NMRA Receptor Hypofunction in the Prelimbic Cortex Increases Sensitivity to the Rewarding Properties of Opiates via Dopaminergic and Amygdalar Substrates

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The medial prefrontal cortex (mPFC) plays a significant role in associative learning and memory formation during the opiate addiction process. Various lines of evidence demonstrate that glutamatergic (GLUT) transmission through the N-methyl D-aspartate (NMDA) receptor can modulate neuronal network activity within the mPFC and influence dopaminergic signaling within the mesocorticolimbic pathway. However, little is known about how modulation of NMDA receptor signaling within the mPFC may regulate associative opiate reward learning and memory formation. Using a conditioned place preference (CPP) procedure, we examined the effects of selective NMDA receptor blockade directly within the prelimbic cortex (PLC) during the acquisition of associative opiate reward learning. NMDA receptor blockage specifically within the PLC caused a strong potentiation in the rewarding effects of either systemic or intra-ventral tegmental area (intra-VTA) morphine administration. This reward potentiation was dose dependently blocked by coadministration of dopamine D1 or D2 receptor antagonists and by blockade of presynaptic GLUT release. In addition, pharmacological inactivation of the basolateral amygdala (BLA) also prevented intra-PLC NMDA receptor blockade-induced potentiation of opiate reward signals, demonstrating a functional interaction between inputs from the VTA and BLA within the PLC, during the encoding and modulation of associative opiate reward information.

Keywords: addiction, dopamine, glutamate, memory, morphine, NMDA receptor, prefrontal cortex

Introduction

Opiate-class drugs represent potently addictive substances, producing rapid dependence and highly aversive states of withdrawal and craving (Nestler 2001; Vanderschuren et al. 2001). In addition to their euphoric effects, opiates powerfully modulate associative learning and memory processes and act as potent associative memory cues. A cardinal feature of human opiate addiction involves compulsive drug seeking in the face of adverse consequences (Cowen et al. 2001). Indeed, evidence of compulsive prefrontal cortical circuits is a well-known neuropathological clinical correlate of addiction in human abusers (Jentsch and Taylor 1999; Dolan et al. 2008). Nevertheless, the neurobiological and neuroanatomical substrates within the prefrontal cortex that may regulate sensitivity to the rewarding and addictive properties of opiate-class drugs have not been identified.

In the mammalian brain, the medial prefrontal cortex (mPFC), ventral tegmental area (VTA), and basolateral nucleus of the amygdala form a neural triumvirate of functionally interconnected regions that are involved importantly in the processing of associative reward learning and memory encoding during the addiction process. For example, activation of prefrontal cortical networks is correlated with the expression of associative memories linked to opiate-related cues during opiate-seeking and relapse-related behavioral phenomena (Daglish et al. 2001; Luo et al. 2001; Schmidt et al. 2005; Koya et al. 2006; Langleben et al. 2008). Behavioral evidence implicates an important role for glutamatergic (GLUT) transmission through both N-methyl D-aspartate (NMDA) and L-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) receptor substrates in the processing and recall of drug-related associative cues (Conrad et al. 2008; Van den Oever et al. 2008); however, the potential role of NMDA receptor transmission within the mPFC during the acquisition of associative opiate reward learning and memory is not known.

The mPFC, and in particular, the NR2B subunit of the NMDA complex, show high expression levels within mPFC (Goebel and Poosch 1999). Several lines of evidence demonstrate NMDA receptor-dependent modulation of dopamine (DA) transmission within the mesocorticolimbic system. In vivo microdialysis studies have shown that NMDA receptor agonists can increase DA release and increase extracellular concentrations of γ-aminobutyric acid (GABA) directly within the mPFC (Del Arco and Mora 1999, 2002). Paradoxically, administration of NMDA antagonist drugs such as ketamine and phencyclidine (PCP), which are highly addictive in and of themselves, lead to increased GLUT and DA release within the mPFC (Moghaddam et al. 1997; Lorrain et al. 2003), presumably by modulating inhibitory versus excitatory influences upon presynaptic GLUT and DA terminal fields (Jackson et al. 2004; Homayoun and Moghaddam 2007). In addition, administration of NMDA antagonists strongly attenuates inhibitory feedforward drive to mPFC pyramidal neurons by decreasing the spontaneous activity of mPFC GABAergic neurons (Homayoun and Moghaddam 2007), providing a mechanism by which NMDA antagonists may amplify prefrontal cortical activity. Given the importance of mPFC NMDA receptor transmission in the modulation of mesocorticolimbic DA, we performed a series of behavioral pharmacology studies to examine the role of mPFC GLUT transmission during associative opiate reward learning. Our results demonstrate that NMDA receptor blockade specifically within the prelimbic (PLC) region of the mPFC strongly potentiates the rewarding associative properties of both systemic and intra-VTA opiate administration and that this NMDA receptor-mediated modulation of opiate reward learning depends upon functional input from the basolateral amygdala (BLA), presynaptic GLUT release, and DA receptor transmission directly within the PLC.

Materials and Methods

Surgical Procedure

Male Sprague-Dawley rats (Charles River; 350–400 g at the start of experiments) were anesthetized with a ketamine (80 mg/ml)-xylazine...
(6 mg/ml) mixture and placed into a stereotaxic device. Stainless steel guide cannulae (22 gauge; Plastics One) were bilaterally implanted using the following stereotaxic coordinates (in mm). For the PLC (15° angle): from bregma, AP +2.9, L 7.9, and from the dural surface, V -3.0. For the infralimbic (IFL) cortex (15° angle): from bregma, AP +3.2, L 7.2, and from the dural surface, V -4.2. For the anterior cingulate cortex (ACC) (15° angle): from bregma, AP +2.0, L 7.1, and from the dural surface, V -1.2. For the VTA (10° angle): from bregma, AP -5.0, L 7.2, and from the dural surface, V -8.0. At the conclusion of the experiments, animals were deeply anesthetized and transcardially perfused with isotonic saline followed by 10% formalin. Brain sections were stained with cresyl violet and mPFC or VTA cannulae placements were verified with light microscopy according to the anatomical boundaries defined by Paxinos and Watson (2005). Animals found to have cannulae placements outside the boundaries of the target regions were excluded from analysis.

**Drug Treatments**

Morphine sulfate (Macfarlan Smith), cis(Z)-flupenthixol-dihydrochloride (Sigma), amino-5-phospho-pentaanoic acid (AP-5; Sigma), SCH 23390 (Tocris), eticlopride (Sigma), muscimol hydrobromide (Tocris), Ro 04-5595 hydrochloride (Tocris), and lamotrigine (LAMO; Tocris) were dissolved in physiological saline (pH adjusted to 7.4). Bilateral intra-PLC, IFL cortex, ACC, or VTA microinjections (0.5 µl volume per infusion) were performed over 1 min. Injectors were then left in place for an additional 1 min to ensure adequate diffusion from the injector tip. For intra-IFL muscimol experiments, BLA microinjections were performed immediately prior to intra-PLC microinusions. For intra-PLC morphine trials, intra-PLC microinusions were performed immediately prior to VTA microinusions. For intra-PLC coadministration of AP-5, x-ful, SCH 23390, eticlopride, or LAMO, animals received drugs simultaneously (in the same suspension). All brain microinusions were performed immediately prior to systemic injections of morphine or saline and animals were placed into the assigned place conditioning environments immediately following systemic injection of morphine or saline.

**Place Conditioning**

Animals were conditioned using an unbiased, fully counterbalanced place conditioning procedure, as described in detail previously (Laviolette et al. 2002; Laviolette and van der Kooy 2003). Briefly, conditioning took place in 1 of 2 environments, which differed in color, texture, and smell. One environment was white with a wire mesh floor, covered in wood chips. The other environment was black with smooth Plexiglass floor, wiped down with 2% acetic acid solution before each conditioning session. Animals display no baseline preference for either of these environments (Laviolette and van der Kooy 2003). Animals receive 4 drug-environment and 4 saline-environment conditioning session in a fully counterbalanced order. At testing, animals are placed on a narrow, gray zone that separates the 2 compartments and times spent in each environment are digitally recorded and scored separately for each animal over a 10-min test session.

**Data Analysis**

All data were analyzed with 1-, 2-, or 3-way analysis of variance (ANOVA) or student's t-tests where appropriate. Post hoc analyses were performed with Newman-Keuls or Fisher's least significant difference tests where appropriate.

**Results**

**NMDA Receptor Blockade Specifically within the PLC Potentiates Opiate Reward Magnitude**

To examine the potential role of NMDA receptor modulation in the mPFC during associative opiate reward encoding, bilateral microinusions of the specific and competitive NMDA receptor antagonist, AP-5 (0.1–1.0 µg/µl), were performed in the PLC division of the mPFC with anatomical control infusions performed in the ACC or IFL cortex subregions (see Materials and Methods). Histological analysis revealed bilateral cannulae placements located within the anatomical boundaries of these structures (PLC, ACC, or IFL cortex) as defined by Paxinos and Watson (2005). Cannulae placements found outside these boundaries resulted in the animal being excluded from experimental analyses. In Figure 1A, we show a representative microphotograph showing a typical intra-PLC microinfusion site. In Figure 1B, we present a schematic illustration demonstrating intra-PLC microinfusion sites from representative experimental groups. Schematic illustrations demonstrating bilateral cannulae placements for anatomical control microinusion in either the ACC or IFL cortex are presented in Figures 1C and 1D, respectively. In our initial study, we examined the effects of bilateral intra-PLC AP-5 administration on the acquisition of CPP for a subreward threshold dose of systemic morphine (0.05 mg/kg; intraperitoneal [ip]). Results of CPP experiments using a subreward threshold dose of systemic morphine (0.05 mg/kg; ip) revealed that bilateral intra-PLC microinusions of the NMDA receptor antagonist AP-5 (0.1–1.0 µg/0.5 µl) dose dependently potentiated morphine reward learning as demonstrated by a strongly potentiated morphine CPP response (Fig. 2A). A 2-way ANOVA revealed a significant group (intra-PLC saline vs. AP-5) × treatment (saline vs. morphine) interaction on times spent in conditioning environments (F1,45 = 3.8; P < 0.05). Post hoc comparisons revealed that intra-PLC microinusions of AP-5 dose dependently increased times spent in morphine-paired environments, relative to saline-paired environments at the higher AP-5 dose (1.0 µg/0.5 µl; P < 0.01; n = 7) (P < 0.01) but not the lower dose (0.1 µg/0.5 µl; P > 0.05; n = 6). In addition, times spent in morphine-paired environments in animals receiving the higher dose of intra-PLC AP-5 (1.0 µg/0.5 µl) were significantly increased relative to times spent in morphine environments in saline control animals (n = 10; P < 0.05). To rule out the possibility that the effective dose of intra-PLC AP-5 alone may be producing motivational effects, we ran a separate control experiment wherein rats received the effective dose of intra-PLC AP-5 (1.0 µg/0.5 µl) in one environment and saline in the alternate environment (Fig. 2A; right column, n = 6). Statistical analysis revealed no behavioral conditioning effects with intra-PLC alone (neither CPP nor aversion) (F1,9 = 2.1; P > 0.05). Having determined the effective dose of intra-PLC AP-5, we next tested 2 suprareward threshold doses of systemic morphine (0.5–5.0 mg/kg; ip) versus intra-PLC AP-5 (1.0 µg/0.5 µl). Results of CPP experiments revealed that intra-PLC AP-5 microinusions significantly potentiated the rewarding properties of both doses of systemic morphine (0.5 mg/kg; ip; Fig. 2B; 5.0 mg/kg; ip; Fig. 2C). For experimental groups receiving 0.5 mg/kg morphine (Fig. 2B), 2-way ANOVA revealed a significant group (intra-PLC saline vs. AP-5) × treatment (saline vs. morphine) interaction on times spent in conditioning environments (F1,25 = 13.4; P < 0.0001). Post hoc comparisons revealed that animals receiving intra-PLC microinusions of AP-5 (1.0 µg/0.5 µl, n = 7) or saline (n = 6) spent significantly more time in morphine-paired environments, relative to saline-paired environments (P values < 0.05 and 0.01, respectively). However, times spent in morphine-paired environments in animals receiving intra-PLC AP-5 (1.0 µg/0.5 µl) was significantly increased relative to times spent in morphine environments in saline control animals (P < 0.01). For experimental groups receiving 5.0 mg/kg morphine (Fig. 2C), a 2-way ANOVA
revealed a significant group (intra-PLC saline vs. AP-5) × treatment (saline vs. morphine) interaction on times spent in conditioning environments ($F_{1,33} = 43.4; P < 0.0001$). Post hoc comparisons revealed that animals receiving intra-PLC microinfusions of AP-5 (1.0 µg/0.5 µl, $n = 7$) or saline ($n = 6$) spent significantly more time in morphine-paired environments, relative to saline-paired environments ($P$ values < 0.05 and 0.01, respectively). However, times spent in morphine-paired environments in animals receiving intra-PLC AP-5 (1.0 µg/0.5 µl) was significantly increased relative to times spent in morphine environments in saline control animals ($P < 0.01$).

A 2-way ANOVA comparing only “difference scores” (time in drug- minus saline-paired environments) between intra-PLC saline versus AP-5 groups revealed a significant effect of group ($F_{1,46} = 32.7; P < 0.001$) on times spent in morphine-relative to saline-paired environments (Fig. 2D). Post hoc analysis revealed that difference scores obtained at testing for all doses of systemic morphine (0.05–5.0 mg/kg; ip) were significantly elevated relative to saline control groups (all $P$ values < 0.01). Thus, bilateral intra-PLC microinfusions of AP-5 rendered a subreward threshold dose of systemic morphine (0.05 mg/kg; ip) rewarding (Fig. 2A) and significantly potentiated the magnitude of morphine CPP over an order of magnitude dose range of suprareward threshold systemic morphine doses (0.5–5.0 mg/kg; ip; Fig. 2C,D).

To determine the anatomical specificity of the effects of AP-5 on associative opiate reward learning in the PFC, separate control groups received intra-ACC ($n = 8$) or -IFL ($n = 6$) microinfusions of the previously established effective dose of AP-5 (1 µg/0.5 µl) prior to receiving a subthreshold systemic dose of morphine (0.05 mg/kg; ip; see Materials and Methods). Neither intra-ACC ($t_x = 0.5; P > 0.05$) nor intra-IFL ($t_y = 1.6; P > 0.05$) AP-5 microinfusions caused a potentiation in opiate reward learning (Fig. 2E) as neither group displayed a preference for morphine-paired environments. Thus, pharmacological blockade of NMDA receptor transmission specifically within the PLC division of the PFC dose dependently amplified the rewarding properties of systemic morphine over an order of magnitude dose range, whereas producing no motivational effects in and of itself.

**Blockade of the NR2B Subunit of the NMDA Receptor Is Sufficient for the Potentiation of Associative Opiate Reward Learning**

To more specifically examine the role of the NMDA receptor complex in modulation associative opiate reward magnitude,
we performed additional experiments using a specific antagonist of the NR2B subunit of the NMDA receptor. Thus, we performed bilateral microinfusions of the specific and competitive NR2B receptor subunit antagonist, Ro-04-5595 (0.1–2.0 µg/0.5 µl) into the PLC, immediately prior to administration of a subreward threshold dose of morphine (0.05 mg/kg; ip). Similar to the effects observed with AP-5, we found that intra-PLC administration of Ro-04-5595 produced a dose-dependent potentiation of associative morphine reward, as demonstrated by the expression of significant CPP for environments paired with normally subreward threshold doses of morphine (0.05 mg/kg; ip) (Fig. 5A). ANOVA revealed a significant main effect...
of group on times spent in morphine- versus saline-paired environments \((F_{1,63} = 13.9; P < 0.001)\) with post hoc analysis revealing that animals receiving the 2 highest doses of intra-PLC \((1.0 \mu g/0.5 \mu l; P < 0.05; n = 7\) and \(2.0 \mu g/0.5 \mu l; P < 0.01; n = 7\) Ro-04-5595 displaying significant morphine CPP relative to saline controls or animals receiving a lower dose of intra-PLC Ro-04-5595 \((0.1 \mu g/0.5 \mu l; P > 0.05; n = 8)\) or intra-PLC saline \((P > 0.05; n = 10)\). To confirm that intra-PLC Ro-04-5595 was not producing any motivational effects in and of itself, a separate control experiment was performed in which rats \((n = 6)\) received intra-PLC microinfusions of either the highest effective dose of Ro-04-5595 \((2.0 \mu g/0.5 \mu l)\) or saline. Behavioral results revealed that intra-PLC Ro-04-5595 did not produce any motivational effects in and of itself as rats displayed no significant difference in times spent in environments paired with saline \((t_s = 0.054; P > 0.05)\) (Fig. 3A, right column).

**Blockade of Glutamate Release in the PLC Blocks the Effects of NMDA Receptor Hypofunction on Morphine Reward Potentiation**

Given that systemically applied NMDA receptor antagonists potentiate increase GLUT release within the mPFC (Moghadam et al. 1997; Lorrain et al. 2003), we next examined the effects of GLUT release blockade on the morphine reward-potententiating effects of intra-PLC AP-5. Coadministration of the GLUT release blocker, LAMO, dose dependently blocks the effects of intra-PLC AP-5 \((1.0 \mu g/0.5 \mu l)\) on the conditioning effects of a subreward threshold dose of systemic morphine \((0.05 mg/kg; ip; Fig. 3B)\). A 2-way ANOVA revealed a significant group \((intra-PLC saline vs. LAMO (0.01–0.1 \mu g/0.5 \mu l)/AP-5)\) × treatment \((saline vs. morphine)\) interaction on times spent in conditioning environments \((F_{5,59} = 7.02; P < 0.001)\). Post hoc comparisons revealed that animals receiving intra-PLC coadministration of the effective dose of AP-5 \((1.0 \mu g/0.5 \mu l)\) with a lower dose of LAMO \((0.01 \mu g/0.5 \mu l; n = 7)\) or saline vehicle alone \((n = 7)\) showed significant CPP for morphine-paired environments \((P values < 0.01 and 0.05\), respectively \((Fig. 3B)\). However, animals receiving coadministration of the effective dose of AP-5 \((1.0 \mu g/0.5 \mu l)\) with a higher dose of LAMO \((0.1 \mu g/0.5 \mu l; n = 8)\) failed to show a CPP for morphine-paired environments, demonstrating that blockade of GLUT release with LAMO dose dependently prevents the morphine reward-potententiating effects of intra-PLC AP-5. A separate control experiment was performed to determine if the previously determined effective dose of LAMO \((0.1 \mu g/0.5 \mu l)\) was specifically blocking the functional effects of intra-PLC AP-5 or nonspecifically blocking the rewarding and/or associative properties of morphine. Accordingly, a control group received intra-PLC LAMO \((0.1 \mu g/0.5 \mu l; n = 7)\) pretreatment prior to conditioning with a suprareward threshold dose of morphine \((5.0 mg/kg; ip)\). CPP testing revealed that the previously determined effective doses of LAMO failed to block morphine reward learning as this group displayed a significant CPP for the morphine-paired environment \((t_s = 4.7; P < 0.01; Fig. 3B, right column)\). Thus, intra-PLC LAMO does not induce nonspecific learning or memory deficits during conditioning but rather dose dependently blocks the behavioral effects of morphine in the presence of intra-PLC AP-5. To confirm that intra-PLC LAMO was not producing any motivational effects in and of itself, a separate control experiment was performed in which rats \((n = 6)\) received intra-PLC microinfusions of either the highest effective dose of LAMO \((0.1 \mu g/0.5 \mu l)\) or saline. Behavioral results revealed that intra-PLC LAMO did not produce any motivational effects in and of itself as rats displayed no significant difference in times spent in environments paired previously with LAMO, relative to environments paired with saline \((t_s = 0.32; P > 0.05)\) (Fig. 3B, right column).

**Intra-mPFC NMDA Receptor Blockade Potentiates Associative Opiate Reward Signals from the Ventral Tegmental Area**

The VTA serves as a critical neural region for the processing of opiate reward signals (Nader and van der Kooy 1997; Laviolette et al. 2004). Given that intra-PLC NMDA receptor blockade strongly potentiated the associative rewarding properties of systemic opiates (Fig. 2), we next examined the potential role of PLC NMDA receptor modulation during the encoding of associative opiate reward learning via direct

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**Figure 3. Effects of intra-PLC GLUT release blockade on NMDA antagonist-mediated potentiation of morphine reward and the effects of intra-PLC NR2B subunit blockade on opiate reward. (A)** Similar to the effects observed with AP-5, intra-PLC administration of Ro-04-5595 \((0.1–2.0 \mu g/0.5 \mu l)\), a competitive and specific antagonist of the NR2B subunit of the NMDA receptor complex, dose dependently potentiates the associative rewarding properties of a subreward threshold dose of systemic morphine \((0.5 mg/kg; ip)\). Microinfusions of the highest effective dose of intra-PLC Ro-04-5595 \((2.0 \mu g/0.5 \mu l)\) produced neither preference nor aversion for drug- versus saline-paired environments (right column). **(B)** Coadministration of a GLUT release blocker, LAMO \((0.01–0.1 \mu g/0.5 \mu l)\); see Materials and Methods) dose dependently blocks the ability of the NMDA antagonist, AP-5 \((1.0 \mu g/0.5 \mu l)\), to potentiate the rewarding properties of a subreward threshold dose of systemic morphine \((0.05 mg/kg; ip)\). Intra-PLC administration of the effective dose of LAMO alone versus saline has no effect on the rewarding properties of a suprareward threshold dose of systemic morphine \((5.0 mg/kg; ip)\) demonstrating that LAMO does not induce any learning impairments during morphine CPP conditioning. Microinfusions of the highest effective dose of intra-PLC LAMO \((0.1 \mu g/0.5 \mu l)\) produced neither preference nor aversion for drug- versus saline-paired environments (right column).
microinfusions of morphine directly into the VTA, following intra-PLC microinfusions of the effective dose of AP-5. In Figure 4A, a microphotograph shows a representative bilateral placement of intra-VTA cannulae and these intra-VTA microinfusion locations are presented schematically in Figure 4B. Based upon previously published reports (Nader and van der Kooy 1997; Laviolette et al. 2004), we selected sub- (250 ng/0.5 μl) and suprareward threshold (500 ng/0.5 μl) doses of intra-VTA morphine and challenged these doses of intra-VTA morphine against the previously established effective dose of intra-PLC AP-5 (1.0 μg/0.5 μl) (Fig. 5). Group analysis revealed a significant interaction between group (intra-PLC AP-5 or saline) and treatment (intra-VTA saline or morphine) ($F_{1,51} = 8.97; P < 0.01$) on times spent in saline- versus morphine-paired environments. Post hoc analysis revealed that rats receiving intra-PLC saline versus a subthreshold conditioning dose of intra-VTA morphine displayed no CPP for morphine-paired environments ($n = 8; P > 0.05$). In contrast, rats receiving intra-PLC AP-5 (1.0 μg/0.5 μl) versus this same dose of subreaward threshold intra-VTA morphine demonstrated a significant CPP for morphine-paired environments ($n = 7; P < 0.05$) (Fig. 5A). Rats receiving intra-PLC saline versus a suprathereshold conditioning dose of intra-VTA morphine (500 ng/0.5 μl) displayed a significant CPP for environments paired with this dose of intra-VTA morphine ($n = 8; P < 0.05$); however, in rats receiving intra-PLC AP-5 (1.0 μg/0.5 μl), the magnitude of CPP for the morphine-paired environment was significantly greater ($n = 8; P < 0.01$) than saline controls ($P < 0.05$) (Fig. 5B). Thus, similar to the effects observed with systemic morphine administration (Fig. 2), blockade of NMDA receptors in the PLC strongly potentiated the associative rewarding properties of morphine reward signals originating directly within the VTA.

**NMDA Receptor Modulation of Opiate Reward Learning Depends upon Dopaminergic Transmission within the mPFC**

Given the finding that modulation of intra-PLC NMDA receptor transmission potentiates intra-VTA morphine reward (Fig. 5), we next examined the potential role of functional interactions between DAergic and NMDA receptor transmission within the PLC in the processing of these behavioral effects. In order to determine whether the potentiation of morphine reward learning by AP-5 is dependent upon an interaction between glutamate and DAergic receptor transmission within the PLC, we challenged the opiate reward-potentiating effects of the effective dose of AP-5 (1.0 μg/0.5 μl) with a broad spectrum (D1 like and D2 like) DA receptor antagonist, α-flupenthixol (α-flu) (see Materials and Methods) over a wide concentration range 0.03–3.0 μg/0.5 μl (Fig. 6A). Similar to prior results, intra-PLC saline-treated control rats ($n = 7$) failed to demonstrate a significant CPP for a subreaward threshold dose of morphine (0.05 mg/kg, ip; $k = 0.32; P > 0.05$, Fig. 6A). In contrast, a 2-way ANOVA comparing groups receiving intra-PLC AP-5 (1.0 μg/0.5 μl) alone ($n = 7$) and groups receiving coadministation of this dose of AP-5 with α-flu (0.03, 0.3, or 3.0 μg/0.5 μl) revealed a significant effect of treatment (saline or morphine; $F_{1,65} = 8.599; P < 0.01$) on times spent in saline-versus morphine-paired environments. Post hoc analyses revealed that whereas rats receiving intra-PLC AP-5 ($n = 8; 1.0 μg/0.5 μl$) in the absence of α-flu demonstrated a strong potentiation in associative opiate reward learning relative to saline controls ($P < 0.05; n = 7$) coadministration of α-flu with AP-5 dose dependently blocked this potentiation of associative opiate reward learning. Thus, higher doses of α-flu (0.3–3.0 μg/0.5 μl) completely blocked AP-5-mediated potentiation of morphine reward learning, demonstrated by a lack of morphine CPP in these groups ($n$ values = 8 and 7, respectively, $P$ values > 0.05). However, a lower dose of α-flu (0.3 μg/0.5 μl) did not block the reward-potentiating effects of intra-PLC AP-5 as this group demonstrated a significant CPP for the morphine-paired environment, relative to saline controls (Fig. 6A; $n = 7; P < 0.05$). In order to control for the possibility that intra-PLC α-flu may induce nonspecific learning deficits, we examined the effects of our highest effective dose of intra-PLC α-flu (3.0 μg/0.5 μl) by itself versus a suprareward threshold dose of systemic morphine (5.0 mg/kg, ip) ($k = 7.1; P < 0.001$). This high dose of α-flu with AP-5 did not block suprathereshold morphine reward learning, indicating that intra-PLC α-flu does not induce nonspecific learning or memory deficits in the context of associative opiate reward (Fig. 6A). Given our finding that specific blockade of the NR2B subunit of the NMDA receptor potentiates also subreaward threshold morphine (Fig. 3A), we examined the ability of our previously established effective

![Figure 4](http://cercor.oxfordjournals.org/)
dose of α-flu to block the opiate reward-potentiating effects of the specific NR2B subunit antagonist, Ro 04-5595. Accordingly, we coadministered our previously established effective dose of intra-PLC Ro 04-5595 (2 µg/0.5 µl) with α-flu (3.0 mg/0.5 ml; n = 6) prior to administration of a subreward threshold dose of systemic morphine (0.05 mg/kg; ip) (Fig. 6B). Relative to control groups receiving either intra-PLC saline (n = 10) or intra-PLC Ro 04-5595 (n = 7), ANOVA revealed a significant group × treatment interaction (F(1,54) = 4.9; P < 0.01) on times spent in morphine- versus saline-paired environments. Post hoc analysis revealed that whereas animals receiving intra-PLC saline demonstrated no significant CPP for environments paired with this subthreshold dose of morphine (P > 0.05), animals receiving intra-PLC Ro 04-5595 alone demonstrated a significant CPP for morphine-paired environments (P < 0.01), whereas animals receiving coadministration of intra-PLC Ro 04-5595 with α-flu displayed no CPP for environments paired with subreward threshold morphine (P > 0.05); thus, similar to our results observed with AP-5, NR2B receptor antagonist-mediated potentiation of associative opiate reward learning depends upon DA receptor transmission within the PLC (Fig. 6B).

**NMDA Receptor Modulation of Opiate Reward Learning Depends upon Both DA D1 and D2 Receptor Subtype Transmission within the mPFC**

Given that nonspecific (D1 and D2) DA receptor blockade experiments with α-flu prevented intra-PLC AP-5-mediated opiate reward potentiation (Fig. 6A,B), we next performed more specific DA receptor blockade experiments to determine if NMDA receptor-mediated opiate reward modulation was dependent specifically upon either the DA D1 or D2 DA receptor subtype transmission. Accordingly, we coadministered either a selective DA D1-like receptor antagonist, SCH 23390 (0.1-1.0 µg/0.5 µl) or a selective DA D2-like receptor antagonist, eticlopride (0.1-1.0 µg/0.5 µl) with the previously established effective dose of AP-5 (1.0 µg/0.5 µl). Coadministration of AP-5 with either SCH 23390 or eticlopride was performed bilaterally into the PLC, prior to administration of a subreward threshold dose of systemic morphine (0.05 mg/kg; ip). Coadministration of SCH 23390 dose dependently blocked the ability of intra-PLC AP-5 to potentiate the associative rewarding effects of a subthreshold dose of morphine (Fig. 6C). ANOVA revealed a significant interaction between group and treatment (F(2,45) = 22.1; P < 0.0001) with post hoc analyses revealing that whereas animals receiving intra-PLC AP-5 (1.0 µg/0.5 µl) alone (n = 7) or a lower dose of SCH 23390 (n = 7; 0.1 µg/0.5 µl) showed significant CPP for morphine-paired environments (P values < 0.01), animals receiving a higher dose of SCH 23390 (n = 7; 1.0 µg/0.5 µl) failed to show a significant CPP for morphine-paired environments (P > 0.05) in the presence of DA D1 receptor blockade. Coadministration of the selective D2 receptor antagonist, eticlopride, similarly caused a dose-dependent block of intra-PLC AP-5-associated opiate reward potentiation (Fig. 6C). ANOVA revealed a significant interaction between group and treatment (F(2,45) = 8.4; P < 0.001) with post hoc analyses revealing that whereas animals receiving intra-PLC AP-5 (1.0 µg/0.5 µl) alone (n = 7) or a lower dose of eticlopride (n = 7; 0.1 µg/0.5 µl) showed significant CPP for morphine-paired environments (P values < 0.01 and 0.05, respectively), animals receiving a higher dose of eticlopride (n = 7; 1.0 µg/0.5 µl) failed to show a significant CPP for morphine-paired environments (P > 0.05) in the presence of DA D2 receptor blockade. Thus, blockade of either the DA D1 or D2 receptor subtypes was sufficient to prevent the ability of intra-PLC NMDA receptor blockade to potentiate subthreshold associative opiate reward encoding, indicating an essential role for both D1 and D2 receptor transmission in the opiate reward modulation effects of NMDA receptor transmission in the PLC. To confirm that intra-PLC microinfusions of our selected DA receptor antagonists (α-flu, SCH 23390, eticlopride) were not producing any motivational effects in and of themselves, separate control experiments were performed in which rats (n = 6 per group) received intra-PLC microinfusions of the highest effective doses of α-flu (3.0 µg/0.5 µl), SCH 23390 (1.0 µg/0.5 µl), or eticlopride (1.0 µg/0.5 µl) versus saline. Behavioral results revealed that intra-PLC α-flu did not produce any motivational effects in and of itself as rats displayed no significant difference in times spent in environments paired previously with α-flu, relative to environments paired with saline (t(6) = 0.33; P > 0.05). Similarly, intra-PLC eticlopride did not produce any motivational effects in and of itself as rats displayed no significant difference in times spent in environments paired previously with eticlopride, relative to environments paired with saline (t(6) = 0.66; P > 0.05). Finally, intra-PLC SCH 23390 did not produce any motivational effects in and of itself as rats displayed no significant difference in times spent in environments paired previously with SCH 23390, relative to environments paired with saline (t(6) = 0.41; P > 0.05) (Fig. 6D).
Inactivation of the Basolateral Amygdala Prevents mPFC NMDA Receptor-Mediated Modulation of Opiate Reward Learning

GLUT inputs arising from the amygdala to the mPFC are involved critically in the modulation of emotional associative learning and memory both behaviorally and in single mPFC neurons (Laviolette et al. 2005; Laviolette and Grace 2006). Although there exist multiple neuroanatomical sources of GLUT inputs to the mPFC, we next tested whether functional connections between the BLA and PLC region may be involved in the modulatory effects of intra-PLC NMDA receptor transmission during opiate reward processing. Accordingly, we...
performed an additional experiment wherein rats received bilateral microinfusions of the GABA$_A$ receptor agonist, muscimol (500 ng/0.5 μl) directly into the BLA (Fig. 7A,B) prior to receiving the effective dose of bilateral intra-PLC AP-5 (1.0 μg/0.5 μl; see Materials and Methods) and challenged these manipulations with a subthreshold dose of systemic morphine (0.05 mg/kg; ip). This dose of intra-BLA muscimol has previously been reported to block associative neuronal learning both behaviorally and at the level of the single neuron within the mPFC via direct, monosynaptic BLA → mPFC projections (Laviolette et al. 2005; Laviolette and Grace 2006). ANOVA revealed a significant interaction between group and treatment on times spent in morphine- versus saline-paired conditioning environments ($F_{1,23} = 5.03; P < 0.05$). Post hoc analysis revealed that whereas rats receiving intra-BLA saline and intra-PLC AP-5 (1.0 μg/0.5 μl; $n = 6$) displayed a significant CPP ($P < 0.01$) for environments paired with a subthreshold dose of morphine (0.05 mg/kg; ip), rats receiving intra-BLA muscimol (500 ng/0.5 μl; $n = 6$) with intra-PLC AP-5 (1.0 μg/0.5 μl) demonstrated no CPP for environments paired with this same dose of morphine (Fig. 7C) ($P > 0.05$). To control for the possibility that intra-BLA muscimol induced nonspecific block of associative opiate reward learning, we ran a separate control group ($n = 8$) that received bilateral intra-BLA muscimol alone (500 ng/0.5 ml) immediately prior to receiving a suprareward threshold dose of morphine (5 mg/kg; ip). Intra-BLA muscimol (500 ng/0.5 μl) failed to block suprathreshold associative opiate reward learning as control animals displayed a significant CPP for environments paired with morphine ($t = 4.75; P < 0.001$) demonstrating that although muscimol-induced inactivation of the BLA is sufficient to prevent intra-PLC NMDA receptor blockade--induced potentiation of subreward threshold morphine, it is not sufficient to induce a blockade of associative opiate reward learning at suprareward threshold morphine doses (Fig. 7C). Thus, intra-PLC NMDA receptor substrates interacting with GLUT inputs from the BLA may serve to modulate associative opiate reward encoding by amplifying subthreshold reward stimuli but do not appear to be necessary for the encoding of suprareward threshold opiate signals.

**Discussion**

Evidence from both human and animal studies implicates the mPFC in the encoding and expression of associative opiate reward memory (Daglish et al. 2001; Volkow et al. 2004; Van den Oever et al. 2008). The present results demonstrate that NMDA receptor hypofunction within the prelimbic subdivision (PLC) of the PFC can strongly increase sensitivity to the rewarding properties of both systemic and intra-VTA opiates through DAergic receptor substrates and functional BLA connections within the PLC.

**NMDA Receptor Transmission in the PLC Modulates the Encoding Amplitude of Associative Opiate Reward Learning and Memory**

NMDA receptors play a key functional role in the neuronal network dynamics of the PFC and strongly influence the balance of DA and GLUT concentrations within this region of the brain (Moghaddam et al. 1997). The functional consequences of in vivo application of various NMDA antagonists such as ketamine and PCP include increased release of both DA and GLUT within the PFC (Moghaddam and Adams 1998; Adams and Moghaddam 2001; Lorrain et al. 2003). In addition, NMDA receptor antagonists induce strong release of DA within the nucleus accumbens (NAc), further demonstrating that mPFC NMDA receptors have profound effects on mesocorticolimbic network activity (Del Arco and Mora 2008). Although the neuronal mechanisms underlying these effects are currently not understood, electrophysiological evidence suggests that NMDA antagonists alter the balance of PFC neuronal activity by inhibiting feedforward inhibitory substrates resulting in a net increase in activity of mPFC pyramidal neurons (Del Arco and Mora 1999, 2002; Homayoun and Moghaddam 2007). Thus, NMDA antagonist--induced attenuation of feedforward inhibition may remove inhibition on presynaptically located GLUT or DA inputs, resulting in increased release of these neurotransmitters. The present results demonstrate that direct intrapl-CLC blockade of NMDA receptors, although producing no motivational effects alone, can strongly amplify the associative reinforcing properties of normally subreward threshold opiate conditioning stimuli.

In addition to the reward-potentiating effects of general NMDA receptor blockade, we found that specific blockade of the NR2B subunit was sufficient to potentiate the associative rewarding properties of subreward threshold morphine doses,
through a DA-dependent mechanism. Of all NMDA receptor subunits, the NR2B subtype demonstrates the highest relative expression levels within the rodent mPFC (Goebel and Poosch 1999) and is preferentially localized to GABAergic interneurons (Rosenblum et al. 1997). Blockade of this particular NMDA subunit has been shown to block reinstatement of opiate-related reward information (Ma et al. 2007), further supporting a role for this subunit in the encoding of opiate-related reward information. Given the localization of NR2B containing NMDA receptors primarily to inhibitory interneurons, one possibility is that administration of NMDA or NR2B-specific antagonists block excitatory inputs to these normally inhibitory feedforward neurons, which may increase spontaneous activity of PFC pyramidal output neurons, as suggested by previous in vivo studies (Homayoun and Moghaddam 2007).

In control studies, we observed no rewarding effects following intra-PLC administration of our behaviorally effective dose of AP-5. Interestingly, Carlezon and Wise (1996) have reported that rats will self-administer several NMDA antagonists, including PCP and MK-801, directly into both the mPFC and NAc. These findings have important implications for the present results for several reasons. First, they demonstrate that NMDA receptor antagonism within either NAc or mPFC can produce reinforcing effects in and of themselves, consistent with the well-established addictive properties of several NMDA antagonist compounds, including PCP and ketamine. This also raises the possibility that in the present study, morphine may be potentiating the reinforcing effects of intra-PLC NMDA receptor antagonism, which would suggest that μ-opiate receptors and intra-PLC NMDA receptor substrates may form an integrative reward circuit, capable of feeding back between the VTA and mPFC during the processing of opiate-related reward information. Nevertheless, this is an unlikely possibility given that our effective dose of AP-5 produced no rewarding effects in and of itself. Second, Carlezon and Wise (1996) reported that the reinforcing effects of intra-NAc NMDA antagonist administration were not blocked by DA receptor antagonism, suggesting that these effects were mediated via DA-independent reward circuitry. In the present study, intra-PLC coadministration of either DA D1 or D2 antagonists blocked the effects of AP-5 on morphine reward conditioning. Although the previously reported study examined only the effects of DA receptor blockade on intra-NAc (but not mPFC) NMDA antagonist self-administration, this difference may suggest that although NMDA hypofunction within the mPFC may amplify reward processing via DA-dependent mechanisms (present results), the ability of NMDA antagonists to serve as primary reinforcers directly within the NAc shell may take place through non-DA reward circuits.

**NMBA Receptor Blockade in the PLC Potentiates Opiate Reward Signaling via the Ventral Tegmental Area**

The VTA is a critical neural substrate for the mediation of opiate reward signaling (Laviolette et al. 2004; Syparaki et al. 1983) where drugs such as morphine can produce DA-dependent or DA-independent rewarding effects through D1-ergic or non-D1-ergic VTA neuronal populations (Laviolette et al. 2004). Opiates are self-administered directly into the VTA and 6-hydroxydopamine lesion-induced destruction of the mesocorticolimbic pathway strongly attenuates the reinforcing effects of opiates (Syparaki et al. 1983; Wise 1989). The present results are the first demonstration that modulation of NMDA receptor transmission extrinsic to the VTA can amplify the motivational reward value of opiates. We observed that subreward threshold doses of intra-VTA morphine were strongly potentiated by NMDA receptor blockade in the PLC. However, given the bidirectional modulatory relationship between ascending D1-ergic and GABAergic inputs from the VTA to the mPFC and descending GLUTergic inputs to the VTA from the mPFC, the directionality of this effect is not entirely clear. Thus, descending GLUT inputs from the mPFC to VTA appear to more selectively target subpopulations of VTA DA neurons that project in return to the mPFC (mesocortical DA neurons) (Carr and Sesack 2000a). Conversely, DA inputs from the VTA to the mPFC appear to more selectively target subpopulations of mPFC pyramidal neurons that send descending inputs to the NAc (Carr et al. 1999). Although future studies are required to examine these issues, given the known ability of NMDA receptor antagonists to potentiate DA release within the NAc (Del Arco and Mora 2008), one possibility is that intra-PLC NMDA antagonists may potentiate morphine-related D1-ergic signals from the VTA to the NAc, which may in turn amplify associative reward signals converging on the NAc from mPFC and the VTA.

Although the present study did not examine animals in a state of chronic opiate exposure and withdrawal, it is interesting that a considerable body of research has demonstrated a functional role for NMDA receptor transmission during the development and expression of opiate dependence, tolerance, and withdrawal. For example, many GLUT receptor antagonists, including dizocilpine (MK-801) and ketamine, can decrease or prevent the development of tolerance and somatic symptoms of physical dependence to opiates in rodents (Gonzalez et al. 1997). In addition, GLUT receptor antagonists have been demonstrated to block the aversive effects of opiate withdrawal as measured in a conditioned place aversion procedure in mice (Kawasaki et al. 2005). Although the precise neural substrates mediating the aversive properties of opiate withdrawal are not entirely understood, considerable evidence demonstrates that chronic opiate exposure is associated with alterations in GLUT receptor expression (including AMPA and specific NMDA receptor subunits) within the mesocorticolimbic system, including the amygdala, NAc, and PFC (Bajo et al. 2006; Van den Oever et al. 2008). Although future studies are required to address these issues, this evidence, together with the present findings, suggests that disturbances in GLUT transmission may be involved not only in the neuroplastic alterations following chronic opiate exposure leading to dependence and the aversive effects of withdrawal but also in contributing to increased vulnerability to the rewarding and addictive properties of opiates during early exposure.

**NMBA Receptor Modulation of Associative Opiate Reward Learning Requires Dopamnergic Receptor Transmission**

Considerable evidence suggests important functional modulation of mesocorticolimbic DA transmission via NMDA receptor substrates within the mPFC (Jackson and Moghaddam 2001; Homayoun and Moghaddam 2007). Anatomically, the mPFC receives direct D1-ergic and non-D1-ergic projections from the VTA (Carr and Sesack 2000b) and it has been suggested that VTA > mPFC projections may be critical for the processing of drug-related associative learning and conditioned memory...
Within the mPFC, DA transmission is involved in higher order cognitive tasks and executive function (Floresco et al. 2006) and various studies have reported that administration of NMDA receptor antagonists such as PCP or ketamine can strongly disrupt mPFC-dependent behaviors, likely related to a disruption of prefrontal cortical synchrony (Kargieman et al. 2007). Interestingly, such effects are reversed with DAergic antagonists (Verma and Moghaddam 1996), further demonstrating a functional link between mPFC DAergic transmission and NMDA signaling. The present results demonstrate an important functional link between mPFC NMDA and DA receptor substrates during the encoding of opiate-related reward information. Molecular evidence has identified functional interactions between both the D2 and D1 receptor subtypes with the NMDA receptor complex (Pei et al. 2004; Liu et al. 2006). Indeed, stimulation of D2 receptors leads to inhibition of NR2B containing NMDA receptors, an effect which leads to augmented behavioral sensitivity to cocaine (Liu et al. 2006). Although the present study examined only morphine-related effects, both drugs potently increase DA release. Although future studies are required to address this issue, one possibility is that NR2B blockade-induced morphine reward potentiation may act through a similar DA-mediated mechanism to amplify the behavioral effects of morphine.

Evidence that NMDA Receptor Modulation of Opiate Reward Acts on Presynaptic GLUT Input from the Basolateral Amygdala

During the encoding of emotionally salient associative information, the mPFC receives functional GLUTergic inputs from subcortical limbic structures, including the BLA (Laviolette et al. 2005; Laviolette and Grace 2006). Indeed, pharmacological inactivation of the BLA prevents the encoding of associative emotional information in single neurons of the mPFC (Laviolette et al. 2005; Laviolette and Grace 2006). Given our findings that AP-5-induced potentiation of opiate reward was dependent upon presynaptic GLUT release, we similarly examined the potential role of the BLA > mPFC circuit by inactivating the BLA prior to administering intra-PLC AP-5, in order to examine if the actions of NMDA antagonism on presynaptic GLUT release may depend upon inputs from the BLA. We found that prior pharmacological inactivation of the BLA (using the same pharmacological inactivation protocol previously reported; Laviolette et al. 2005; Laviolette and Grace 2006) was sufficient to block the opiate reward-potentiating effects of intra-PLC AP-5, suggesting that NMDA receptor-mediated modulation of opiate reward signals depends on GLUT inputs arising from the BLA. Interestingly, inactivation of the BLA alone did not block the encoding of suprathreshold morphine reward signals, demonstrating that although associative opiate reward learning can take place in the absence of BLA input, modulation of these inputs via NMDA receptor blockade can nevertheless powerfully influence the magnitude of opiate reward encoding within the mPFC. This is in contrast to previous reports examining the encoding of fear-related associative memory within the PFC which found that inactivation of the BLA was sufficient to block PFC neuronal encoding of associative fear information (Laviolette et al. 2005; Laviolette and Grace 2006). Although future studies are required to address this issue, this apparent dichotomy may suggest that although input from the BLA is required during PFC encoding of emotionally aversive information, in the context of reward-related associative learning, inputs from the BLA may be more critical as a modulator of the magnitude of associative reward memory within PFC neuronal circuits. In addition, BLA neuronal activity may be relatively more important for encoding or maintaining already established associative opiate-related memories or opiate-seeking reinstatement cues (Fuchs and See 2002; Milekic et al. 2006) and/or for the formation of memories linked to the experience of opiate withdrawal (Hellems et al. 2006).

In the present study, we found that blockade of GLUT release within the PLC with a GLUT release inhibitor and anticonvulsant compound, LAMO, was sufficient to prevent the opiate reward-potententiating effects of NMDA receptor antagonism while producing no motivational effects in and of itself. Interestingly, several studies have reported that LAMO can attenuate the psychotomimetic effects of other NMDA antagonists. Thus, Gozzi et al. (2008) reported that LAMO suppressed corticolimbic activation patterns following administration of psychotomimetic doses of PCP. Furthermore, Deakin et al. (2008) reported that LAMO pretreatment in humans effectively blocked both cortical activation patterns and clinical psychosis ratings following ketamine administration. Although these results further suggest that the behavioral effects of NMDA receptor blockade can be attenuated with GLUT release inhibition, it is important to note that LAMO has been reported to produce pharmacological actions beyond the block of presynaptic GLUT release. For example, using in vitro recordings, Cunningham and Jones (2000) reported that while LAMO can effectively block GLUT release in rat entorhinal cortex, the same doses could increase spontaneous GABA release. Furthermore, Lee et al. (2008) found that in dentate gyrus slice preparations, LAMO blocks GLUT release but can also inhibit postsynaptic AMPA receptors. Thus, the observed effects of intra-PLC LAMO on blockade of NMDA antagonist-induced opiate reward potentiation could be mediated via non-GLUTergic mechanisms, for example, by blocking incoming GLUT signals post-synaptically at AMPA receptors or by increasing GABA release from intrinsic cortical GABAergic interneuron populations which may in turn decrease activity of PLC pyramidal neurons, thereby counteracting the ability of NMDA antagonism to increase cortical pyramidal neuron activity (Homayoun and Moghaddam 2007). Although further studies are required to fully characterize the functional role of BLA GLUT inputs to the PLC during opiate reward encoding, the present findings add further evidence that both rewarding and aversive emotional information (Laviolette et al. 2005; Laviolette and Grace 2006) may depend critically upon functional GLUT transmission along the BLA > mPFC pathway.

Conclusions

The present results demonstrate a critical modulatory role for intra-PLC NMDA receptor transmission during the encoding of associative opiate reward learning. Both general blockade of the NMDA receptor complex and specific blockade of the NR2B subunit was sufficient to potentiate the associative rewarding properties of normally subreward threshold doses of either systemic or intra-VTA opiate reward signals, via DA D1- or D2 receptor-dependent mechanisms. In light of previous reports demonstrating primary reinforcing and addictive effects of various NMDA antagonists in both humans (Crider 1986) and other animals (Balster and Woolverton 1980;
Carlezon and Wise 1996), the present results may suggest a more general mechanism whereby hypofunction of NMDA receptor transmission within the PLC, a possible correlate of prefrontal hypofrontality, may serve to amplify the rewarding stimulus properties and increase the abuse liability of psychotropic drugs.

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Notes
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