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**Critical role of cholinergic transmission from the laterodorsal tegmental nucleus to the ventral tegmental area in cocaine-induced place preference**

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## **Abstract**

Conditioned place preference (CPP) is widely used to investigate the rewarding properties of cocaine. Various brain regions and neurotransmitters are involved in developing cocaine CPP. However, the contribution of cholinergic transmission in the ventral tegmental area (VTA) to cocaine CPP remains largely unexplored. Here, we examined the role of cholinergic input arising from the laterodorsal tegmental nucleus (LDT) to the VTA in the acquisition and expression of cocaine CPP in rats. Intra-LDT injection of carbachol, which hyperpolarizes LDT neurons, and of NMDA and AMPA receptor antagonists before cocaine conditioning blocked and attenuated cocaine CPP, respectively, indicating the necessity of LDT activity for acquiring the CPP. Additionally, intra-VTA injection of scopolamine or mecamylamine before cocaine conditioning also attenuated cocaine CPP, demonstrating the contribution of cholinergic transmission via muscarinic and nicotinic acetylcholine receptors in CPP acquisition. Furthermore, intra-VTA injection of scopolamine or mecamylamine immediately before the test attenuated cocaine CPP, indicating that cholinergic signaling is also associated with the expression of CPP. These results suggest that cholinergic transmission from the LDT to the VTA is critically involved in both acquiring and retrieving cocaine-associated memories in cocaine CPP.

*Keywords:* laterodorsal tegmental nucleus; ventral tegmental area; conditioned place preference; addiction; acetylcholine; mesocorticolimbic system

## 1. Introduction

Conditioned place preference (CPP) is widely used to study the rewarding properties of drugs of abuse, including cocaine. Accumulating evidence demonstrates that various brain regions are involved in the acquisition and expression of CPP induced by systemic cocaine injection (for a review, see Tzschentke, 2007). Among these regions, the ventral tegmental area (VTA), nucleus accumbens (NAc), and medial prefrontal cortex (mPFC), which constitute the mesocorticolimbic system, are considered critical. Elevated dopamine (DA) levels in the NAc are required for developing cocaine CPP. As cocaine inhibits DA transporters, the DA elevation after systemic cocaine administration may be attributable to accumulated DA, which is generally released spontaneously or in an action potential-dependent manner. Nevertheless, intra-NAc cocaine injection fails to elicit CPP (Hemby et al., 1992). These findings raise the possibility that tonic firing of DA neurons is insufficient to increase DA levels necessary for developing cocaine CPP, and some other mechanisms might explain how systemic cocaine injections excite DA neurons robustly enough to release a large amount of DA.

Glutamatergic transmission, particularly through NMDA receptors (NMDARs), plays an important role in activating DA neurons (Cherqui et al., 1993) and is implicated in cocaine CPP; both intra-VTA injection of an NMDAR antagonist (Harris and Aston-Jones, 2003) and selective knockout of NMDARs from VTA DA neurons (Zweifel et al., 2008) suppress cocaine CPP. On the other hand, acetylcholine receptor (AChR) agonists also enhance DA neuronal activity (Gronier and Rasmussen, 1998; Zhang et al., 2005), and conditioning with intra-VTA injection of carbachol (CCh), an AChR agonist, induces place preference (Ikemoto and Wise, 2002; Yeomans et al., 1985), suggesting the crucial contribution of cholinergic transmission in regulating DA neuronal activity and establishing place preference.

The laterodorsal tegmental nucleus (LDT) has recently emerged as a brain region relevant to reward-related behavior (Lammel et al., 2012) and drug addiction (Kurosawa et al., 2013; Schmidt et

al., 2009; Szelecki et al., 2013). The LDT consists of cholinergic, glutamatergic, and GABAergic neurons (Wang and Morales, 2009). Projections from the LDT to the VTA are necessary for activating and generating burst firings of VTA DA neurons (Lodge and Grace, 2006). Additionally, electrical stimulation of the LDT elicits a bulk release of DA in the NAc, which is blocked by intra-VTA injection of AChR antagonists (Forster and Blaha, 2000; Lester et al., 2010). This indicates the significance of cholinergic transmission from the LDT to the VTA in regulating NAc DA levels. However, it remains unclear whether cholinergic signaling to the VTA is associated with establishing cocaine CPP. To address this issue, we performed intracranial microinjections of drugs at different time points during CPP in order to examine the involvement of cholinergic transmission in the acquisition and expression of cocaine CPP.

## **2. Materials and methods**

### *2.1. Animals*

Male Sprague-Dawley rats (weighing 170–250 g at the beginning of behavioral tests) were maintained in a temperature-controlled ( $22 \pm 1^\circ\text{C}$ ) 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 University. All efforts were made to minimize the number and suffering of animals used in the experiments.

### *2.2. Drugs*

Cocaine hydrochloride (Takeda Pharmaceutical, Osaka, Japan) was dissolved in saline. Carbamoylcholine chloride (carbachol [CCh]; an AChR agonist), (-)-scopolamine hydrobromide trihydrate (a muscarinic AChR [mAChR] antagonist), mecamylamine hydrochloride (a nicotinic AChR [nAChR] antagonist), dl-AP5 (an NMDAR antagonist), and CNQX disodium salt hydrate (an

AMPA receptor antagonist) were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.1 M PBS (pH = 7.4). The doses of these drugs were determined on the basis of previous studies: CCh (3.0 µg/0.2 µL/side; Lodge and Grace, 2006), dl-AP5/CNQX cocktail (0.2 and 0.05 µg/0.2 µL/side; Mahler et al., 2012), scopolamine (5 and 50 µg/0.5 µL/side; Chapman et al., 1997) and mecamylamine (5 and 50 µg/0.5 µL/side; Chen et al., 2006). The doses of all drugs (including cocaine) were expressed in terms of their salt weight.

### *2.3. 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) or VTA (5.8 mm caudal, 1.0 mm lateral, 8.5 mm ventral to bregma) (Paxinos and 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 or VTA. Bilateral infusions were performed at a volume of 0.2 (LDT) or 0.5 µL (VTA) in each side at a rate of 0.2 or 0.5 µL/min, respectively, and the injection cannulae were left in place for an additional 1 min after microinjection to prevent backflow.

### *2.4. Conditioned place preference*

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. The CPP chambers were set in sound-attenuating boxes equipped with a ventilating fan (Muromachi Kikai, Tokyo, Japan). On days 1 (habituation) and 2 (pretest), 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), which were positioned on the top cover of each compartment (Kaneko et al., 2007; Kitanaka et al., 2006). Rats that spent >80% (>720 s) of the total time (900 s) in one side on day 2 or showed a difference of >200 s in the time spent in one side between days 1 and 2 were eliminated from subsequent procedures. We used a bias-like protocol (Tzschentke, 1998) and designated the compartment in which each rat spent less time on day 2 (pretest) as their drug-paired compartment. On days 4–9 (conditioning), rats were given alternating injections of cocaine (20 mg/kg, i.p.) or saline (1 mL/kg, i.p.) and confined to one compartment for 30 min on 6 consecutive days. On cocaine-conditioning days, rats were given a bilateral intra-LDT or a bilateral intra-VTA microinjection 5 min before their cocaine injection. On day 11 (posttest), rats were allowed to explore the two compartments freely for 900 s, and the time spent in each compartment during the exploratory period and locomotor activity were measured. When the effects of drugs on CPP expression were examined, rats were given a bilateral intra-VTA microinjection 15 min before the start of the posttest. The CPP scores were calculated by subtracting the time spent in the cocaine-paired compartment during the pretest from the time spent in this compartment during the posttest.

## 2.5. *Histology*

After the CPP tests, to confirm the placements of the drug injection, a mixture of thionin (1.0%) and cresyl violet (1.0%) was microinjected into the LDT or VTA in a volume of 0.2 (LDT) or 0.5 (VTA)  $\mu$ L/side at a rate of 0.2 (LDT) or 0.5 (VTA)  $\mu$ L/min immediately before decapitation. The brains were then rapidly removed and frozen in powdered dry ice. Coronal sections (50  $\mu$ m) of the LDT or

VTA were prepared using a cryostat, thaw-mounted onto slides, stained with neutral red, and examined under a microscope ( $\times 40$ ). Data obtained from rats with extensive tissue damage and with misplacement of both (for the VTA) or either (for the LDT and VTA) of the bilateral injection cannulae were excluded from statistical analyses, with the exception of the animal data presented in Figures 1 and 2.

## 2.6. Statistical Analyses

Data are expressed as means  $\pm$  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 when comparing only two groups.

## 3. Results

### 3.1. Inhibition of LDT neuronal activity suppresses acquisition of cocaine CPP

We first addressed whether the LDT is involved in acquiring cocaine CPP. For this, we inhibited LDT neuronal activity by microinjecting CCh, which is known to hyperpolarize LDT neurons primarily by stimulating M2 and M4 mAChRs (Kohlmeier et al., 2012; Leonard and Llinás, 1994), into the LDT before each cocaine conditioning session (Fig. 1A). Figure 1B depicts the drug or vehicle injection sites. We analyzed data obtained from three groups, including control (bilateral vehicle injection into the LDT), CCh (bilateral CCh injection into the LDT), and CCh miss (bilateral CCh injection near the LDT) groups. As shown in Fig. 1C, one-way ANOVA indicated a significant difference in CPP scores among these groups ( $F_{2,21} = 5.81$ ,  $P = 0.0098$ ). A *post hoc* Holm-Sidak test revealed that the CPP scores after the intra-LDT CCh microinjection were significantly smaller than those after vehicle injection (vehicle,  $156.4 \pm 20.8$  s,  $n = 7$ , CCh,  $32.2 \pm 27.7$  s,  $n = 7$ ,  $P = 0.0186$ ; Fig. 1C). Further, the time spent in the cocaine-paired chamber after intra-LTD CCh injection was



not statistically different between pretest and posttest sessions (pretest,  $335.1 \pm 28.2$  s, posttest,  $367.3 \pm 37.2$  s,  $n = 7$ ,  $t_6 = 1.165$ ,  $P = 0.2881$ , paired  $t$ -test), demonstrating that LDT inhibition by CCh blocked cocaine CPP acquisition. In contrast, there was no significant difference in CPP scores between the CCh miss group and vehicle-injected group (CCh miss,  $156.5 \pm 31.3$  s,  $n = 10$ ,  $P = 0.9986$ , one-way ANOVA with a *post hoc* Holm-Sidak test; Fig. 1C), indicating that the blocking effect of CCh was exerted by LDT inhibition.

We further examined whether intra-LDT CCh injection by itself induces place aversion or preference. In this experiment, cocaine was substituted by saline during the conditioning; i.e., saline was injected in every conditioning session (6 days), and CCh was microinjected into the LDT 5 min prior to confinement in one compartment every other day (3 days). Under this condition, the time spent in the intra-LDT CCh injection-paired compartment during the posttest was not significantly different from that during the pretest (pretest,  $365.9 \pm 13.1$  s, posttest,  $415.4 \pm 39.9$  s,  $n = 6$ ,  $t_5 = 1.702$ ,  $P = 0.1495$ , paired  $t$ -test; Fig. 1D), demonstrating that the intra-LDT CCh microinjection does not induce place aversion or preference. Taken together, these findings indicate that inhibition of LDT neuronal activity during conditioning disrupts the acquisition of cocaine CPP.

### 3.2. Blockade of glutamate receptors in the LDT attenuates acquisition of cocaine CPP

The above results showed that LDT neuronal activity is necessary for cocaine CPP acquisition. Next, we addressed whether glutamatergic input contributes to the activation of LDT neurons during cocaine conditioning by microinjecting a cocktail of an NMDAR antagonist AP5 and an AMPA receptor antagonist CNQX into the LDT before each cocaine conditioning session (Fig. 2A). Figure 2B shows the drug or vehicle injection sites. We analyzed data obtained from three groups: the control (bilateral vehicle injection into the LDT), AP5/CNQX (bilateral AP5/CNQX cocktail injection into the LDT), and AP5/CNQX miss (bilateral AP5/CNQX injection near the LDT) groups. As shown in Fig. 2C, one-way ANOVA indicated a significant difference in CPP scores among these

groups ( $F_{2,16} = 8.68$ ,  $P = 0.0028$ ). *Post hoc* comparison using a Holm-Sidak test revealed that, compared with vehicle injection, intra-LDT microinjection of the AP5/CNQX cocktail significantly attenuated cocaine CPP (vehicle,  $156.4 \pm 20.8$  s,  $n = 7$ , AP5/CNQX cocktail,  $68.5 \pm 20.2$  s,  $n = 6$ ,  $P = 0.0078$ ; Fig. 2C). The time spent in the cocaine-paired chamber after intra-LTD cocktail injection during the posttest was longer than that during the pretest (pretest,  $346.9 \pm 14.9$  s, posttest,  $415.4 \pm 13.3$  s,  $n = 6$ ,  $t_5 = 3.395$ ,  $P = 0.0194$ , paired *t*-test), demonstrating that the antagonist cocktail reduced, but did not prevent, cocaine CPP acquisition. The effect of the AP5/CNQX cocktail did not result from the diffusion of the cocktail outside the LDT, because the CPP scores of the AP5/CNQX miss and vehicle-injected groups were not significantly different (AP5/CNQX miss,  $172.7 \pm 12.8$  s,  $n = 6$ ,  $P = 0.5427$ , one-way ANOVA with a *post hoc* Holm-Sidak test; Fig. 2C).

We tested whether conditioned place aversion or preference is induced by intra-LDT injection of the AP5/CNQX cocktail *per se* by substituting saline conditioning for cocaine conditioning. Under this condition, there was no significant difference between the time spent in the cocktail injection-paired compartment on the posttest and the pretest sessions (pretest,  $318.0 \pm 10.9$  s, posttest,  $378.6 \pm 46.9$  s,  $n = 5$ ,  $t_4 = 1.207$ ,  $P = 0.2941$ , paired *t*-test; Fig. 2D), indicating that the intra-LDT microinjection of the cocktail induces neither place aversion nor preference. These results demonstrate that glutamatergic transmission to the LDT may be, at least partly, responsible for activating LDT neurons, which is a prerequisite for acquiring CPP.

### 3.3. Blockade of muscarinic and nicotinic AChRs in the VTA attenuates acquisition of cocaine CPP

Although previous studies have demonstrated that glutamatergic transmission to the VTA is critical for acquiring cocaine CPP (Harris and Aston-Jones, 2003; Zweifel et al., 2008), it remains unknown whether cholinergic input also contributes to cocaine CPP acquisition. The results presented above suggest the involvement of cholinergic input to the VTA in acquiring CPP, since the LDT is the major source of cholinergic input to the VTA (Oakman et al., 1995). To test this, we inhibited VTA

mAChRs or nAChRs by an intra-VTA microinjection of scopolamine or mecamylamine, respectively, before each cocaine conditioning session (Fig. 3A). Figure 3B shows the drug or vehicle injection sites. As shown in Fig. 3C, one-way ANOVA indicated that intra-VTA injection of scopolamine reduced cocaine CPP in a dose-dependent manner ( $F_{2,17} = 10.80$ ,  $P = 0.0009$ ). *Post hoc* Holm-Sidak test revealed that compared to vehicle injection, intra-VTA microinjection of a high, but not a low, dose of scopolamine significantly decreased the CPP score (vehicle,  $184.6 \pm 22.6$  s,  $n = 7$ , scopolamine 5  $\mu\text{g}/\text{side}$ ,  $144.3 \pm 32.4$  s,  $n = 6$ ,  $P = 0.4799$ , scopolamine 50  $\mu\text{g}/\text{side}$ ,  $28.8 \pm 21.1$  s,  $n = 7$ ,  $P = 0.0006$ , Fig. 3C). In addition, one-way ANOVA demonstrated that intra-VTA mecamylamine injection also dose-dependently reduced cocaine CPP ( $F_{2,18} = 3.579$ ,  $P = 0.00491$ ; Fig. 3D). *Post hoc* Holm-Sidak test revealed that compared to vehicle injection, intra-VTA microinjection of a high, but not a low, dose of mecamylamine significantly decreased CPP score (vehicle,  $184.6 \pm 22.6$  s,  $n = 7$ , mecamylamine 5  $\mu\text{g}/\text{side}$ ,  $129.0 \pm 42.6$  s,  $n = 6$ ,  $P = 0.2916$ , mecamylamine 50  $\mu\text{g}/\text{side}$ ,  $85.8 \pm 20.2$  s,  $n = 8$ ,  $P = 0.0310$ , Fig. 3D).

We further examined whether intra-VTA scopolamine or mecamylamine injection by itself affects place preference. This experiment was performed similarly to the experiments shown in Figs. 1D and 2D. The time spent in the scopolamine (50  $\mu\text{g}/\text{side}$ ) or mecamylamine (50  $\mu\text{g}/\text{side}$ ) injection-paired compartment was not significantly different between the posttest and pretest sessions (scopolamine, pretest,  $341.5 \pm 22.6$  s, vs. posttest,  $297.3 \pm 27.0$  s,  $n = 6$ ,  $t_5 = 1.364$ ,  $P = 0.2308$ , paired *t*-test; mecamylamine, pretest,  $339.4 \pm 10.1$  s, vs. posttest,  $358.3 \pm 39.5$  s,  $n = 6$ ,  $t_5 = 0.492$ ,  $P = 0.6436$ ; Fig. 3E), demonstrating that intra-VTA microinjection of neither scopolamine nor mecamylamine induces place aversion or preference. These findings indicate that cholinergic transmission to the VTA via muscarinic and nicotinic AChRs during conditioning is crucial for acquiring cocaine CPP.

#### 3.4. Blockade of muscarinic and nicotinic AChRs in the VTA attenuates expression of cocaine CPP

Finally, we tested whether cholinergic transmission is also involved in the expression of cocaine CPP. For this, we microinjected scopolamine or mecamylamine into the VTA immediately before the posttest session (Fig. 4A). Figure 4B summarizes the drug or vehicle injection sites. Two-way ANOVA revealed that both scopolamine and mecamylamine significantly attenuated cocaine CPP (Fig. 4C, scopolamine effect,  $F_{1,26} = 12.13$ ,  $P = 0.0018$ ; mecamylamine effect,  $F_{1,26} = 9.774$ ,  $P = 0.0043$ ). Further, compared to vehicle injection, injection of only scopolamine and of only mecamylamine reduced CPP scores (CPP score; vehicle,  $196.2 \pm 24.4$  s,  $n = 8$ , scopolamine,  $105.6 \pm 25.5$  s,  $n = 8$ , mecamylamine,  $94.1 \pm 28.9$  s,  $n = 7$ ), although *post hoc* Holm-Sidak tests showed these were not significantly different. On the other hand, intra-VTA microinjection of the antagonist mixture significantly reduced the cocaine CPP ( $-16.6 \pm 48.9$  s,  $n = 7$ ,  $P = 0.0005$ , two-way ANOVA with a *post hoc* Holm-Sidak test) compared to vehicle injection. Two-way ANOVA also revealed that the interaction between scopolamine and mecamylamine was not statistically significant ( $F_{1,26} = 0.0979$ ,  $P = 0.7568$ ), suggesting that scopolamine and mecamylamine may independently act on mAChRs and nAChRs, respectively. Microinjections of the antagonists before the posttest sessions did not affect the counts measured by infrared sensors during the posttest sessions (vehicle,  $3459 \pm 123$ , scopolamine,  $3830 \pm 196$ , mecamylamine,  $4004 \pm 456$ , scopolamine + mecamylamine,  $3783 \pm 174$ , two-way ANOVA, scopolamine effect,  $F_{1,26} = 0.9301$ ,  $P = 0.3437$ ; mecamylamine effect,  $F_{1,26} = 0.0839$ ,  $P = 0.7743$ ; interaction,  $F_{1,26} = 1.305$ ,  $P = 0.2637$ ), confirming that the observed effects of these antagonists were not due to their non-specific locomotor effects. Thus, our findings indicate that cholinergic input, probably derived from the LDT, to the VTA contributes to the expression of cocaine CPP.

#### 4. Discussion

The main findings of the present study were that the acquisition of cocaine CPP requires LDT neuronal activity, glutamatergic transmission to the LDT, and cholinergic transmission to the VTA

via muscarinic and nicotinic AChRs. In addition, cholinergic transmission to the VTA was found to contribute to the expression of cocaine CPP. To the best of our knowledge, these results provide the first evidence of the critical involvement of the LDT and its cholinergic transmission to the VTA in developing cocaine CPP.

We found that intra-LDT CCh injection significantly reduced cocaine CPP. CCh can hyperpolarize the majority of LDT neurons, including cholinergic neurons, by acting at M2 and M4 mAChRs that open G-protein-coupled inwardly rectifying potassium channels (Kohlmeier et al., 2012; Leonard and Llinás, 1994); however, it depolarizes the remaining minorities by stimulating mAChRs other than M2/M4 subtypes (Kohlmeier et al., 2012). CCh also acts on nAChRs, causing depolarization of LDT neurons (Ishibashi et al., 2009). However, this depolarizing effect might be obscured by mAChR-mediated hyperpolarization and may persist only for a short time because of rapid desensitization of nAChRs (Albuquerque et al., 2009). Thus, the effect of intra-LDT CCh injection on cocaine CPP is possibly mediated by its inhibitory effect on LDT neuronal activity. This is further supported by a previous study showing that intra-LDT CCh injection reproduced the inhibitory effect of intra-LDT injection of a baclofen/muscimol mixture on VTA DA neuronal activity (Lodge and Grace, 2006).

Previous reports have shown that systemic passive- and self-administrations of cocaine induce Fos expression in LDT cholinergic neurons (Geisler et al., 2008; Zahm et al., 2010), indicating the activation of these neurons following cocaine administrations. We think that one possible mechanism for this might be excitation by glutamatergic afferents to the LDT, because we observed attenuation of cocaine CPP acquisition due to intra-LDT AP5/CNQX injection. The LDT receives glutamatergic inputs from the mPFC, lateral hypothalamus (LH), lateral habenula, pedunclopontine tegmental nucleus (PPT), and LDT itself (Kohlmeier et al., 2012; Satoh and Fibiger, 1986; Semba and Fibiger, 1992). Some of these regions, such as the mPFC and LH, are

associated with cocaine CPP (Harris et al., 2005; Tzschentke and Schmidt, 1998). In addition, systemic cocaine injections have been reported to induce expression of Fos or Fos-related antigens in these regions (Harris et al., 2005; Pich et al., 1997). Therefore, glutamatergic inputs from these regions might activate LDT neurons during cocaine conditioning.

Systemic cocaine injection suppresses DA neuronal activity under anesthesia (Einhorn et al., 1988; Hinerth et al., 2000), but increases the firing rate and bursting of most DA neurons in awake rats (Koulchitsky et al., 2012). Burst firings of VTA DA neurons are critical for cocaine CPP acquisition; selective knockout of NMDARs in VTA DA neurons, which cannot express burst firings, prevents the acquisition of cocaine CPP (Zweifel et al., 2008). Moreover, conditioning with phasic photo-stimulation of channelrhodopsin-2 expressing DA neurons induces place preference (Tsai et al., 2009), indicating that burst firing is sufficient for acquisition of behavioral conditioning. The LDT is required for inducing burst firings in VTA DA neurons (Lodge and Grace, 2006), where NMDAR-mediated transmission is considered critical. Along with this, intra-VTA injection of an NMDAR antagonist inhibits cocaine CPP acquisition (Harris and Aston-Jones, 2003). On the other hand, cholinergic input has been suggested to contribute to burst firings in DA neurons (Gronier and Rasmussen, 1998; Kitai et al., 1999; Zhang et al., 2005). In line with this, we have provided evidence that cholinergic transmission to the VTA is involved in acquiring cocaine CPP. Consistent with these findings, previous studies have shown that conditioning with intra-VTA CCh injection elicits place preference (Ikemoto and Wise, 2002; Yeomans et al., 1985).

The primary sources of cholinergic input to the VTA are the LDT and PPT, although the latter mainly innervates the substantia nigra pars compacta (Oakman et al., 1995). PPT stimulation with NMDA induces burst firings in VTA DA neurons; however, this effect requires LDT activity (Lodge and Grace, 2006). Additionally, DA release evoked by intra-VTA injection of neostigmine, a cholinesterase inhibitor, is significantly reduced by lesions of the LDT, but not of the PPT,

suggesting a larger contribution of cholinergic afferents from the LDT, than of those from the PPT, to DA neuronal activity (Blaha et al., 1996). Thus, considering the suppressive effects of LDT inhibition by CCh and the AP5/CNQX cocktail, the attenuating effects of intra-VTA injection of the AChR antagonists on cocaine CPP acquisition may be primarily attributable to the blockade of cholinergic input arising from the LDT. These results suggest that the acquisition of cocaine CPP requires enhanced DA neuronal activity through activation of LDT cholinergic neurons that is, at least partially, attributable to glutamate afferents. This idea is further supported by the finding that intra-NAc cocaine injection, which activates neither DA nor LDT cholinergic neurons, fails to induce CPP (Hemby et al., 1992).

The expression of cocaine CPP may be dependent on cocaine experience memory, which is retrieved when the animals enter or are placed in the cocaine-conditioned chamber in the posttest session. Our results showed that cholinergic transmission, arising probably from the LDT, to the VTA might be involved in this process. Given that the cholinergic transmission is critical for enhancing DA neuronal activity, activation of VTA DA neurons via cholinergic input as well as glutamatergic input and subsequent DA signaling might be important for cocaine CPP expression. In line with this hypothesis, D1- and D2-like DA receptor-mediated signaling is suggested to be associated with cocaine CPP expression (Liao et al., 1998; but see also Cervo and Samanin, 1995). In addition, intra-VTA injection of scopolamine or mecamylamine suppresses cocaine-primed reinstatement of cocaine seeking after extinction of cocaine self-administration (Schmidt et al., 2009), implying the critical involvement of cholinergic transmission to the VTA in cocaine-related memory retrieval. Moreover, a recent study has demonstrated that cholinergic signaling in the VTA regulates phasic DA release and cue-induced cocaine-seeking during early cocaine withdrawal (Solecki et al., 2013). Thus, these findings strongly suggest that, similar to the acquisition, the expression of cocaine CPP involves cholinergic signaling from the LDT to the VTA.

Cocaine exposure induces numerous forms of synaptic plasticity, including the long-term potentiation of glutamatergic transmission in VTA DA neurons (Chen et al., 2008; Ungless et al., 2001). Additionally, we have recently found that repeated cocaine exposure increases glutamatergic input onto LDT cholinergic neurons via a presynaptic mechanism (Kurosawa et al., 2013). Although further studies are necessary to examine whether such synaptic plasticity in LDT cholinergic neurons is induced under the cocaine-conditioning paradigm used in the present study, it is likely that plastic changes in LDT cholinergic neurons result in increased cholinergic transmission to the VTA. Given that the blockade of AChRs in the VTA significantly reduced the expression of cocaine CPP (Fig. 4), this plastic increase in cholinergic transmission to the VTA might contribute to elevating DA neuronal activity, which might have been triggered in response to the cocaine-experienced context during the posttest session and be a prerequisite process for retrieving cocaine-associated memories.

In conclusion, we have provided evidence that cholinergic transmission from the LDT to the VTA via muscarinic and nicotinic AChRs is critical for both the acquisition and expression of cocaine CPP. These findings suggest that cholinergic signaling might be required for the rewarding effect of cocaine and for memory retrieval that drives cocaine experience-dependent motivated behaviors.

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## Conflict of interest

The authors declare no conflict of interest.

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## Figure legends

### Figure 1

Inhibition of LDT neuronal activity suppresses CPP acquisition. A, Timeline of drug treatments and the behavioral paradigm. Groups of rats received saline (Sal, 1.0 mL/kg, i.p.) and cocaine (Coc, 20 mg/kg, i.p.) conditioning once daily for 6 days. On Coc-conditioning days, rats received a bilateral intra-LDT or extra-LDT (CCh-miss) microinjection of carbachol (CCh, 3.0  $\mu$ g/0.2  $\mu$ L/side) or a bilateral intra-LDT microinjection of vehicle (Veh, 0.2  $\mu$ L/side) 5 min before each Coc injection. On Sal-conditioning days, rats received an injection of Sal alone. B, The placements of the tips of the microinjection cannulae for CCh injection into the LDT (square) and outside the LDT (diamond) and for Veh injected into the LDT (circle). The numbers beside each panel represent the approximate AP distance (mm) from bregma. C, Summary graph of CPP scores. \* $P < 0.05$  compared to Veh (one-way ANOVA with Holm-Sidak *post hoc* test). D, The columns show the time spent in the CCh-paired side during the pretest (white column) and posttest (black column) sessions. During conditioning, rats received Sal injections every day with or without intra-LDT microinjection of CCh. Data in C and D are expressed as means  $\pm$  S.E.M.

### Figure 2

Blockade of glutamate receptors in the LDT attenuates CPP acquisition. A, Timeline of drug treatments and the behavioral paradigm. On cocaine (Coc)-conditioning days, rats received a bilateral intra-LDT or extra-LDT (AP5/CNQX-miss) microinjection of the AP5/CNQX cocktail (AP5/CNQX, 0.2 and 0.05  $\mu$ g/0.2  $\mu$ L/side) or a bilateral intra-LDT microinjection of the vehicle (Veh, 0.2  $\mu$ L/side) 5 min before injection of Coc. On saline (Sal)-conditioning days, rats received an

injection of Sal alone. B, The placements of the tips of the microinjection cannulae for the AP5/CNQX cocktail injection into the LDT (square) and outside the LDT (diamond) and for Veh injected into the LDT (circle). The numbers beside each panel represent the approximate AP distance (mm) from bregma. C, Summary graph of CPP scores.  $^{***}P < 0.01$  compared to Veh (one-way ANOVA with Holm-Sidak *post hoc* test). D, The columns show the time spent in the AP5/CNQX cocktail-paired side during the pretest (white column) and posttest (black column) sessions. During conditioning, rats received Sal injections every day with or without intra-LDT microinjection of the AP5/CNQX cocktail. Data in C and D are expressed as means  $\pm$  S.E.M.

### Figure 3

Blockade of muscarinic or nicotinic acetylcholine receptors in the VTA attenuates CPP acquisition. A, Timeline of drug treatments and the behavioral paradigm. On cocaine (Coc)-conditioning days, rats received a bilateral intra-VTA microinjection of scopolamine (Sco, 5 or 50  $\mu\text{g}/0.5 \mu\text{L}/\text{side}$ ), mecamylamine (Mec, 5 or 50  $\mu\text{g}/0.5 \mu\text{L}/\text{side}$ ), or vehicle (Veh, 0.5  $\mu\text{L}/\text{side}$ ) 5 min before Coc injection. On saline (Sal)-conditioning days, rats received an intraperitoneal injection of Sal alone. B, The placements of the tips of the microinjection cannulae for Sco (5  $\mu\text{g}/\text{side}$ , gray triangle; 50  $\mu\text{g}/\text{side}$ , black triangle), Mec (5  $\mu\text{g}/\text{side}$ , gray square; 50  $\mu\text{g}/\text{side}$ , black square) and Veh (black circle) injection into the VTA. The numbers beside each panel represent the approximate AP distance (mm) from bregma. C, D, Summary graphs of CPP scores. \*,  $^{***}P < 0.05, 0.001$  compared with Veh (one-way ANOVA with Holm-Sidak *post hoc* test). E, The columns show the time spent in Sco- or Mec-paired side during the pretest (white columns) and posttest (black columns) sessions. During conditioning, rats received Sal injections every day with or without intra-VTA microinjection of Sco (50  $\mu\text{g}/\text{side}$ ) or Mec (50  $\mu\text{g}/\text{side}$ ). Data in C–E are expressed as means  $\pm$  S.E.M.

### Figure 4

Blockade of muscarinic or nicotinic acetylcholine receptors in the VTA attenuates CPP expression. A, Timeline of drug treatments and the behavioral paradigm. On the posttest day, rats received a bilateral intra-VTA microinjection of scopolamine (Sco, 50  $\mu\text{g}/0.5 \mu\text{L}/\text{side}$ ), mecamylamine (Mec, 50  $\mu\text{g}/0.5 \mu\text{L}/\text{side}$ ), Sco + Mec (Sco/Mec, each 50  $\mu\text{g}/0.5 \mu\text{L}/\text{side}$ ), or vehicle (Veh, 0.5  $\mu\text{L}/\text{side}$ ) 15 min before the start of the posttest session. B, The placements of the tips of the microinjection cannulae for Sco (triangle), Mec (square), Sco/Mec (pentagon), and Veh (circle) injection into the VTA. The numbers beside each panel represent the approximate AP distance (mm) from bregma. C, Summary graph of CPP scores.  $***P < 0.001$  compared to Veh (two-way ANOVA with Holm-Sidak *post hoc* test). D, The columns show the locomotor activity counts during the posttest session. There was no significant difference among the groups. Data in C and D are expressed as means  $\pm$  S.E.M.









