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Amphetamine-induced conditioned place preference and changes in mGlu1/5 receptor expression and signaling in the rat medial prefrontal cortex

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Abstract

The medial prefrontal cortex (mPFC) is implicated in the rewarding effect of psychostimulants, although molecular mechanisms underlying the rewarding properties of stimulants in this region are poorly understood. Group I metabotropic glutamate (mGlu) receptors (mGlu1/5 subtypes) are believed to be critical in this event. We thus in this study investigated changes in mGlu1/5 receptor expression and function in the rat mPFC in response to conditioned place preference (CPP) induced by amphetamine. Repeated amphetamine administration (2.5 mg/kg, once daily on alternate days for 10 days) induced reliable CPP. In the mPFC, surface expression of mGlu5 receptors was elevated in rats after amphetamine conditioning. mGlu5 receptors were also increased at synaptic and extrasynaptic sites in amphetamine-conditioned rats. Expression of mGlu1 receptors was stable in surface and synaptic compartments, while it was elevated in the extrasynaptic location. In mPFC neurons, the mGlu1/5 agonist-stimulated phosphoinositide signaling pathway was upregulated in its efficacy following amphetamine conditioning. The mGlu1/5 agonist-stimulated Src kinase phosphorylation was also augmented in rats treated with amphetamine. These results demonstrate the sensitivity of mPFC mGlu1/5 receptors to amphetamine-induced CPP. Amphetamine conditioning results in the upregulation of mGlu1/5 receptor expression at subcellular and/or subsynaptic levels and mGlu1/5-mediated postreceptor signaling in mPFC neurons.

Keywords

mGlu1; mGlu5; metabotropic glutamate receptor; amphetamine; conditioned place preference; mPFC

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Conflict of interest

The authors declare that there are no potential conflicts of interest.

Introduction

Repeated administration of the psychostimulant amphetamine (AMPH) induces long-lasting rewarding and motivational effects. Conditioned place preference (CPP) is an animal model that is widely used to measure the preference of animals for a stimulant such as AMPH (Bardo and Bevins, 2000; Tzschentke, 2007). By measuring the amount of time spent in a chamber that has been previously associated with the AMPH experience, one can evaluate the reinforcement, extinction, and reinstatement of a conditioned stimulus (Tzschentke, 2007). Various brain regions have been implicated in AMPH effects. Early studies focus on the striatum and reveal that a striatal structure, i.e., the nucleus accumbens (NAc), is the central substrate of AMPH and is critical for AMPH CPP (Spiraki et al., 1982; Carr and White, 1986; McBride et al., 1999). In addition to the striatum, the medial prefrontal cortex (mPFC) plays a pivotal role. This region is composed of (from ventral to dorsal) infralimbic, prelimbic, and anterior cingulate cortex (Kolb, 1984; McDonald, 1991). Like the striatum, the mPFC is one of major dopamine responsive regions in the limbic reward system. It receives dopaminergic projections from the ventral tegmental area (VTA) and projects back to the VTA and NAc (Tzschentke, 2000; Heidbreder and Groenewegen, 2003; de Almeida et al., 2008). Available evidence links the mPFC to a variety of common neuropsychiatric disorders (Bissonette et al., 2013; Morici et al., 2015) and the stimulant-induced CPP (Tzschentke, 2000; Ventura et al., 2003). However, compared to the NAc, the mPFC has been less studied for its roles in processing AMPH CPP.

Group I metabotropic glutamate (mGlu) receptors (mGlu1 and 5 subtypes) are G protein-coupled receptors. Via $G_{\alpha q}$ proteins, mGlu1/5 receptors activate phospholipase C β 1 and thereby hydrolyze phosphoinositide into inositol-1,4,5-triphosphate (IP $_3$) and diacylglycerol, which trigger the Ca $^{2+}$ and protein kinase C signaling pathways, respectively (Niswender and Conn, 2010). Both mGlu1a and mGlu5 receptors are expressed in pyramidal and/or interneurons within the mPFC and are principally postsynaptic (Takumi et al., 1999; Lopez-Bendito et al., 2002; Muly et al., 2003). Thus, mGlu1/5 receptors are considered to play a role in the regulation of mPFC functions and the pathogenesis of mPFC-associated psychiatric disorders. In fact, increasing evidence implicates a pool of mGlu5 receptors in the mPFC in cocaine effects. Intra-mPFC injection of the mGlu5 antagonist MTEP prevented behavioral sensitization to cocaine, while intra-mPFC injection of DHPG, an mGlu1/5 agonist, induced an mGlu5-dependent cross-sensitization to cocaine (Timmer and Stekete, 2012). The mGlu5 mRNA expression in the prefrontal cortex was elevated in cocaine-sensitized mice (Blanco et al., 2014). Thus, it is likely that the mPFC mGlu5 receptor is sensitive to AMPH. As a critical element within excitatory synapses, mGlu5 receptors may undergo adaptive changes in response to chronic AMPH exposure and regulate the long-lasting synaptic plasticity related to the rewarding properties of stimulants, although plastic responses of mPFC mGlu5 receptors to AMPH are incompletely studied at present.

In this study, we thus investigated alterations in mGlu1/5 expression and function in the mPFC in an AMPH CPP paradigm in adult rats. Enduring CPP induced by repeated AMPH administration is particularly useful for exploring long-lasting neuroplastic changes

associated with rewarding and reinforcing effects of stimulants. Thus, in rats that developed CPP in response to repeated AMPH, changes in subcellular and subsynaptic expression of mGlu1/5 receptors and in the mGlu1/5-associated signaling events were examined in mPFC neurons.

Experimental procedures

Animals

Animals used in this study were Wistar male rats (Charles River, New York, NY). After arriving, 49 rats (220–240 g) were acclimated to the facility for at least 5 days before the experiment. At a constant temperature of 23°C and humidity of $50 \pm 10\%$, rats were provided with food and water *ad libitum*. Rooms in the our animal facility was kept on a 12-h/12-h light/dark cycle (lights on between 7 a.m. and 7 p.m.). Animal use and care were in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Institutional Animal Care and Use Committee.

CPP

We chose unbiased CPP in 44 rats based on the fact that CPP is a well-established and widely-used model for assessing the reinforcing properties of stimulant experience (Bardo and Bevins, 2000; Tzschentke, 2007). We used a three-chamber apparatus (AccuScan Instruments, Columbus, OH) which consisted of two side conditioning chambers (25.4 cm L x 30.48 cm W x 30.48 cm H) and a middle chamber (10.16 cm L x 30.48 cm W x 30.48 cm H) with two removable partitions (guillotine doors) to separate them (Rademacher et al., 2006; Shen et al., 2006). One side chamber has horizontal white stripes on the wall, whereas the other has vertical white lines on the wall. Two side chambers contain different floor textures to provide differential tactile cues. During a pre-conditioning phase, as shown in Fig. 1A, rats were acclimated to the CPP apparatus by placing them into the middle chamber and allowing them to explore the entire apparatus freely for 45 min (once a day for 3 days). At the final day (day 3), rats were pre-tested for their time spent in each chamber. To this end, rats were placed in the middle chamber and allowed free access to all chambers. Time spent in each chamber was recorded over a 15-min period. Three animals displayed a severe bias (> 70% of total time towards one of the two conditioning chambers) at the pre-conditioning stage and were removed to ensure an unbiased design (Rademacher et al., 2006). After pre-conditioning, rats were randomly assigned to two conditioning treatments: 1) AMPH, and 2) saline. For AMPH conditioning, a 10-day treatment regimen (days 4–13) was used. Rats received an intraperitoneal (i.p.) injection of AMPH (2.5 mg/kg) and were paired with one conditioning chamber while a saline injection (1 ml/kg, i.p.) was paired with the other with partitions closed for 45 min. We chose a dose of 2.5 mg/kg because AMPH at this dose induced significant and reliable CPP (Leri and Franklin, 2000; Latagliata et al., 2017). The AMPH-paired chamber was assigned in a counterbalanced order. Five pairings of AMPH and 5 pairings of saline with the respective chamber were carried out on alternate days, totaling a 10-day period of conditioning. Saline control rats received saline injections paired with both chambers. In details, control rats after saline injections were confined to one side chamber on days 4, 6, 8, 10, and 12 (considering “drug-paired side”) and confined

to the opposite side on days 5, 7, 9, 11, and 13 (saline-paired side or drug-unpaired side) (Shen et al., 2006). The middle chamber was not used during conditioning and was blocked by partitions. On day 16 (3 days after the last conditioning injection), drug-free rats were post-tested for their time spent in each chamber for 15 min as described for the pre-conditioning test. After the test, rats were anesthetized and sacrificed for the subsequent neurochemical assays (see below). We chose a 3-day time point because AMPH-induced CPP was fully developed and the mGlu5 antagonist MPEP inhibited expression of AMPH CPP in rats at this time point (Hoffman and Beninger, 1989; Herzig et al., 2005; Rademacher et al., 2006). The degree of place preference was determined by comparing the time spent in the drug-paired chamber between pre-tests and CPP tests. All rats were habituated to the test room in home cages for 1 h before placement into CPP test chambers.

mPFC slice preparation

We prepared rat mPFC slices as described previously (Jin et al., 2013). Briefly, after rats were anesthetized and decapitated, we removed brains which were then cut using a Leica VT1200S vibratome. Slices were preincubated at 30°C in artificial cerebrospinal fluid containing (in mM) 10 glucose, 124 NaCl, 3 KCl, 1.25 KH₂PO₄, 26 NaHCO₃, 2 MgSO₄, and 2 CaCl₂, bubbled with 95% O₂-5% CO₂, pH 7.4, followed by an additional preincubation for 10–20 min. Drugs were added and incubated at 30°C.

Surface protein biotinylation

We performed surface protein biotinylation on brain slices as described previously (Kato et al., 2012; Knackstedt et al., 2013; Pabba et al., 2014). Briefly, coronal brain slices (300 µm) were cut from 20 rats and the mPFC was dissected, including the anterior cingulate, prelimbic and infralimbic cortex (Heidbreder and Groenewegen, 2003). The mPFC slices were incubated in ice-cold artificial cerebrospinal fluid with 1 mg/ml EZ-LINK™-Sulfo-NHS-SS-Biotin (ThermoFisher, Rockford, IL) for 45 min at 4°C. After unreacted biotinylation reagent was removed by washes, slices were quenched by glycine (100 mM). Slices were then homogenized in a lysis buffer containing (mM) 25 HEPES, 150 NaCl, 1% Triton X-100, 0.5% sodium dodecyl sulfate, and a protease/phosphatase inhibitor cocktail (ThermoFisher). Homogenates were centrifuged for 10 min (1,000 g, 4°C). The supernatant was collected as a pool of total proteins. An equal amount of total proteins was aliquoted and incubated with ThermoFisher neutrAvidin resin overnight (4°C). Biotinylated proteins were precipitated by centrifugation, eluted with a lithium dodecyl sulfate sample buffer, and used as surface proteins, while the supernatant that was separated from neutrAvidin beads by centrifugation was used as unbiotinylated proteins representing the intracellular protein fraction. Protein concentrations were determined and were analyzed by immunoblot.

Fractionation of synaptic and extrasynaptic proteins

This fractionation was conducted according to a procedure that we used previously (Mao et al., 2013; Mao and Wang, 2018). Briefly, the mPFC slices were prepared from 12 rats and homogenized in isotonic sucrose homogenization buffer: 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, and a protease/phosphatase inhibitor cocktail (ThermoFisher). The homogenate was centrifuged twice at 760 g (10 min, 4°C) and the combined supernatant from two centrifugations was then centrifuged (10,000 g, 15 min, 4°C). The pellet contained

crude synaptosomal plasma membranes and was washed and centrifuged to produce the final pellet. This final pellet was resuspended in Triton X-100 (0.5%, v/v)/sucrose homogenization buffer with gentle rotation (20 min, 4°C). Centrifugation (20 min, 32,000 *g*) produced the pellet enriched with Triton X-100-insoluble synaptic membranes and the supernatant enriched with Triton X-100-soluble extrasynaptic membranes. Synaptic and extrasynaptic membranes were solubilized in the sucrose-Triton buffer containing 1% sodium dodecyl sulfate and a protease/phosphatase inhibitor cocktail (ThermoFisher). Samples after protein concentrations were determined were stored at –80°C until use.

Western blot analysis

This analysis was performed as described previously (Jin et al., 2013; Mao et al., 2018). Briefly, proteins (with no heat) were loaded with lithium dodecyl sulfate buffer and separated on 4–12% Tris-glycine gels (Invitrogen, Carlsbad, CA). After transferred to polyvinylidene fluoride membranes, proteins on membranes were incubated with a primary antibody overnight (4°C). A horseradish peroxidase-conjugated secondary antibody (1:2,000–5,000) was incubated for 1 h at room temperature. The enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ) was used for development. The immunoblot density was measured using the NIH ImageJ (Bethesda, MD). We normalized synaptic and extrasynaptic samples to total protein loading in Ponceau S stained membranes (Gustin et al., 2010; Eagleson et al., 2013; Bermejo et al., 2014). The phosphorylated tyrosine 416 (pY416) levels were normalized to the total Src protein levels. No reducing agents (such as dithiothreitol) and antioxidants were used for mGlu1a and mGlu5 receptor immunoblots.

IP₃ Assays

This assay was performed to measure cellular IP₃ levels in mPFC slices from 18 rats using a CUSABIO rat IP₃ enzyme-linked immunosorbent assay Kit (Wuhan, China) according to the manufacturer's instructions.

Antibodies and pharmacological agents

We used the following primary antibodies: a mouse antibody against mGlu1a (BD Biosciences, San Jose, CA) or transferrin receptors (TfR, ThermoFisher), or a rabbit antibody against mGlu5 (MilliporeSigma), pY416 (Cell Signaling, Danvers, MA), Src (Cell Signaling), or β -actin (MilliporeSigma). Pharmacological agents used in this study include D-amphetamine sulfate (MilliporeSigma) and (*RS*)-3,5-dihydroxyphenylglycine (DHPG) (Tocris, Minneapolis, MN). At the day of experiments, all drugs were freshly prepared.

Statistics

Data were tested and found to be normally distributed with equal variances. GraphPad Prism 6 was used to analyze data (GraphPad software, La Jolla, CA). One- or two-way analysis of variance (ANOVA) followed by *post doc* analysis (corrected for multiple comparisons) or a two-tailed unpaired Student's *t*-test was conducted. We considered a *P* value < 0.05 as a statistical significance.

Results

CPP

Rat CPP was evaluated by comparing the time spent in the drug-paired chamber between pre-tests versus CPP tests (both for 15 min). We found that repeated AMPH administration (2.5 mg/kg, i.p.; once daily for five alternate days) induced significant preference for the AMPH-paired chamber in CPP tests compared to pre-tests [two-way ANOVA: pre- and CPP-test ($F_{(1,36)} = 4.137$, $P = 0.049$), treatment ($F_{(1,36)} = 2.604$, $P = 0.115$), and interaction ($F_{(1,36)} = 5.661$, $P = 0.023$)]. *Post hoc* analyses revealed a significant increase in time spent in the drug-paired chamber in CPP tests over pre-tests in AMPH-treated rats (Fig. 1B). In contrast, treatment with saline alone did not induce preference for one test over the other. These data demonstrate the establishment of CPP in response to repeated AMPH administration.

Subcellular expression of mGlu1/5 receptors in rats showing AMPH-induced CPP

We used a biotinylation method on brain slices to assay the surface versus intracellular expression of mGlu1a, a long-form splice variant of mGlu1 receptors, and mGlu5 receptors in the mPFC. A predominant amount of mGlu1a and mGlu5 receptors is expressed as dimers (250–280 kDa), a major functional form of mGlu1/5 receptors in neurons *in vivo* (Shaffer et al., 2010). We thus focused on dimers of mGlu1a and mGlu5 receptors in their changes in expression in response to AMPH conditioning. At three days after the last conditioning injection, the surface fraction of mGlu1a in the mPFC showed a tendency of an increase in rats treated with AMPH, although it did not reach a statistically significant level as compared to saline controls (Fig. 2A, Student's *t*-test, $t = 1.292$, $P = 0.213$). The intracellular fraction of mGlu1a and total mGlu1a proteins showed little change in AMPH-treated rats (intracellular: $t = 0.114$, $P = 0.910$; total: $t = 1.502$, $P = 0.150$). As a result, the surface to intracellular (S:I) ratio of mGlu1a expression was not significantly altered (Fig. 2B). Unlike mGlu1a, mGlu5 receptors seemed to be sensitive to AMPH. As shown in Fig. 2C, AMPH conditioning produced a small but significant increase in the surface pool of mGlu5 receptors ($t = 3.090$, $P = 0.006$). In contrast, AMPH seemed to reduce mGlu5 expression in the intracellular pool (although $t = 1.838$, $P = 0.083$). Thus, the surface to intracellular ratio was increased in AMPH-treated rats relative to saline-treated rats (Fig. 2D, $t = 3.529$, $P = 0.002$). In addition to mGlu1a/5 receptors, we analyzed changes in a surface marker (TfR) and an intracellular marker (β -actin) as a surface and intracellular protein control, respectively. The biotinylation method that selectively crosslinks surface proteins was validated by the finding that TfRs were mainly shown in surface fractions whereas β -actin was lacking in surface fractions and was expressed in intracellular fractions (Fig. 3A and 3B). There were no significant changes in surface expression of TfRs (Fig. 3A, $t = 1.180$, $P = 0.253$) and intracellular expression of β -actin (Fig. 3B, $t = 0.115$, $P = 0.910$) in the mPFC of rats treated with AMPH compared to rats treated with saline. These results indicate that AMPH conditioning enhances mGlu5 receptor expression in the mPFC, which primarily occurs in the surface compartment of mPFC neurons.

Subsynaptic expression of mGlu1/5 receptors in rats after AMPH conditioning

We next wanted to determine whether AMPH conditioning alters expression of mGlu1a/5 receptors at synaptic versus extrasynaptic sites. Using a pre-validated subsynaptic fractionation method (Mao et al., 2013; Mao and Wang, 2018), we found that mGlu1a proteins in the mPFC were expressed at both synaptic and extrasynaptic sites with a higher level in synaptic than extrasynaptic fractions (Fig. 4A). Similarly, mGlu5 receptors were present in synaptic fractions more abundantly than that in extrasynaptic locations (Fig. 4B). Thus, in the mPFC, mGlu1a/5 receptors are concentrated at synaptic sites, while a significant amount of mGlu1a/5 also exists at extrasynaptic sites. In rats treated with AMPH conditioning, little change was seen in mGlu1a expression at synaptic sites (Fig. 4C, $t = 0.763$, $P = 0.463$). Notably, a significant increase in mGlu1a abundance was seen at extrasynaptic sites ($t = 2.436$, $P = 0.035$). The amount of mGlu5 proteins in the mPFC was elevated in synaptic fractions in AMPH-treated rats relative to saline-treated rats (Fig. 4D, $t = 3.107$, $P = 0.011$). Similarly, mGlu5 levels were enhanced in extrasynaptic fractions ($t = 2.560$, $P = 0.028$). These results indicate that AMPH conditioning increases mGlu1a expression at extrasynaptic sites and mGlu5 expression in both synaptic and extrasynaptic locations.

Effects of AMPH conditioning on mGlu1/5-mediated IP₃ signaling

Extrasynaptic expression of mGlu1a receptors and surface and synaptic/extrasynaptic expression of mGlu5 receptors were upregulated after AMPH conditioning (see above). We thus wanted to examine whether changes in these receptor expression patterns can translate to modifications of the receptor function. Group I receptors are known to activate the G_{αq}-coupled pathway to yield IP₃ (Niswender and Conn, 2010). The cytosolic IP₃ level was therefore measured as a readout of the best characterized signaling pathway of mGlu1/5 receptors. We measured IP₃ levels on mPFC slices from rats that were sacrificed 3 days after the final conditioning injection. Adding the mGlu1/5 agonist DHPG (50 μM, 20 s) to mPFC slices of rats conditioned with saline induced a typical increase in IP₃ levels (Fig. 5). In rats conditioned with AMPH, an increase in IP₃ levels was also induced by DHPG. This increase was noticeably greater in its degree than that seen in saline rats [two-way ANOVA: vehicle versus DHPG ($F_{(1,30)} = 49.04$, $P < 0.0001$), saline versus AMPH ($F_{(1,30)} = 4.590$, $P = 0.040$), and interaction ($F_{(1,30)} = 4.218$, $P = 0.048$)]. These data indicate that AMPH conditioning enhances mGlu1/5 function in mPFC neurons in terms of the agonist-stimulated IP₃ formation.

Effects of AMPH conditioning on mGlu1/5-mediated Src signaling

Group I mGlu receptors are linked to another signaling effector Src, a member of the Src family of non-receptor tyrosine kinases (Roskoski, 2005; Okada, 2012). The group I mGlu agonist DHPG and the mGlu5 agonist CHPG consistently elevated autophosphorylation of Src at a conserved activation site (Y416) in mouse cultured cortical neurons (Heidinger et al., 2002) and rat cultured hippocampal neurons (Takagi et al., 2012). Similarly, we observed that adding DHPG (100 μM, 15 min) to normal rat mPFC slices induced a significant increase in pY416 levels ($t = 3.690$, $P = 0.006$), while total Src protein levels remained stable (Fig. 6A). Thus, pharmacological stimulation of group I mGlu receptors activates the

Src pathway by triggering its autophosphorylation at Y416. We therefore tested the Y416 phosphorylation as an integrative measure of group I mGlu activity. In rats conditioned with saline, applying DHPG (50 μ M, 15 min) to mPFC slices elevated Src Y416 phosphorylation (Fig. 6B). In rat conditioned with AMPH, DHPG also induced an increase in Y416 phosphorylation in mPFC slices. Moreover, the degree of the upregulation of Y416 phosphorylation in AMPH-conditioned rats was greater than that seen in saline-conditioned rats [two-way ANOVA: vehicle versus DHPG ($F_{(1,18)} = 39.360$, $P < 0.0001$), saline versus AMPH ($F_{(1,18)} = 7.395$, $P = 0.014$), and interaction ($F_{(1,18)} = 4.823$, $P = 0.041$)]. Thus, AMPH conditioning upregulates group I mGlu activity in mPFC neurons in terms of the agonist-stimulated Src Y416 phosphorylation.

Discussion

This study focused on the mPFC and investigated adaptive changes in mGlu1/5 receptor expression in a CPP model in response to intermittent administration of a dopamine stimulant. We found that AMPH (2.5 mg/kg, i.p.; once daily on alternate days for 10 days) induced reliable CPP behavior. Remarkably, in rats showing CPP, mGlu5 receptor expression in mPFC neurons was elevated in surface membranes. The number of mGlu5 receptors at synaptic and extrasynaptic sites was also increased in AMPH-conditioned rats. While mGlu1a receptor expression in surface and intracellular compartments showed little change, mGlu1a distribution to extrasynaptic sites was elevated following AMPH conditioning. In addition, AMPH conditioning enhances the efficacy of the mGlu1/5 agonist in stimulating IP₃ production and Src autophosphorylation in the mPFC. These results indicate that AMPH CPP has a significant impact on mGlu1/5 receptors in their subcellular and subsynaptic expression and function in mPFC neurons.

The mPFC is a major dopamine responsive region in the limbic system. Its implications in the addictive properties of drugs of abuse have been well documented in recent studies using animal models of behavioral sensitization and self-administration (Van den Oever et al., 2010; Moorman et al., 2015). As to the CPP paradigm, while studies directly assessing the involvement of the mPFC in reward mechanisms are limited, available evidence indicates a pivotal role of the mPFC in the conditioned effect of various drugs (Tzschentke, 2000; Hsu and Packard, 2008; Peters et al., 2009). In fact, a number of studies have established the importance of the mPFC for expression and/or extinction of CPP induced by cocaine, methamphetamine, opiates, and other drugs (Tzschentke, 2000; Hsu and Packard, 2008; Peters et al., 2009). Regarding AMPH, prefrontal cortical norepinephrine depletion induced the absence of AMPH-induced CPP in mice (Ventura et al., 2003). AMPH conditioning induced c-fos expression (a marker for neuronal activation) in the mouse mPFC (Mead et al., 1999). In this study, significant changes in mGlu1/5 receptor expression at the subcellular and/or subsynaptic levels were observed in rats after AMPH conditioning. Thus, the mPFC is believed to be a sensitive site in the limbic system involved in molecular mechanisms underlying the rewarding properties of AMPH. Future studies need to investigate changes in mGlu1/5 receptor expression and function in the mPFC in response to other natural or pharmacological stimuli in a CPP model.

Changes in mGlu5 receptor expression in the mPFC in response to AMPH-induced CPP have not been documented until this study, to our knowledge. Several reports have described the response of mGlu5 receptors in mPFC neurons to other drugs in different drug treatment regimens. A single injection of AMPH induced a transient increase in mGlu5 proteins in the mPFC (Shaffer et al., 2010). Repeated cocaine administration (15 mg/kg, i.p.; once daily for 7 days) at a 3-week period of withdrawal had no effect on mGlu5 proteins in the rat prefrontal cortex (Swanson et al., 2001). Chronic administration of methamphetamine at a 14-day withdrawal did not alter mGlu5 levels in the mPFC (Herrold et al., 2013a). Cocaine self-administration also showed no effect on mGlu5 protein expression in the mPFC (Ben-Shahar et al., 2009). Of note, these studies basically analyzed expression of mGlu5 receptors in a total cellular homogenate preparation. Consistent with these reports, no significant change in total mGlu5 expression was observed in the mPFC following AMPH-induced CPP in this study. However, noticeably, we observed significant changes when mGlu5 expression was assayed at subcellular and subsynaptic levels. Moreover, these changes in expression were accompanied by alterations in the efficacy of the mGlu1/5 agonist-induced signaling activity. These results support that the mGlu5 receptor in the mPFC is a substrate of AMPH and may play a role in the rewarding effect of AMPH.

A large number of studies have reported the effectiveness of mGlu5 antagonism in inhibiting CPP. An mGlu5 antagonist (MTEP or MPEP) attenuated CPP induced by AMPH (Herzig et al., 2005), methamphetamine (Herrold et al., 2013b), cocaine (McGeehan and Olive, 2003; Brown et al., 2012; although Rutten et al., 2011), nicotine (Yarabas et al., 2010), or ethanol (Kotlinska et al., 2011). MPEP also reduced the conditioned effect of morphine (Popik and Wrobel, 2002; Aoki et al., 2004; Herzig and Schmidt, 2004). The effect of mGlu5 antagonists may be achieved in part by interacting with mGlu5 receptors in the mPFC in addition to the NAc (Martinez-Rivera et al., 2013). It is likely that AMPH conditioning induces long-lasting mGlu5 receptor plasticity in mPFC neurons, which as a critical element participates in the remodeling of glutamatergic corticostriatal synapses in the reward limbic system and results in enduring CPP behavior. In support of this notion, group I mGlu receptor activation contributes to the increased excitability of pyramidal neurons in the mPFC of cocaine-sensitized rats (Timmer and Steketee, 2013). Future studies will define the role of mGlu1/5 receptors in the subregions of the mPFC in the rewarding effect of AMPH.

Studies on the linkage of mPFC mGlu1 receptors to AMPH CPP are limited. The other drug such as cocaine after repeated or self administration had no effect on total mGlu1 receptor expression in the prefrontal cortex (Swanson et al., 2001; Ben-Shahar et al., 2009). Similarly, we did not observe a significant change in total mGlu1 receptor expression in the mPFC after AMPH conditioning. However, a significant increase in mGlu1 abundance was seen in the extrasynaptic location following AMPH conditioning. This seems to imply that mGlu1 receptors may also play a role in some aspects of the rewarding properties of AMPH. Indeed, the mGlu1 antagonist EMQMCM significantly attenuated ethanol CPP (Kotlinska et al., 2011), although the mGlu1 antagonist JNJ16259685 did not influence the maintenance of methamphetamine-induced CPP (Herrold et al., 2013b). Future studies will elucidate whether and how mGlu1 receptors in the mPFC participate in glutamate receptor plasticity related to the rewarding effect of AMPH.

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Abbreviations:

AMPH	amphetamine
ANOVA	analysis of variance
CPP	conditioned place preference
DHPG	(<i>RS</i>)-3,5-dihydroxyphenylglycine
IP₃	inositol-1,4,5-triphosphate
mGlu	metabotropic glutamate
MPEP	2-methyl-6-(phenylethynyl)pyridine
mPFC	medial prefrontal cortex
MTEP	3-((2-methyl-4-thiazolyl)ethynyl)pyridine
NAc	nucleus accumbens
TfR	transferrin receptor
VTa	ventral tegmental area

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- Amphetamine (AMPH) induced conditioned place preference (CPP) in rats.
- Expression of mGlu1/5 receptors was elevated in the medial prefrontal cortex (mPFC) of CPP rats.
- Efficacy of mGlu1/5 signaling was increased in the mPFC after AMPH conditioning.
- AMPH conditioning also augmented the mGlu1/5 agonist-stimulated Src kinase phosphorylation in mPFC neurons.

Figure 1. Repeated AMPH administration induces CPP in rats.

(A) Timeframe for an AMPH-induced CPP model. Following a 3-day pre-conditioning period (days 1–3), rats underwent a conditioning phase (days 4–13). Rats, after an AMPH injection (2.5 mg/kg, i.p.), were confined to one chamber. Same rats were treated with saline on alternate days and were confined to an opposite chamber. Three days (day 16) after the final conditioning injection, rats were tested for CPP and sacrificed for mPFC tissue collection. (B) Effects of AMPH on the time spent in the drug-paired chamber during pre- and post-conditioning tests. Note that AMPH significantly elevated the time spent in the paired chamber during a post-test (CPP test) relative to a pre-test. Data are presented as mean $\pm$ SEM ($n = 9$ in saline group, $n = 11$ in AMPH group). * $P < 0.05$ versus the pre-test.

Figure 2. Effects of AMPH conditioning on surface and intracellular expression of mGlu1a/5 receptors in the rat mPFC.

(**A** and **B**) Effects of AMPH conditioning on surface and intracellular (Intra) expression of mGlu1a receptors and on the surface to intracellular (S:I) ratio of mGlu1a receptors. (**C** and **D**) Effects of AMPH conditioning on surface and intracellular expression of mGlu5 receptors and on the S:I ratio of mGlu5 receptors. Note that AMPH increased surface mGlu5 expression and the S:I ratio of mGlu5 receptors. Representative immunoblots (IB) are shown left to the quantified data (A and C). Rats were conditioned with AMPH (2.5 mg/kg, i.p.; once daily on alternate days for 10 days) or saline and were sacrificed 3 days after the last conditioning. Data are presented as means $\pm$ SEM ($n = 9$ in saline group, $n = 11$ in AMPH group). * $P < 0.05$ versus saline.

Figure 3. Effects of AMPH conditioning on surface TfR and intracellular β -actin expression in the rat mPFC.

(A) Effects of AMPH conditioning on surface expression of TfRs. (B) Effects of AMPH conditioning on intracellular (Intra) expression of β -actin. Representative immunoblots (IB) are shown left to the quantified data. Rats were conditioned with AMPH (2.5 mg/kg, i.p.; once daily on alternate days for 10 days) or saline and were sacrificed 3 days after the last conditioning. Data are presented as means $\pm$ SEM ($n = 9$ in saline group, $n = 11$ in AMPH group).

Figure 4. Effects of AMPH conditioning on synaptic and extrasynaptic expression of mGlu1a/5 receptors in the rat mPFC.

(A) Distribution of mGlu1a receptors in synaptic (Syn) and extrasynaptic (Extrasyn) compartments. (B) Distribution of mGlu5 receptors in synaptic and extrasynaptic compartments. (C and D) Effects of AMPH conditioning on synaptic versus extrasynaptic expression of mGlu1a (C) and mGlu5 (D) receptors. Note that AMPH increased extrasynaptic expression of mGlu1a receptors and synaptic and extrasynaptic expression of mGlu5 receptors. Representative immunoblots (IB) are shown left to (A and B) or above (C and D) the quantified data. Rats were conditioned with AMPH (2.5 mg/kg, i.p.; once daily on alternate days for 10 days) or saline and were sacrificed 3 days after the last conditioning. Data are presented as means $\pm$ SEM ($n = 6$ in saline group, $n = 6$ in AMPH group). * $P < 0.05$ versus saline.

Figure 5. Effects of AMPH conditioning on DHPG-stimulated IP₃ production in the rat mPFC. Note that the degree of the DHPG-induced IP₃ formation was augmented in AMPH-treated rats compared to saline-treated rats. Rats were conditioned with AMPH (2.5 mg/kg, i.p.; once daily on alternate days for 10 days) or saline and were sacrificed 3 days after the last conditioning for preparing mPFC slices. DHPG (50 μ M) or vehicle was applied to slices for 20 s. Data are presented as means $\pm$ SEM ($n = 9$ in saline group, $n = 9$ in AMPH group). * $P < 0.05$ versus vehicle.

Figure 6. Effects of AMPH conditioning on DHPG-stimulated Src Y416 phosphorylation in the rat mPFC.

(A) Effects of DHPG on Src Y416 phosphorylation. (B), Effects of AMPH conditioning on the DHPG-stimulated Y416 phosphorylation. Note that DHPG increased Y416 phosphorylation to a greater extent in AMPH- than saline-treated rats. Representative immunoblots (IB) are shown left to the quantified data. Rats were conditioned with AMPH (2.5 mg/kg, i.p.; once daily on alternate days for 10 days) or saline and were sacrificed 3 days after the last conditioning for preparing mPFC slices. DHPG at 100 (A) or 50 (B) μ M was added to slices for 15 min prior to slice collections for Western blot. Data are presented as means $\pm$ SEM ($n = 6$ in saline group, $n = 6$ in AMPH group). * $P < 0.05$ versus vehicle.