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Rapid and sustained GluA1 S845 phosphorylation in synaptic and extrasynaptic locations in the rat forebrain following amphetamine administration

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Abstract

The α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is a major ionotropic glutamate receptor subtype in the mammalian brain. Like other glutamate receptors, the AMPA receptor is regulated by phosphorylation. By phosphorylating specific serine residues in AMPA receptor subunits (GluA1 and GluA2), various protein kinases regulate subcellular/subsynaptic expression and function of the receptor. In this study, we conducted a time course study to evaluate the temporal property of responses of phosphorylation at those sites to dopamine stimulation with the psychostimulant amphetamine in the adult rat striatum and medial prefrontal cortex (mPFC) *in vivo*. We focused on biochemically-enriched AMPA receptors from synaptic and extrasynaptic compartments. We found that acute injection of amphetamine induced a rapid and relatively sustained increase in GluA1 S845 phosphorylation at both synaptic and extrasynaptic sites in the striatum. Similar results were observed in the mPFC. In contrast to S845, amphetamine did not induce a significant change in GluA1 S831 phosphorylation in synaptic and extrasynaptic pools in the striatum and mPFC. GluA2 S880 phosphorylation in synaptic and extrasynaptic fractions in the two brain regions also remained stable in response to amphetamine. These results support S845 to be a principal site on AMPA receptors sensitive to acute stimulant exposure. Its phosphorylation levels are rapidly upregulated by amphetamine in the two defined subsynaptic microdomains (synaptic versus extrasynaptic locations) in striatal and cortical neurons.

Keywords

Striatum; basal ganglia; prefrontal cortex; dopamine; glutamate; GluR; excitatory amino acid; addiction

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1. Introduction

Protein phosphorylation is an important posttranslational process modifying protein expression and function in mammalian cells. Glutamate receptors in the central nervous system are a typical example for such phosphorylation modification. All ionotropic and metabotropic glutamate receptors are subject to the regulation by phosphorylation (Wang et al., 2006; Mao et al., 2011). Among different glutamate receptor subtypes, the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor draws particular attention as it typically carries out the fast synaptic transmission in excitatory synapses. This receptor, like other ionotropic glutamate receptors, is assembled into a functional heterotetrameric complex from four subunits (GluA1-4 or formerly GluR1-4) (Hollmann and Heinemann, 1994). These subunits can therefore be regulated by phosphorylation. Two common phosphorylation sites were identified in intracellular C-terminal tails of GluA1 subunits: serine 831 (S831) and serine 845 (S845) (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997; Serulle et al., 2007). Two sites are phosphorylated by protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (for S831) and by protein kinase A (PKA) and cyclic guanosine monophosphate-dependent protein kinase II (for S845). The phosphorylation level at these sites is directly linked to surface trafficking and synaptic delivery of GluA1 and physiological properties of GluA1/AMPA receptors (Mao et al., 2011). Additionally, serine 880 (S880) in the intracellular C-terminus of GluA2 is phosphorylated by PKC (Matsuda et al., 1999; Chung et al., 2000; McDonald et al., 2001). This site phosphorylation regulates the interaction of GluA2 with PDZ (postsynaptic density 95/Discs large/zona occludens 1) domain-containing proteins and thereby promotes the internalization of AMPA receptors and the expression of long-term depression (LTD) (Chung et al., 2000; Xia et al., 2000; Kim et al., 2001).

AMPA receptors are highly expressed in dopamine-innervated brain regions, including the striatum and the medial prefrontal cortex (mPFC) (Bernard et al., 1997; Kondo et al., 2000; Hu et al., 2004). In the striatum, GluA1/A2 receptors are the most common composition subtypes (Reimers et al., 2011) and are expressed in all medium spiny projection neurons and most interneurons of the striatum (Bernard et al., 1997). These receptors in their phosphorylation levels have been demonstrated to be sensitive to dopamine stimulation according to a number of early studies (Wang et al., 2006). Activating dopamine D1 receptors with selective agonists elevated GluA1 phosphorylation at S845, although not S831, in striatal or cortical neurons (Price et al., 1999; Snyder et al., 2000; Chao et al., 2002b; Swayze et al., 2004). Dopamine stimulation with the psychostimulants, such as cocaine and amphetamine (AMPH), following a systemic or a local injection into the nucleus accumbens (NAc) increased S845 phosphorylation in the striatum (Snyder et al., 2000; Li et al., 2011). These data indicate that S845 is a sensitive site on GluA1 subjected to the upregulation by phosphorylation in response to stimulant exposure.

AMPA receptors are membrane-bound proteins and are mostly postsynaptic. At the subsynaptic level, these receptors are concentrated at synaptic sites, while some extrasynaptic AMPA receptors are also present (Bernard et al., 1997; Ferrario et al., 2011b). Of note, most early studies have prepared tissue lysates to assay S831 and S845 phosphorylation in response to stimulants. Recently, we used enriched synaptic versus extrasynaptic membranes to probe S831 and S845 phosphorylation in a dose-dependent study with AMPH (Mao et al., 2013a). In this study, we expanded on this work by carrying out a time course experiment to profile the temporal property of changes in GluA1 S831 and S845 phosphorylation in the striatum and mPFC following acute injection of AMPH. In addition, GluA2 S880 phosphorylation was evaluated given the fact that little is known on this site phosphorylation in terms of its sensitivity to acute administration of stimulants.

2. Experimental procedures

2.1. Animals

Adult male Wistar rats (200–446 g; Charles River, New York, NY) were housed in a controlled environment at a constant temperature of 23°C and humidity of 50 ± 10% with food and water available *ad libitum*. The animal room was on a 12-h/12-h light/dark cycle. Rats were allowed 5–7 days of habituation to the animal colony. All animal use and procedures were in strict accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Systemic drug injection

A single dose of d-amphetamine sulfate (Sigma-Aldrich, St. Louis, MO) was given to rats via an intraperitoneal (i.p.) injection. The dose of 5 mg/kg was calculated as the salt and was chosen because the drug at this dose caused typical motor stimulation (Wang and McGinty, 1995). To carry out a time course study, rats were sacrificed at different time points (10, 15, 30, or 60 min) after AMPH injection for the subsequent neurochemical analysis. Age-matched rats were given an acute injection of saline (1 ml/kg, i.p.) and were sacrificed immediately after injection to serve as controls.

2.3. Fractionation of synaptic and extrasynaptic membranes

Procedures for enriching synaptic and extrasynaptic membranes have been outlined in our recent report (Mao et al., 2013b). These procedures were developed based on the insolubility of the postsynaptic density and synaptic junctions in Triton X-100 as described previously (Davies et al., 2007; 2008; Goebel-Goody et al., 2009; Ferrario et al., 2011b). Briefly, rats were decapitated immediately after rats were anesthetized with equithesin (5 ml/kg, i.p.). Brains were removed and the striatum and mPFC were dissected on ice and frozen immediately. The striatum tissue contains the caudate putamen and nucleus accumbens and the mPFC tissue includes the anterior cingulate, prelimbic, and infralimbic cortex (Mao et al., 2013a). The subsequent fractionation procedures were performed at 4°C. The brain tissue was homogenized in isotonic sucrose homogenization buffer (0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM ethylenediaminetetraacetic acid) containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Thermo Scientific, Rochester, NY) and was then centrifuged (760 g, 10 min). The supernatant was centrifuged (10,000 g, 30 min). The pellet 2 (P2) containing crude synaptosomal plasma membranes was washed and centrifuged at 10,000 g (30 min). The washed P2 was resuspended in the sucrose homogenization buffer with 0.5% Triton X-100 using a motorized pestle. The suspension was incubated (20 min) and centrifuged (20 min) at 32,000 g to yield the Triton X-100-insoluble pellet enriched with synaptic membranes and the Triton X-100-soluble supernatant enriched with extrasynaptic membranes. Synaptic and extrasynaptic membranes were solubilized in sucrose-Triton buffer containing 1% sodium dodecyl sulfate (SDS), a protease inhibitor cocktail (Thermo Scientific), and a phosphatase inhibitor cocktail (Thermo Scientific). Protein concentrations were determined. Samples were stored at –80°C until use.

2.4. Western blot analysis

SDS NuPAGE Novex 4–12% gels (Invitrogen, Carlsbad, CA) were used to separate proteins as described previously (Mao et al., 2013a). Separated proteins on gels were then transferred to the polyvinylidene fluoride membrane (Invitrogen). The membrane was blocked in a blocking buffer (5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20) for 1 h, and was washed and incubated in the blocking buffer containing a primary antibody (1:1,000) overnight at 4°C. This was followed by 1 h incubation in a horseradish peroxidase-

linked secondary antibody against the rabbit (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:5,000. Immunoblots were developed with the enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ). MagicMark XP Western protein standards (Invitrogen) were used for protein size determination. Immunoblots were measured using NIH gel analysis software. Primary antibodies used in this study include rabbit polyclonal antibodies against GluA1 with phosphorylated S831 (pS831) (PhosphoSolutions, Aurora, CO), GluA1 pS845 (PhosphoSolutions), GluA2 pS880 (PhosphoSolutions), GluA1 (Millipore, Billerica, MA), or GluA2 (PhosphoSolutions).

2.5. Statistics

The results are presented as means $\pm$ SEM, and were evaluated using a one-way analysis of variance followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means. Probability levels of < 0.05 were considered statistically significant.

3. Results

3.1. AMPH did not alter GluA1 S831 phosphorylation

To examine the effect of AMPH on GluA1 S831 phosphorylation in a time course study, we subjected rats to a single dose of AMPH (5 mg/kg, i.p.). We then sacrificed rats at different time points (10, 15, 30, or 60 min) after drug injection. Striatal tissue containing both the caudate putamen and NAc was removed and used for preparing synaptic and extrasynaptic membranes. In the following Western blot analysis in which synaptic membranes were first tested, we did not observe a significant change in pS831 protein levels at any of the four time points surveyed (Fig. 1A). Similarly, no significant changes in pS831 signals in extrasynaptic fractions were seen in rats treated with AMPH relative to saline (Fig. 1B). The amount of GluA1 proteins in synaptic and extrasynaptic pools remained stable at all different survival times after AMPH. These data support that GluA1 S831 phosphorylation in synaptic and extrasynaptic locations is not sensitive to AMPH in striatal neurons.

Like the striatum, the mPFC is a key site in the mesocorticolimbic reward circuit and is involved in processing stimulant effects (Steketee, 2003; Van den Oever et al., 2010). We therefore targeted the mPFC in our next time course study to investigate the impact of AMPH on GluA1 S831 phosphorylation in this region. Similar to what we have seen in the striatum, S831 phosphorylation in the mPFC was not changed in AMPH-treated rats during the entire course studied. As shown in Fig. 2, mPFC pS831 protein levels showed no significant changes in synaptic (Fig. 2A) and extrasynaptic (Fig. 2B) membranes in AMPH-treated rats compared to saline-treated rats at 10, 15, 30, or 60 min after drug injection. GluA1 protein levels in AMPH-treated rats did not differ from that seen in saline-treated rats. Thus, the mPFC is another site where S831 phosphorylation is not sensitive to AMPH.

3.2. AMPH induced a rapid and prolonged change in GluA1 S845 phosphorylation

We next analyzed S845 phosphorylation in the same samples from the striatum. In contrast to S831, S845 phosphorylation was altered by AMPH. In synaptic membranes, as shown in Fig. 3A, a significant increase in pS845 protein levels was seen at an early time point (10 min after AMPH injection). This increase persisted at the rest of time points. The positive response of S845 phosphorylation to AMPH was not confined to synaptic sites. In extrasynaptic membranes, similar increases were seen in AMPH-treated animals compared to saline-treated rats (Fig. 3B). These data demonstrate that AMPH can induce a rapid and relatively sustained increase in GluA1 S845 phosphorylation, and phosphorylation at S845 in the two subsynaptic compartments seems to be equally sensitive to AMPH. Since total GluA1 protein levels in the two fractions remained stable (Fig. 3A and 3B), increased pS845

protein levels in these fractions are reasoned to result from accelerated phosphorylation reactions at this specific amino acid site.

In the mPFC, enhanced GluA1 S845 phosphorylation was also observed in synaptic and extrasynaptic pools. As shown in Fig. 4, rapid increases in pS845 protein levels in the two fractions occurred 10 min after AMPH administration. The increase remained at 15 and 30 min. A tendency for increased pS845 levels to decline was seen at 60 min, even though the increase in pS845 at this time point was still significantly different in AMPH-treated rats from saline-treated rats. These results indicate the ability of AMPH to stimulate S845 phosphorylation in mPFC neurons in a time-sensitive fashion.

Effects of AMPH on GluA2 S880 phosphorylation—GluA2 S880 phosphorylation was next investigated in the two forebrain regions. In the striatum, AMPH had no effect on synaptic S880 phosphorylation as pS880 protein levels in synaptic membranes of AMPH-treated rats did not differ from those in saline-treated rats at all four time points (Fig. 5A). Similar results were observed in extrasynaptic membranes (Fig. 5B). GluA2 protein levels in synaptic and extrasynaptic membranes were not altered following AMPH administration. Thus, S880 phosphorylation of GluA2 is not a sensitive event subjected to the regulation by AMPH in striatal neurons.

In the mPFC, S880 phosphorylation exhibited a minimal change in synaptic fractions in AMPH-treated rats relative to saline-treated rats (Fig. 6A). GluA2 pS880 protein levels in extrasynaptic fractions were not different between the two groups of animals (Fig. 6B). Total GluA2 levels in synaptic and extrasynaptic membranes were not significantly altered in AMPH- versus saline-treated animals. Thus, like the striatum, the mPFC contains GluA2 with S880 phosphorylation less sensitive to AMPH.

4. Discussion

This study was aimed to investigate the temporal property of AMPH effects on AMPA receptor phosphorylation in specific subsynaptic microdomains. Three common phosphorylation sites on AMPA receptor subunits were chosen to be profiled. In the two dopamine responsive forebrain regions, i.e., the striatum and mPFC, acute injection of AMPH induced a rapid and persistent increase in GluA1 phosphorylation at S845 in both synaptic and extrasynaptic locations. In contrast, AMPH had no effect on GluA1 S831 and GluA2 S880 phosphorylation in synaptic and extrasynaptic compartments of striatal and mPFC neurons. Synaptic and extrasynaptic levels of GluA1 and GluA2 in the two brain areas were not altered by AMPH. These results support S845 to be a selective phosphorylation site of AMPA receptors which is subjected to the dynamic regulation by AMPH in the two subsynaptic locations.

A number of early studies have documented the effect of dopamine stimulation on AMPA receptor phosphorylation (Wang et al., 2006). Pharmacological activation of dopamine D1 receptors with the selective agonist elevated GluA1 S845 phosphorylation, while the agonist did not alter S831 phosphorylation, in striatal or cortical neurons (Price et al., 1999; Snyder et al., 2000; Chao et al., 2002b; Swayze et al., 2004). Similarly, acute systemic injection of cocaine and methamphetamine or intra-NAc injection of AMPH increased S845 but not S831 phosphorylation in the striatum (Snyder et al., 2000; Li et al., 2011). Chronic cocaine administration caused behavioral sensitization and at the same time enhanced pS845 but not pS831 levels in the NAc (Schierberl et al., 2011). Cocaine self-administration was also effective in stimulating S845 phosphorylation in the NAc (Ferrario et al., 2011a) and S845 but not S831 phosphorylation in the ventral tegmental area (VTA) (Choi et al., 2011). These results indicate that S845 is a highly sensitive site while S831 is relatively less sensitive to

the dopamine regulation. A recent study using enriched synaptic and extrasynaptic samples as opposed to early studies usually using homogenates or synaptosomes also supports this view (Mao et al., 2013a). S845 but not S831 phosphorylation in the synaptic pool of rat striatal neurons was dose-dependently enhanced by AMPH (Mao et al., 2013a). In this study, we expanded the experiment to a time course analysis. We found that S845 phosphorylation in either synaptic or extrasynaptic locations can be rapidly induced by AMPH. The stimulated S845 phosphorylation largely remained up to 60 min after drug injection. In contrast, S831 phosphorylation in either pool was not altered throughout the time course. Thus, in the range of our time course assays, S845 remains as a major phosphorylation site on AMPA receptors regulated by AMPH. Behaviorally, acute AMPH is known to induce a time-dependent increase in rat motor activity. At a dose of 5 or 5.53 mg/kg (i.p.), AMPH induced a rapid motor response which remained at 1 h and gradually declined 2–3 h after drug injection (Wang et al., 1994a; 1994b; Wang and McGinty, 1995). Apparently, the AMPH-stimulated S845 phosphorylation temporally parallels with the motor response to the drug.

The intracellular signaling pathway linking dopamine receptors to AMPA receptor phosphorylation has been dissected. As a specific site known to be phosphorylated by PKA (Roche et al., 1996), the D1/cAMP/PKA pathway mediated the dopamine-stimulated S845 phosphorylation in the striatum (Snyder et al., 2000; Chao et al., 2002b; Mangiavacchi et al., 2004). With regard to S845 phosphorylation and receptor function, the phosphorylation at this site seems to accelerate surface expression of GluA1 (Chao et al., 2002a; Mangiavacchi et al., 2004; Man et al., 2007; Sun et al., 2008) probably in a region close to extrasynaptic sites (Sun et al., 2005; Oh et al., 2006; Gao et al., 2006). This may facilitate insertion of GluA1/AMPA receptors to synapses (Esteban et al., 2003; Sun et al., 2005) and potentiate AMPA receptor-mediated currents (Roche et al., 1996). In our study, we have not observed an increase in the amount of GluA1 in synaptic fractions as a result of increased S845 phosphorylation in response to acute injection of AMPH. However, there might be a subtle increase in GluA1 in a confined synaptic microdomain. This highly specific pool of affected GluA1 might not be effectively enriched by our fractionation method for detection. Of note, chronic drug exposure induces an increase in S845 phosphorylation accompanied by an increase in GluA1 expression. For example, an increased amount of surface and extrasynaptic pools of pS845 was seen in the rat NAc in response to self-administration of cocaine, which was associated with increased surface expression of GluA1 (Ferrario et al., 2011a). Moreover, cocaine self-administration elevated pS845 and GluA1 in the rat VTA, and overexpression of PKA-resistant GluA1 (S845A) in this region reduced self-administration of cocaine (Choi et al., 2011).

GluA2 S880 phosphorylation modulates synaptic plasticity in the form of LTD. In this stepwise process, PKC phosphorylates GluA2 S880, which in turn regulates the interaction of GluA2 with PDZ domain-containing proteins such as GRIP/ABP (glutamate receptor-interacting protein/AMPA receptor-binding protein) and PICK1 (protein interacting with C kinase 1) (Matsuda et al., 1999; Chung et al., 2000). In cerebellar and hippocampal neurons, this led to the internalization trafficking of AMPA receptors and the expression of LTD (Chung et al., 2000; Xia et al., 2000; Kim et al., 2001; Seidenman et al., 2003; Steinberg et al., 2006). In our study, we did not observe a significant change in striatal and cortical S880 phosphorylation after acute injection of AMPH. However, S880 phosphorylation was increased in the NAc shell of rats showing cocaine priming-induced reinstatement of cocaine seeking (Famous et al., 2008). This indicates that trafficking of GluA2/AMPA receptors in NAc neurons may be modified as a result of altered S880 phosphorylation, which may contribute to cocaine seeking. Apparently, more studies need to be carried out in the future to characterize adaptive changes in S880 phosphorylation and to link these changes to excitatory synaptic plasticity and stimulant addiction.

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Highlights

Amphetamine increases GluA1 S845 phosphorylation in the rat striatum and prefrontal cortex.

This increase was sustained throughout the time course surveyed (10–60 min after drug injection)

Amphetamine did not alter GluA1 S831 and GluA2 S880 phosphorylation in the striatum and cortex.

Figure 1. Effects of AMPH on GluA1 S831 phosphorylation in the rat striatum

(A) Effects of AMPH on S831 phosphorylation in synaptic fractions. (B) Effects of AMPH on S831 phosphorylation in extrasynaptic fractions. Representative immunoblots are shown left to the quantified data. Rats received a single dose of AMPH (5 mg/kg, i.p.) and were sacrificed at different time points (10, 15, 30, or 60 min) after drug injection. Data are presented as means $\pm$ SEM (n = 5 per group).

Figure 2. Effects of AMPH on GluA1 S831 phosphorylation in the rat mPFC

(**A**) Effects of AMPH on S831 phosphorylation in synaptic fractions. (**B**) Effects of AMPH on S831 phosphorylation in extrasynaptic fractions. Data are presented as means $\pm$ SEM ($n = 5$ per group).

Figure 3. Effects of AMPH on GluA1 S845 phosphorylation in the rat striatum

(A) Effects of AMPH on S845 phosphorylation in synaptic fractions. (B) Effects of AMPH on S845 phosphorylation in extrasynaptic fractions. Note that AMPH produced rapid and prolonged increases in pS845 proteins in both synaptic and extrasynaptic fractions. Representative immunoblots are shown left to the quantified data. Rats received a single dose of AMPH (5 mg/kg, i.p.) and were sacrificed at different time points (10, 15, 30, or 60 min) after drug injection. Data are presented as means $\pm$ SEM ($n = 5$ per group). * $P < 0.05$ and ** $P < 0.01$ versus saline.

Figure 4. Effects of AMPH on GluA1 S845 phosphorylation in the rat mPFC

(**A**) Effects of AMPH on S845 phosphorylation in synaptic fractions. (**B**) Effects of AMPH on S845 phosphorylation in extrasynaptic fractions. Data are presented as means $\pm$ SEM ($n = 5$ per group). * $P < 0.05$ and ** $P < 0.01$ versus saline.

Figure 5. Effects of AMPH on GluA2 S880 phosphorylation in the rat striatum

(A) Effects of AMPH on S880 phosphorylation in synaptic fractions. (B) Effects of AMPH on S880 phosphorylation in extrasynaptic fractions. Representative immunoblots are shown left to the quantified data. Rats received a single dose of AMPH (5 mg/kg, i.p.) and were sacrificed at different time points (10, 15, 30, or 60 min) after drug injection. Data are presented as means $\pm$ SEM (n = 5 per group).

Figure 6. Effects of AMPH on GluA2 S880 phosphorylation in the rat mPFC

(**A**) Effects of AMPH on S880 phosphorylation in synaptic fractions. (**B**) Effects of AMPH on S880 phosphorylation in extrasynaptic fractions. Data are presented as means $\pm$ SEM ($n = 5$ per group).