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ABSTRACT

Effects of stress on the reward system are well established in the literature. Although previous studies have revealed that stress can reinstate extinguished addictive behaviors related to cocaine, the effects of stress on the rewarding memory of cocaine are not fully understood. Here, we provide evidence that stress potentiates the expression of rewarding memory of cocaine via the activation of brainstem-reward circuitry using a cocaine-induced conditioned place preference (CPP) paradigm combined with restraint stress in rats. The rats exposed to 30-min restraint stress immediately before posttest exhibited significantly larger CPP scores compared to non-stressed rats. Intra-laterodorsal tegmental nucleus (LDT) microinjection of a β or α2 adrenoceptor antagonist attenuated the stress-induced enhancement of cocaine CPP. Consistent with this observation, intra-LDT microinjection of a β or α2 adrenoceptor agonist before posttest increased cocaine CPP. Additionally, intra-ventral tegmental area (VTA) microinjection of antagonists for the mAChR, nAChR, or glutamate receptors attenuated the stress-induced enhancement of cocaine CPP. Finally, intra-medial prefrontal cortex (mPFC) microinjection of a D1 receptor antagonist also reduced the stress-induced enhancement of cocaine CPP. These findings suggest a mechanism wherein the LDT is activated by noradrenergic input from the locus coeruleus, leading to the activation of VTA dopamine neurons via both cholinergic and glutamatergic transmission and the subsequent excitation of the mPFC to enhance the memory of cocaine-induced reward value.

Keywords addiction, dopamine, laterodorsal tegmental nucleus, medial prefrontal cortex, noradrenaline, ventral tegmental area

INTRODUCTION

Stress can have various effects on behaviors associated with cocaine use. A large body of literature indicates that stress reinstates extinguished cocaine self-administration (Capriles *et al.* 2003; Mantsch *et al.* 2016) and cocaine conditioned place preference (CPP) in rodents (Redila & Chavkin 2008; Mantsch *et al.* 2010). Because extinction therapy for addiction is not necessarily a successful rehabilitation strategy (Conklin & Tiffany 2002), it is crucial to elucidate how stress potentiates the rewarding memory of cocaine in the absence of an extinction procedure. Several studies have investigated this research question using CPP; however, animals in previous studies were typically exposed to stress before the start of conditioning (McLaughlin *et al.* 2003; Kreibich *et al.* 2009; Schindler *et al.* 2012; Montagud-Romero *et al.* 2015). Given that cocaine-associated rewarding memories drive craving, it is also critical to investigate the effects of stress exposure after a preference for cocaine is formed. To this end, Schindler *et al.* (2010) found that κ-receptor activation associated with forced swim stress prior to posttest potentiated the rewarding valence of cocaine. Yet, except for the involvement of the κ-opioid system, the mechanisms underlying this potentiating effect of stress remain unclear.

The noradrenaline (NA) system is critically associated with the stress response. A variety of stressors including foot shock, forced swimming, and restraint activate NA neurons (Abercrombie & Jacobs 1987; Chowdhury *et al*. 2000; Salchner *et al*. 2004; Takase *et al.* 2005) and produce elevations in NA in several brain regions (Jordan *et al*. 1994; Tanaka 1999). These alterations in NA have been previously associated with stress-induced drug seeking behavior (Mantsch *et al.* 2016). Yet, few studies have investigated how stress-induced activation of the NA system affects the rewarding memory of cocaine (Mantsch *et al*. 2014). Understanding these points is critical for developing novel therapeutic strategies to treat cocaine addiction.

We previously reported that the laterodorsal tegmental nucleus (LDT) of the brainstem participated in the acquisition and expression of cocaine CPP (Shinohara *et al.* 2014; Kamii *et al.*

2015). The LDT contains cholinergic, glutamatergic, and GABAergic neurons (Wang & Morales 2009). The cholinergic and glutamatergic afferents to the VTA are crucial for regulating dopamine (DA) neuron activity and reward information processing (Gronier & Rasmussen 1998; Lodge & Grace 2006; Lammel *et al*. 2012). Given previous evidence that stress affects reward-related circuitry (Mantsch *et al*. 2016) and that the LDT receives NA inputs from the adjacently located locus coeruleus (LC) (Cornwall *et al*. 1990; Semba & Fibiger 1992), we hypothesized that stress may affect activity in the LDT and subsequently the VTA by increasing LDT levels of NA, leading to the potentiation of cocaine reward memory. We addressed this hypothesis using CPP paradigm combined with acute restraint stress and pharmacological agents administered immediately prior to posttest of cocaine CPP.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (weighing 170–240 g at the beginning of behavioral tests) were maintained in a temperature-controlled (22 ± 1 °C) room under a 12-h light/dark cycle with food and water available *ad libitum*. All experiments were conducted in accordance with the National Institutes of Health guidelines and performed with the approval of the Institutional Animal Care and Use Committee at Hokkaido and Kanazawa University. All efforts were made to minimize the number and suffering of animals used in the experiments.

Drugs

Cocaine hydrochloride (Takeda Pharmaceutical, Osaka, Japan) was dissolved in saline. Timolol maleate (β adrenoceptor antagonist, 2.0 μg/0.2 μL/side; Colussi-Mas *et al*. 2005), terazosin hydrochloride (α1 adrenoceptor antagonist, 1.0 μg/0.2 μL/side; Lazzaro *et al*. 2010), RX821002 hydrochloride (α2 adrenoceptor antagonist, 3.0 μg/0.2 μL/side; Alves *et al*. 2014), (-)-isoproterenol

(+)-bitartrate salt (β adrenoceptor agonist, 3.0 μg/0.2 μL/side; Pavesi *et al.* 2011), guanfacine hydrochloride (α2 adrenoceptor agonist, 0.01 μg/0.2 μL/side; Abela & Chudasama 2014), scopolamine hydrochloride (mAChR antagonist, 50 μg/0.5 μL/side; Chapman *et al.* 1997), mecamylamine hydrochloride (nAChR antagonist, 50 μg/0.2 μL/side; Chen *et al.* 2006), dl-AP5 (NMDAR antagonist, 0.02 μg/0.2 μL/side; Mahler *et al.* 2012), CNQX disodium salt hydrate (AMPA receptor antagonist, 0.01 μg/0.2 μL/side; Mahler *et al.* 2012), R(+)-SCH23390 hydrochloride (D1 DA receptor antagonist, 1.0 μg/0.5 μL/side; Hall *et al.* 2009) and raclopride (D2 DA receptor antagonist, 3.0 μg/0.5 μL/side; Pardey *et al* 2013) were purchased from Sigma–Aldrich (St. Louis, MO) except for guanfacine (Wako, Osaka, Japan) and raclopride (Tocris Bioscience), and dissolved in 0.1 M PBS (pH = 7.4). The doses of all drugs (including cocaine) except for raclopride were expressed in terms of their salt weight.

Surgery and microinjection

Under sodium pentobarbital anesthesia (50 mg/kg, i.p.), rats were implanted bilaterally with 25-gauge stainless-steel guide cannulae (o.d., 0.5 mm; i.d., 0.22 mm) above the LDT (9.0 mm caudal, 0.83 mm lateral, 7.0 mm ventral to bregma), VTA (5.8 mm caudal, 1.0 mm lateral, 8.5 mm ventral to bregma) or mPFC (3.0 mm rostral, 0.67 mm lateral, 4.0 mm ventral to bregma) (Paxinos & Watson 2007). In the case of LDT surgery, the guide cannulae were implanted at a 22° angle from the vertical axis in the rostrocaudal plane. After surgery, rats were housed individually in their home cages, allowed to recover for 6–9 days, and handled each day for 3 consecutive days before the behavioral experiments. For microinjection, 33-gauge stainless-steel injection cannulae (o.d., 0.2 mm; i.d., 0.08 mm) were inserted bilaterally into the guide cannulae. The injection cannulae protruded 1.5 mm from the tip of the guide cannulae to reach the LDT, VTA or mPFC. Bilateral infusions were performed at a volume of 0.2 (LDT, 0.2 μL/min) or 0.5 μL (VTA, mPFC, 0.5 μL/min) in each side, and the injection cannulae were left in place for an additional 1 min after microinjection

to prevent backflow.

CPP

CPP tests were conducted as described previously (Shinohara *et al.* 2014, Fig. 1A). The CPP chambers consisted of two equally-sized compartments (30 × 30 × 30 cm) with distinct tactile and visual cues (one compartment had a black floor and walls with an equally spaced stainless-steel stripe-like grid on the floor, and the other had a white floor and walls with stainless-steel grid on the floor), which were separated by a removable partition. On days 1 (habituation), 2 (pretest) and 11 (posttest), rats freely explored the two compartments for 900 s, and the time spent in each compartment during the exploratory period and locomotor activity were measured using infrared sensors (Supermex, Muromachi Kikai, Tokyo, Japan), which were positioned on the top cover of each compartment. Rats that spent >80% (>720 s) of the total time (900 s) in one side on day 2 (n = 1) or showed a difference of >200 s in the time spent in one side between days 1 and 2 (n = 1) were excluded from subsequent procedures. We used a biased design (Tzschentke 1998) and designated the compartment in which each rat spent less time on day 2 (pretest) as their cocaine-paired compartment. On days 4–9 (conditioning), rats were given alternating injections of cocaine (5 mg/kg, i.p.) or saline (1 mL/kg, i.p.) and confined to one compartment for 30 min on 6 consecutive days. In some experiments, a higher dose of cocaine (20 mg/kg) was used for conditioning. The CPP scores were calculated by subtracting the time spent in the cocaine-paired compartment during the pretest from that during the posttest.

Restraint stress

On day 11 of CPP test, rats of stressed groups were restrained using plastic bags (DecapiCones, Briantree Scientific) for 15 or 30 min immediately before the posttest. Rats of non-stressed groups were left in their home cages for the same time periods. Intracranial drug injection was conducted 2

min before the restraint stress exposure.

Histology

After the CPP tests, to confirm the placements of the drug injection, the brains were rapidly removed and frozen in powdered dry ice. Coronal sections (50 μm) of the LDT, VTA or mPFC were prepared using a cryostat, thaw-mounted onto slides, stained with thionin (0.25%), and examined under a microscope (×40).

Statistical analyses

Data are expressed as means ± S.E.M. and were compared using a one-way or a two-way analysis of variance (ANOVA) followed by the Holm-Sidak *post hoc* test when comparing more than two groups or paired *t*-test or student's *t*-test when comparing only two groups.

RESULTS

Acute restraint stress enhances cocaine-induced CPP

We first investigated the effects of restraint stress exposed immediately before posttest on cocaine CPP (Fig. 1A). Under the non-stressed condition, low-dose cocaine (5 mg/kg) conditioning did not significantly change the time spent in cocaine-paired compartment during the posttest compared to that during the pretest (non-stress for 15 min, pretest, 363.5 ± 17.7 s, posttest, 469.7 ± 51.1 s, $n = 6$, $t_5 = 1.847$, $P = 0.12$, non-stress for 30 min, pretest, 370.5 ± 10.8 s, posttest, 434.1 ± 45.1 s, $n = 7$, $t_6 = 1.307$, $P = 0.239$, paired *t*-test; Fig. 1B). This indicates that a low dose of cocaine induces a non-significant level of CPP. Then, rats were exposed to restraint stress for either 15 or 30 min immediately before the posttest. Although the 15-min stressed group showed no significant difference between the time spent in cocaine-paired compartment during the pretest and that during the posttest (pretest, 347.3 ± 23.7 s, posttest, 450.0 ± 55.5 s, $n = 6$, $t_5 = 1.472$, $P = 0.20$, paired *t*-test;

Fig. 1B), stress exposure for 30 min significantly increased the time spent in cocaine-paired compartment during the posttest compared to that during the pretest (pretest, 351.0 ± 12.8 s, posttest, 626.2 ± 27.3 s, n = 7, t_6 = 10.70, P < 0.0001, paired t-test; Fig. 1B). As shown in Fig. 1D, a two-way ANOVA revealed a significant interaction between the effects of stress and duration of stress exposure ($F_{1,22}$ = 4.414, P = 0.0473). CPP scores after stress exposure for 30 min were significantly larger than those after non-stress exposure (non-stress for 30 min, 63.64 ± 48.7, n = 7; stress for 30 min, 275.21 ± 25.7, n = 7; P = 0.0119, *post hoc* Holm-Sidak test; Fig. 1D). On the other hand, 15-min stress exposure did not affect CPP scores compared to non-stress exposure (non-stress for 15 min, 106.2 ± 53.2 s, n = 6; stress for 15 min, 102.7 ± 64.6 s, n = 6; P = 0.9633; Fig. 1D). We confirmed that there were no significant differences between the time spent in saline-paired compartment (where rats spent less time during the pretest) during the pretest and that during the posttest in non-stressed and stressed groups when the rats were conditioned with saline only (non-stress, pretest, 344.8 ± 24.9 s, posttest, 347.4 ± 27.6 s, n = 8, t_7 = 0.09657, P = 0.93, stress, pretest, 351.6 ± 13.3 s, posttest, 321.1 ± 51.7 s, n = 6, t_5 = 0.4766, P = 0.65, paired t-test; Fig. 1C). Accordingly, there was no significant difference in CPP scores between these groups (non-stress, −30.5 ± 59.3 s, n = 8; stress, 2.63 ± 27.2 s, n = 6; t_{12} = 0.524, P = 0.6096, Student's t-test; Fig. 1E). These results indicated that 30-min restraint stress significantly enhanced cocaine CPP.

Role of noradrenergic transmission to the LDT in the stress-induced enhancement of cocaine CPP

Stress activates LC neurons and facilitates noradrenergic transmission in several brain regions (Abercrombie & Jacobs 1987; Jordan *et al*. 1994; Tanaka 1999; Chowdhury *et al*. 2000; Salchner *et al*. 2004; Ma & Morilak 2005; Takase *et al.* 2005). Since previous studies have indicated that the LDT is involved in the expression of cocaine CPP (Shinohara *et al.* 2014; Kamii *et al.* 2015) and receives noradrenergic transmission from the LC (Semba & Fibiger 1992), we hypothesized that this

transmission would also be involved in stress-induced enhancement of cocaine CPP. To test this, we performed intra-LDT microinjections of α1, α2, or β adrenoceptor antagonists before the restraint stress procedure (Fig. 2A). In the vehicle or terazosin injection groups, the time spent in cocaine-paired compartment during the posttest was significantly longer than that during the pretest (vehicle, pretest, 350.3 ± 15.4 s, posttest, 677.7 ± 36.3 s, n = 5, t_4 = 8.406, P = 0.0011; terazosin, pretest, 355.3 ± 25.0 s, posttest, 718.7 ± 41.4 s, n = 5, t_4 = 10.57, P = 0.0005, paired t-test; Fig. 2B). On the other hand, in the timolol or RX821002 injection groups, there were no significant differences between the time spent in cocaine-paired compartment during the pretest and that during the posttest (timolol, pretest, 347.1 ± 14.9 s, posttest, 420.1 ± 109.8 s, n = 5, t_4 = 0.751, P = 0.49; RX821002, pretest, 354.7 ± 15.8 s, posttest, 439.0 ± 54.7 s, n = 6, t_5 = 1.472, P = 0.20, paired t-test; Fig. 2B). As shown in Fig. 2C, a one-way ANOVA revealed a significant difference in CPP scores among the groups ($F_{3,17}$ = 5.99, P = 0.0056). *Post hoc* Holm-Sidak tests revealed that, compared to vehicle injection, intra-LDT microinjection of timolol or RX821002 but not terazosin significantly decreased CPP scores (vehicle, 327.4 ± 39.0 s, n = 5; timolol, 73.0 ± 97.2 s, n = 5, P = 0.0335; terazosin, 363.4 ± 34.4 s, n = 5, P = 0.6921; RX821002, 84.3 ± 57.3 s, n = 6, P = 0.0355; Fig. 2C). These results indicated that noradrenergic transmission to the LDT via β and α2 adrenoceptors, but not α1 adrenoceptors, contributed to the stress-induced enhancement of cocaine CPP.

We also examined whether stimulation of β or α2 adrenoceptors in the LDT alone would be sufficient to enhance cocaine CPP. Fig. 2D depicts the drug and vehicle injection sites. Although there was no significant difference between the time spent in cocaine-paired compartment during the pretest and that during the posttest in the vehicle injection group (pretest, 336.5 ± 13.0 s, posttest, 414.1 ± 51.0 s, n = 6, t_5 = 1.653, P = 0.16, paired t-test; Fig. 2E), intra-LDT injection of isoproterenol or guanfacine significantly increased the time spent in cocaine-paired compartment during the posttest compared to that during the pretest (isoproterenol, pretest, 329.1 ± 28.6 s, posttest, 534.3 ± 31.0 s, n = 5, t_4 = 6.974, P = 0.0022; guanfacine, pretest, 341.7 ± 24.7 s, posttest, 564.3 ±

46.7 s, n = 5, t_4 = 6.329, P = 0.0032, paired t-test; Fig. 2E). As shown in Fig. 2F, a one-way ANOVA revealed a significant difference in CPP scores among the groups ($F_{2,13}$ = 4.24, P = 0.0384). As compared to vehicle injection, intra-LDT microinjection of isoproterenol or guanfacine significantly increased CPP scores (vehicle, 77.58 ± 46.9 s, n = 6; isoproterenol, 205.2 ± 29.4 s, n = 5, P = 0.0415; guanfacine, 222.6 ± 35.2 s, n = 5, P = 0.0415; *post hoc* Holm-Sidak test; Fig. 2F). Taken together, these results demonstrated that noradrenergic transmission to the LDT via β and α2 adrenoceptors enhanced cocaine CPP.

We further investigated whether the β and α2 adrenoceptor-mediated effects are selective for stress-induced enhancement of cocaine CPP. To test this, we used a higher dose of cocaine (20 mg/kg) for conditioning and examined the effects of intra-LDT injections of timolol or RX821002 (Fig. 3A) on the expression of cocaine CPP in the absence of restraint stress. In the vehicle, timolol or RX821002 injection groups, the time spent in cocaine-paired compartment during the posttest were significantly longer than that during the pretest (vehicle, pretest, 377.1 ± 5.9 s, posttest, 531.3 ± 36.3 s, n = 8, t_7 = 4.163, P = 0.0042, timolol, pretest, 363.6 ± 11.9 s, posttest, 534.2 ± 37.9 s, n = 7, t_6 = 3.874, P = 0.0082, RX821002, pretest, 344.2 ± 6.9 s, posttest, 515.8 ± 22.9 s, n = 7, t_6 = 7.168, P = 0.0004, paired t-test; Fig. 3B). As shown in Fig. 3C, a one-way ANOVA revealed no significant difference in CPP scores among the groups ($F_{2,19}$ = 0.0755, P = 0.928). These results indicated that the blockade of β and α2 adrenoceptors does not affect the expression of cocaine CPP in the absence of stress exposure and suggest that the noradrenergic contribution is selective for the restraint condition.

Role of cholinergic and glutamatergic transmission to the VTA in the stress-induced enhancement of cocaine CPP

We previously reported that signal transmission from the LDT to the VTA was critical for the expression of cocaine CPP (Shinohara *et al.* 2014; Kamii *et al.* 2015). Given that the LDT contains cholinergic and glutamatergic neurons, we next investigated whether cholinergic or glutamatergic

transmission to the VTA was involved in the stress-induced enhancement of cocaine CPP. First, we administered a cholinergic receptor antagonist directly into the VTA before stress exposure (Fig. 4A). In the vehicle or scopolamine injection groups, the time spent in cocaine-paired compartment during the posttest was significantly longer than that during the pretest (vehicle, pretest, 346.1 ± 27.2 s, posttest, 638.5 ± 66.7 s, n = 5, t_4 = 5.846, P = 0.0043; scopolamine, pretest, 300.6 ± 28.3 s, posttest, 411.7 ± 28.9 s, n = 5, t_4 = 6.988, P = 0.0022, paired t-test; Fig. 4B). On the other hand, in the mecamylamine injection group, there was no significant difference between the time spent in cocaine-paired compartment during the pretest and that during the posttest (pretest, 338.3 ± 18.0 s, posttest, 433.2 ± 59.2 s, n = 5, t_4 = 1.783, P = 0.15, paired t-test; Fig. 4B). As shown in Fig. 4C, a one-way ANOVA revealed a significant difference in CPP scores among the groups ($F_{2,12}$ = 6.46, P = 0.0125). *Post hoc* Holm-Sidak tests revealed that, compared to vehicle injection, intra-VTA microinjection of scopolamine or mecamylamine significantly decreased CPP scores (vehicle, 292.4 ± 50.0 s, n = 5; scopolamine, 111.1 ± 15.9 s, n = 5, P = 0.0142; mecamylamine, 94.9 ± 53.2 s, n = 5, P = 0.0142; Fig. 4C).

We next blocked glutamate receptors in the VTA before stress exposure (Fig. 5A). In the vehicle injection group, the time spent in cocaine-paired compartment during the posttest was significantly longer than that during the pretest (pretest, 346.1 ± 27.2 s, posttest, 638.5 ± 66.7 s, n = 5, t_4 = 5.846, P = 0.0043, paired t-test; Fig. 5B). On the other hand, in the AP5/CNQX injection group, there was no significant difference between the time spent in cocaine-paired compartment during the pretest and that during the posttest (pretest, 352.3 ± 17.5 s, posttest, 363.1 ± 61.3 s, n = 5, t_4 = 0.191, P = 0.86, paired t-test; Fig. 5B). Fig. 5C shows that CPP score after intra-VTA microinjection of AP5/CNQX cocktail was smaller than that after vehicle injection (vehicle, 292.4 ± 50.0 s, n = 5, AP5/CNQX cocktail 10.8 ± 56.6 s, n = 5, t_8 = 3.729, P = 0.0058). These findings indicated that cholinergic transmission via mAChR and nAChR and glutamatergic transmission via AMPA and NMDA receptors to the VTA, which might be derived from the LDT, are involved in the

stress-induced enhancement of cocaine CPP.

Role of dopaminergic transmission to the mPFC in the stress-induced enhancement of cocaine CPP

Both cholinergic and glutamatergic transmission activate VTA dopaminergic neurons (Gronier & Rasmussen 1998; Lodge & Grace 2006). Thus, we examined the possible involvement of dopaminergic transmission from the VTA to the mPFC in the stress-induced enhancement of cocaine CPP. We administered a D1 or D2 receptor antagonist directly into the mPFC by microinjection (Fig. 6A). In all groups, there were significant differences between the time spent in cocaine-paired compartment during the pretest and that during the posttest (vehicle, pretest, 338.8 ± 19.3 s, posttest, 694.7 ± 24.5 s, n = 6, t_5 = 12.68, P < 0.0001; SCH23390, pretest, 330.1 ± 10.2 s, posttest, 523.1 ± 52.3 s, n = 7, t_6 = 3.827, P = 0.0087; raclopride, pretest, 355.6 ± 16.8 s, posttest, 645.2 ± 35.8 s, n = 6, t_5 = 7.134, P = 0.0008, paired t-test; Fig. 6B). As shown in Fig. 6C, a one-way ANOVA revealed a significant difference in CPP scores among the groups ($F_{2,16}$ = 3.89, P = 0.0419). *Post hoc* Holm-Sidak tests revealed that, compared to vehicle injection, SCH23390 injection but not raclopride injection significantly decreased CPP scores (vehicle, 355.8 ± 28.1 s, n = 6; SCH23390, 193 ± 50.4 s, n = 7, P = 0.0277; raclopride, 289.6 ± 40.6 s, n = 6, P = 0.2952; Fig. 6C). These findings indicated that D1 but not D2 dopaminergic transmission to the mPFC was important for the stress-induced enhancement of cocaine CPP.

DISCUSSION

The main findings of the present study were as follows: (1) 30-min restraint stress exposed immediately before posttest enhanced cocaine-induced CPP; (2) stress-induced enhancement of cocaine CPP was attenuated by intra-LDT microinjection of a β or α2 adrenoceptor antagonist; (3) intra-LDT microinjection of a β or α2 adrenoceptor agonist before posttest also enhanced cocaine

CPP; (4) intra-VTA microinjection of antagonists for the mAChR, nAChR, or glutamate receptor attenuated the stress-induced enhancement of cocaine CPP; and (5) intra-mPFC microinjection of a D1 receptor antagonist also reduced the stress-induced enhancement of cocaine CPP. These findings suggest a mechanism wherein the LDT is activated by noradrenergic input from the locus coeruleus, leading to the activation of VTA dopamine neurons via both cholinergic and glutamatergic transmission and subsequent excitation of the mPFC to enhance the rewarding memory of cocaine.

Restraint stress has been reported to induce cFos expression (Chowdhury *et al*. 2000) and increased firing activity (Abercrombie & Jacobs 1987) in the LC, presumably increasing NA transmission. Indeed, NA levels are elevated in many brain regions during stress exposure (Cenci *et al*. 1992; Nakane *et al.* 1994; Saito *et al.* 2002; Ma & Morilak 2005; Del Arco *et al.* 2015). Although no study has directly measured NA levels in the LDT during stress, it is likely that NA is increased in the LDT during restraint stress given its location adjacent to the LC and previous evidence that it receives NA inputs and contains adrenoceptors (Jones 1991). We previously demonstrated that NA activates LDT cholinergic neurons indirectly after repeated cocaine exposure (Taoka *et al.* 2016); an *ex vivo* electrophysiological analysis revealed that, in slices obtained from rats that were treated repeatedly with saline, NA bath application had no effect on inhibitory postsynaptic current (IPSC) amplitude, whereas after repeated cocaine exposure, NA bath application decreased IPSC amplitudes via presynaptic α2 adrenoceptors. Because postsynaptic α2 adrenoceptor-mediated hyperpolarization (Williams & Reiner 1993) was not different between LDT cholinergic neurons obtained from cocaine- and saline-treated animals, changes in IPSCs likely represent the disinhibition of LDT cholinergic neurons. In the present study, we found that intra-LDT injection of RX821002 attenuated the stress-induced enhancement of cocaine CPP, indicating the involvement of α2 adrenoceptors in stress-induced CPP enhancement. Together, these findings suggest that α2 adrenoceptor-mediated disinhibition of LDT cholinergic neurons might at least partially account for the stress-induced enhancement of cocaine CPP.

It has been hypothesized that NA activates LDT GABAergic neurons via two different mechanisms: α1 adrenoceptor-mediated direct depolarization and β adrenoceptor-mediated increases in excitatory postsynaptic potentials (Kohlmeier & Reiner 1999). Thus, the blockade of α1 adrenoceptors may reduce GABAergic neuronal activity. In the present study, intra-LDT injection of terazosin had no effect on the stress-induced enhancement of cocaine CPP, suggesting that GABAergic neuronal activity may not be involved in the underlying mechanism. On the other hand, we found that intra-LDT injection of timolol inhibited the stress-induced enhancement of cocaine CPP. At present, the mechanism underlying the effect of timolol is unclear. Considering the possibility that β adrenoceptor stimulation excites LDT glutamatergic neurons that may have collateral projections to LDT cholinergic neurons and synapse with VTA DA neurons (Kohlmeier *et al.* 2012; Lammel *et al.* 2012), the site of action of timolol might be β adrenoceptors expressed on LDT glutamatergic neurons. Further studies are required to determine the precise mechanism(s) of β adrenoceptor antagonism.

We also found that blockade of glutamatergic transmission in the VTA attenuated the stress-induced enhancement of cocaine CPP. A possible source of glutamatergic input to the VTA derives from the LDT. Yet, because the VTA receives glutamatergic inputs from various brain regions (Qi *et al.* 2014; Brown & Shepard 2016), we cannot exclude the possibility that glutamatergic afferents derived from regions other than the LDT contribute to the enhancing effect of stress on cocaine CPP.

We previously reported that cholinergic transmission from the LDT to the VTA is critical for the expression of cocaine CPP (Shinohara *et al.* 2014). In the present study, cholinergic transmission was similarly necessary for the enhancing effect of stress on cocaine CPP. Thus, the LDT-VTA pathway may play important roles in both cocaine CPP expression and the effect of stress on cocaine CPP. With regard to glutamate, a previous study indicated that glutamatergic transmission was not involved in the expression of cocaine CPP (Sartor & Aston-Jones 2012). Yet, we found that

glutamatergic transmission was required for the stress-induced enhancement of cocaine CPP. Therefore, we hypothesize that cocaine cue-elicited behaviors observed during posttest of cocaine CPP in normal and stressed animals are commonly regulated by the cholinergic activation of VTA DA neurons, while both cholinergic and glutamatergic inputs to the VTA are necessary for the stress-induced enhancement of cocaine CPP.

Our data implicate D1 receptor-mediated transmission in the mPFC in the stress-induced enhancement of cocaine CPP. There are several studies showing the involvement of mPFC D1 receptors in stress-induced cocaine seeking (Capriles *et al.* 2003; McFarland *et al.* 2004) and cocaine CPP expression (Sanchez *et al.* 2003). D1 receptor stimulation depolarizes mPFC neurons (Lavin & Grace 2001; Witkowski *et al.* 2008) and potentiates NMDA-induced responses in mPFC pyramidal cells (Wang & O'Donnell 2001; Chen *et al.* 2004). However, NA and serotonin are also released in the mPFC in response to acute stress exposure (Nakane *et al.* 1994; Yoshioka *et al.* 1995) to excite mPFC neurons (Aghajanian & Marek 1997; Otis *et al.* 2013). The involvement of multiple signaling pathways in the effect of stress on cocaine CPP may account for the partial inhibitory effect of a D1 receptor antagonist on stress-induced cocaine CPP enhancement in our study. Alternatively, dopaminergic transmission in other brain areas such as the nucleus accumbens and basolateral amygdala, which are both associated with the expression of cocaine CPP (Lai *et al.* 2008; Wang *et al.* 2014), might cooperatively contribute to the stress-induced enhancement of cocaine CPP. Further studies are necessary to address this issue.

We found that intra-LDT injection of the β or α2 adrenoceptor antagonist before posttest did not affect the expression of CPP, which was produced by a higher dose of cocaine conditioning in the absence of restraint stress, suggesting the selectivity of noradrenergic transmission to the stressed condition. Considering our previous data showing that both cholinergic transmission in the VTA (Shinohara *et al.* 2014) and dopaminergic transmission in the mPFC (Shinohara *et al.* 2017) are critical for the expression of cocaine CPP, the downstream processes following the noradrenergic

transmission in the LDT, including LDT-VTA-mPFC signaling, might be involved regardless of whether the animals are exposed to restraint stress.

Because we exposed animals to restraint stress just prior to posttest, the potentiating effect of stress on cocaine CPP could not have been due to an enhancement in associative learning during the conditioning sessions (Schindler *et al.* 2010). Rather, we hypothesize that stress augmented the expression of rewarding memory of cocaine, which motivated animals to remain in the conditioned chamber for longer periods. These findings support the notion that stress acts not only as a trigger for reinstating extinguished cocaine CPP expression, but also as a modulator of cue-induced motivated behaviors through effects on the brainstem reward circuitry including the LC-LDT-VTA-mPFC pathway.

Conflict of interest

The authors declare no conflict of interest.

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Authors Contributions

KK is responsible for designing the studies. FS and HK conducted all behavioral studies. FS and KK conducted all statistical analysis. KK drafted the manuscript and MM provided critical revision of the manuscript. All authors reviewed and approved the final version submitted for publication.

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Figure Legends

Figure 1

Acute restraint stress exposed for 30 min but not 15 min before posttest potentiates cocaine-induced place preference. A, Timeline of the behavioral paradigm. B, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). *** $P < 0.001$ (Paired *t*-test). C, The columns show the time spent in the side, in which each rat spent less time on the pretest, during the pretest (white columns) and posttest (black columns). D, Summary graph of conditioned place preference (CPP) scores for cocaine-conditioned rats. * $P < 0.05$ (two-way ANOVA with *post hoc* Holm-Sidak tests). E, Summary graph of CPP scores for saline-conditioned

rats.

Figure 2

Involvement of β and α2 but not α1 adrenoceptors in the laterodorsal tegmental nucleus (LDT) in the stress-induced enhancement of cocaine CPP. A, Microinjection cannula tip placements for timolol (Tim, 2.0 μg/side, light gray), terazosin (Tera, 1.0 μg/side, dark gray), RX821002 (RX, 3.0 μg/side, black), and vehicle (Veh, white) injections into the LDT. B, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). ** $P < 0.01$, *** $P < 0.001$ (paired *t*-test). C, Summary graph of CPP scores. * $P < 0.05$ (one-way ANOVA with *post hoc* Holm-Sidak tests). D, Microinjection cannula tip placements for isoproterenol (Iso, 3.0 μg/side, gray), guanfacine (Gua, 0.01 μg/side, black), and vehicle (Veh, white) injections into the LDT. E, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). ** $P < 0.01$ (paired *t*-test). F, Summary graph of CPP scores. * $P < 0.05$ (one-way ANOVA with *post hoc* Holm-Sidak tests).

Figure 3

Blockade of β or α2 adrenoceptors in the LDT does not affect the expression of CPP induced by higher dose cocaine conditioning in the absence of stress exposure. A, Microinjection cannula tip placements for timolol (Tim, 2.0 μg/side, light gray), RX821002 (RX, 3.0 μg/side, black), and vehicle (Veh, white) injections into the LDT. B, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). ** $P < 0.01$, *** $P < 0.001$ (paired *t*-test). C, Summary graph of CPP scores.

Figure 4

Blockade of muscarinic or nicotinic acetylcholine receptors in the VTA attenuates the stress-induced

enhancement of cocaine CPP. A, Microinjection cannula tip placements for scopolamine (Sco, 50 µg/side, gray), mecamylamine (Mec, 50 µg/side, black) and vehicle (Veh, white) injections into the VTA. B, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). ** $P < 0.01$ (paired *t*-test). C, Summary graph of CPP scores. * $P < 0.05$ (one-way ANOVA with *post hoc* Holm-Sidak tests).

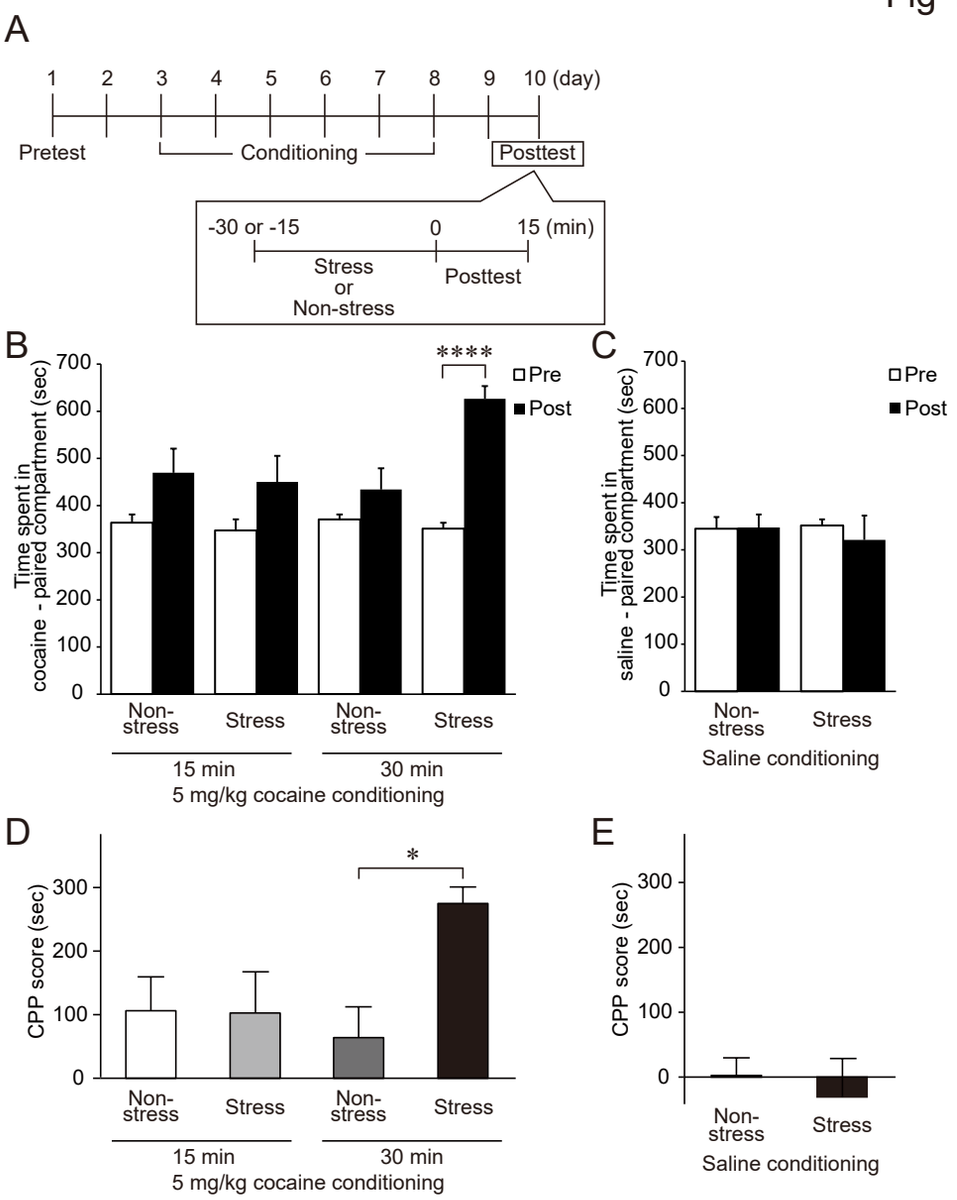
Figure 5

Blockade of glutamate receptors in the VTA attenuates the stress-induced enhancement of cocaine CPP. A, Microinjection cannula tip placements for AP5/CNQX (0.04 and 0.01 µg/side, black) and vehicle (Veh, white) injections into the VTA. B, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). ** $P < 0.01$ (paired *t*-test). C, Summary graph of CPP scores. ** $P < 0.01$ (student's *t*-test).

Figure 6

Blockade of D1, but not D2, dopaminergic receptors in the mPFC attenuates stress-induced enhancement of cocaine CPP. A, Microinjection cannula tip placements for SCH23390 (SCH,1.0 µg/side, gray), raclopride (Rac, 3.0 µg/side, black) and vehicle (Veh, white) injections into the mPFC. B, The columns show the time spent in cocaine-paired side during the pretest (white columns) and posttest (black columns). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (paired *t*-test). C, Summary graph of CPP scores. * $P < 0.05$ (one-way ANOVA with *post hoc* Holm-Sidak tests).

Fig 1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Conditioning
Posttest
-30 or -15
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Stress
or
Non-stress
Posttest
B
Time spent in cocaine - paired compartment (sec)
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Pre
Post
Non-stress
Stress
Non-stress
Stress
15 min
30 min
5 mg/kg cocaine conditioning
C
Time spent in saline - paired compartment (sec)
Pre
Post
Non-stress
Stress
Saline conditioning
D
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E
CPP score (sec)
Non-stress
Stress
Saline conditioning

Fig 2

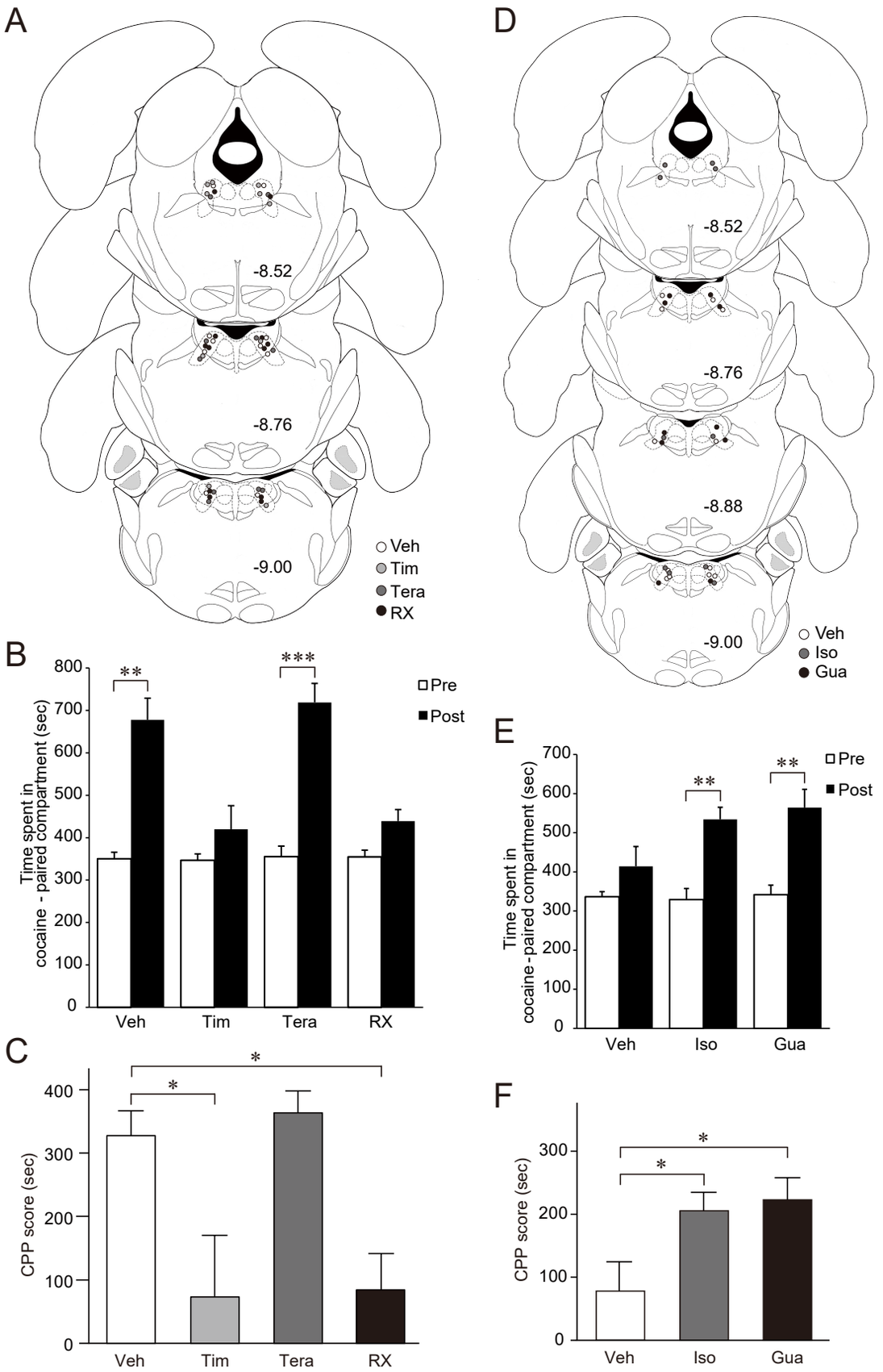
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Fig 3

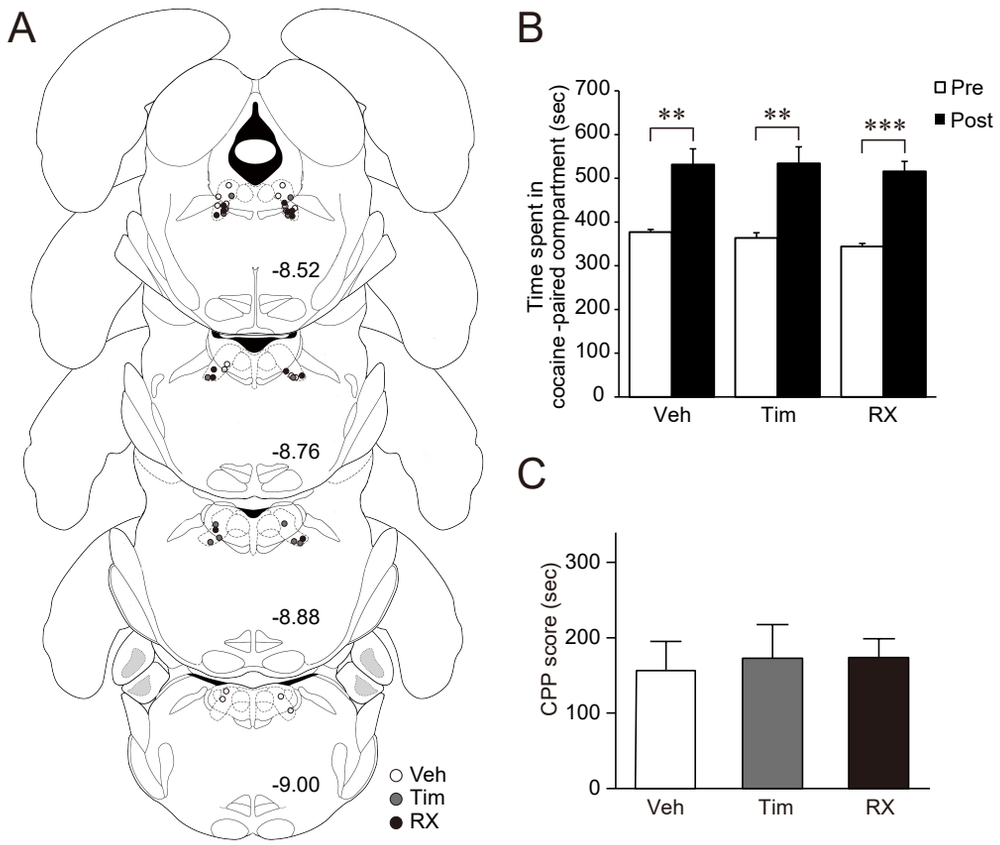
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Fig 4

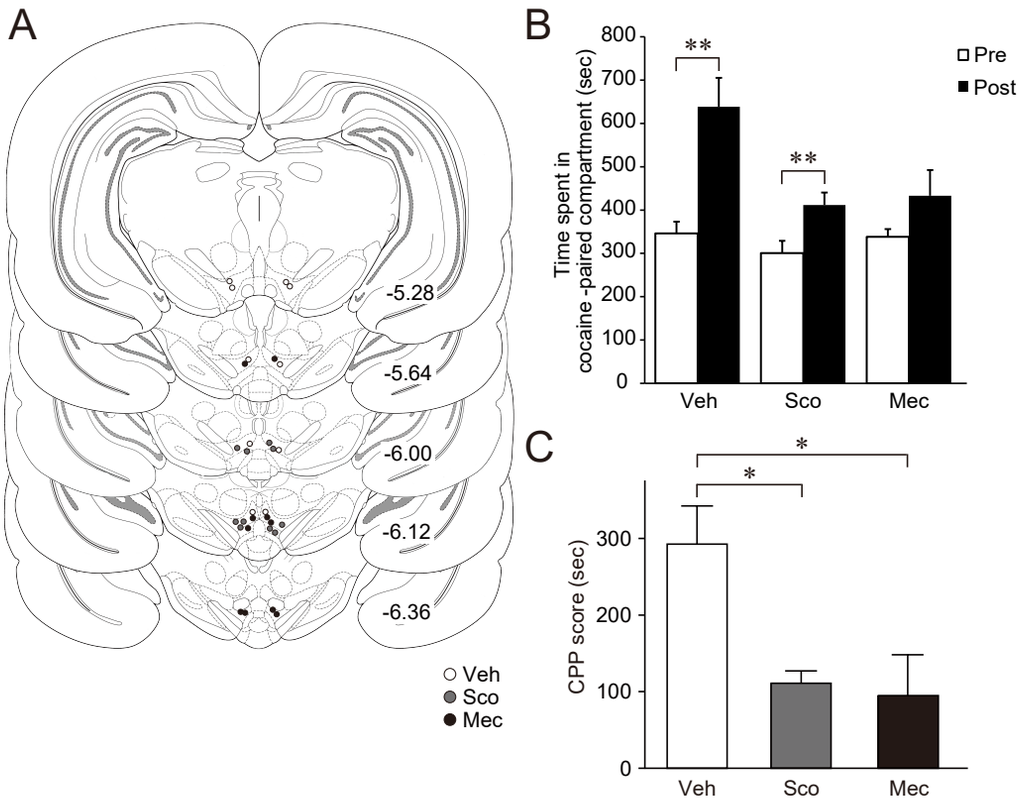
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Fig 5

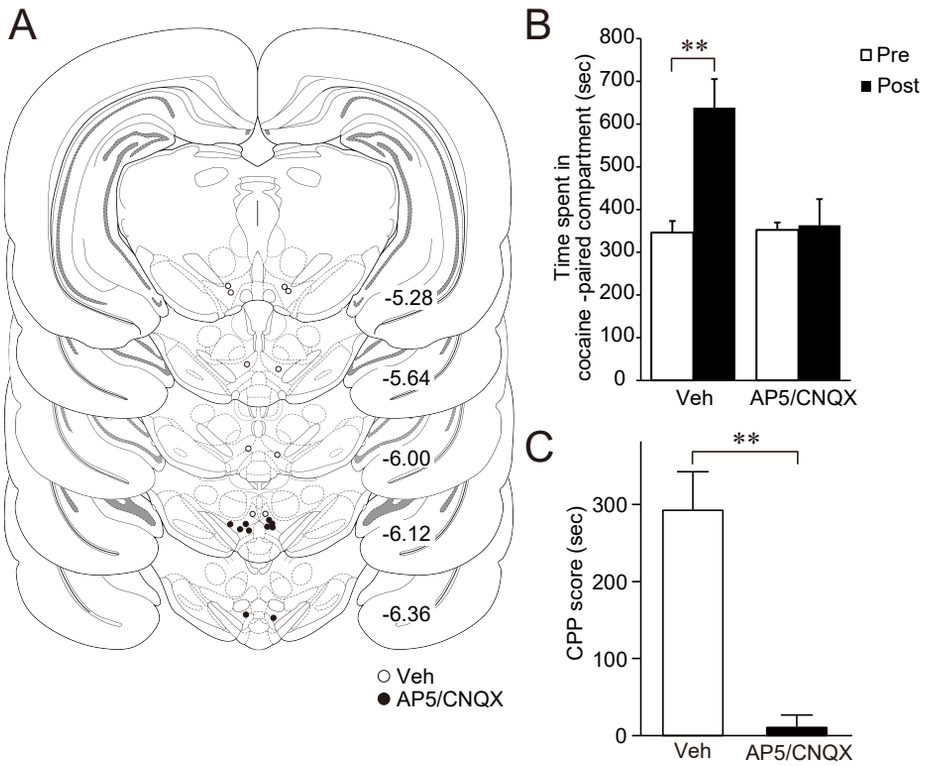
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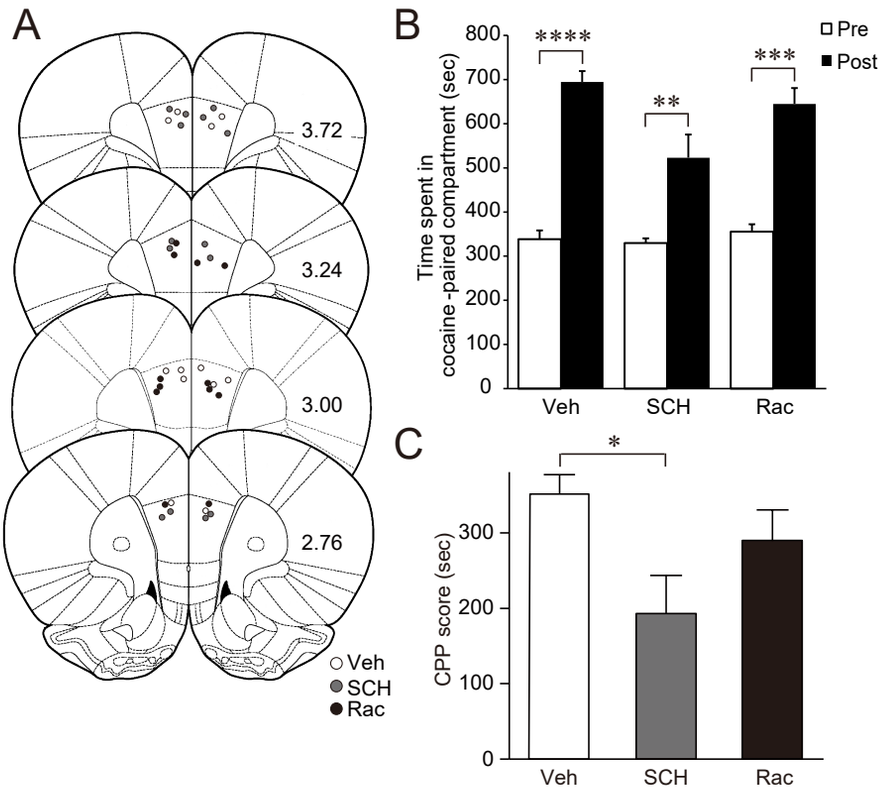
Fig 6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CPP score (sec)
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