonstrated, but understanding putative, drug-induced, or experience-based neuroplasticity in Src-family kinase activity within the DH and other limbic brain regions may aid in the development of effective treatments for drug relapse prevention.

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