European Journal of Pharmacology 715 (2013) 164–171

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

# Regulation of phosphorylation of synaptic and extrasynaptic GluA1 AMPA receptors in the rat forebrain by amphetamine

Li-Min Mao [a,b], Jesus A. Diaz [b], Eugene E. Fibuch [b], John Q. Wang [a,b,*]

[a] Department of Basic Medical Science, School of Medicine, University of Missouri—Kansas City, Kansas City, MO 64108, USA
[b] Department of Anesthesiology, School of Medicine, University of Missouri—Kansas City, Kansas City, MO 64108, USA

ARTICLE INFO

*Article history:*
Received 1 February 2013
Received in revised form
6 May 2013
Accepted 20 May 2013
Available online 4 June 2013

*Keywords:*
Striatum
Basal ganglia
Striatum
Prefrontal cortex
Dopamine
Glutamate
GluR1
Excitatory amino acid
Addiction

ABSTRACT

The AMPA receptor is regulated by phosphorylation. Two major phosphorylation sites (S831 and S845) are located in the intracellular C-terminal tail of GluA1 subunits. The phosphorylation on these sites controls receptor expression and function and is subject to the regulation by psychostimulants. In this study, we further characterized the regulation of S831 and S845 phosphorylation by amphetamine (AMPH) in the adult rat striatum and medial prefrontal cortex (mPFC) *in vivo*. We focused on the specific fraction of GluA1/AMPA receptors enriched from synaptic and extrasynaptic membranes, using a pre-validated biochemical fractionation procedure. We found that acute AMPH administration elevated GluA1 S845 phosphorylation in the defined synaptic membrane from the striatum in a dose-dependent manner. AMPH also induced a comparable increase in S845 phosphorylation in the extrasynaptic fraction of striatal GluA1. Similar increases in S845 phosphorylation in both synaptic and extrasynaptic pools were observed in the mPFC. In contrast, S831 phosphorylation was not altered in synaptic and extrasynaptic GluA1 in striatal neurons and synaptic GluA1 in mPFC neurons in response to AMPH, although a moderate increase in S831 phosphorylation was seen in extrasynaptic GluA1 in the mPFC after an AMPH injection at a high dose. Total synaptic and extrasynaptic GluA1 expression remained stable in the two regions after AMPH administration. Our data demonstrate the differential sensitivity of S845 and S831 phosphorylation to dopamine stimulation. S845 is a primary site where phosphorylation of GluA1 is upregulated by AMPH in striatal and mPFC neurons at both synaptic and extrasynaptic compartments.



## 1. Introduction

The ionotropic AMPA receptor is broadly expressed in the mammalian brain. These receptors typically carry out function in a heterotetrameric complex assembled from four subunits (GluA1—4 or formerly GluR1—4) (Hollmann and Heinemann, 1994). Like other glutamate receptors, AMPA receptors are regulated by posttranslational phosphorylation (Wang et al., 2006; Mao et al., 2011). GluA1 is of particular interest due to its relatively long intracellular C-terminal tails and well-characterized phosphorylation by multiple protein kinases. Two serine residues (S831 and S845) in GluA1 C-termini are the major phosphorylation sites (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997; Serulle et al., 2007). The former (S831) is phosphorylated by protein kinase C (PKC) and $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII), whereas the latter (S845) by protein kinase A (PKA) and cGMP-dependent protein kinase II (cGKII). Altered phosphorylation levels at these sites modulate neurochemical and physiological properties of GluA1/AMPA receptors (Mao et al., 2011).

AMPA receptors are enriched in the striatum (Bernard et al., 1997; Kondo et al., 2000; Hu et al., 2004). In this region, GluA1/A2 receptors are the most common composition subtypes (Reimers et al., 2011) and are seen in all medium spiny projection neurons and most interneurons (Bernard et al., 1997). At the subsynaptic level, transmembrane AMPA receptors are concentrated on the postsynaptic membrane of asymmetric (excitatory) synapses, although extrasynaptic AMPA receptors are also present (Bernard et al., 1997; Ferrario et al., 2011b). The abundant distribution of AMPA receptors in striatal neurons supports their roles in experience-dependent synaptic and behavioral plasticity in response to stimulant exposure (Wolf and Ferrario, 2010).

Phosphorylation of AMPA receptors is a highly regulated event (Wang et al., 2006). The direct $D_1$ dopamine receptor agonist SKF81297 or SKF38393 enhanced GluA1 phosphorylation at the PKA site (S845) although not at the PKC/CaMKII site (S831) in striatal or prefrontal cortical neurons (Price et al., 1999; Snyder

* Corresponding author at: Department of Basic Medical Science, University of Missouri—Kansas City, School of Medicine, 2411 Holmes Street, Kansas City, MO 64108, USA.
*E-mail address:* wangjq@umkc.edu (J.Q. Wang).

0014-2999/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejphar.2013.05.027

et al., 2000; Chao et al., 2002b; Swayze et al., 2004). This enhancement was associated with an increase in surface-expressed AMPA receptors and AMPA receptor-mediated currents. Similarly, dopamine stimulation with an acute systemic injection of the psychostimulants (cocaine and methamphetamine) or a local injection of amphetamine (AMPH) into the nucleus accumbens (NAc) increased S845, but not S831, phosphorylation in the striatum *in vivo* (Snyder et al., 2000; Li et al., 2011). These data consistently identify S845 as a primary phosphorylation site sensitive to stimulant exposure. However, to date, little is known about the two specific pools of GluA1, i.e., GluA1 in synaptic versus extrasynaptic locations, in their S845 and S831 phosphorylation in response to stimulants.

We therefore conducted this study to determine the sensitivity of GluA1 enriched from distinct synaptic and extrasynaptic compartments to AMPH in terms of S831 and S845 phosphorylation. Using a pre-validated subsynaptic fractionation method, we isolated synaptic and extrasynaptic GluA1 proteins from the striatum and medial prefrontal cortex (mPFC) of adult rat brains. Changes in protein levels of phosphorylated GluA1 (pGluA1) at S831 (pS831) or S845 (pS845) were analyzed in the two regions following an acute injection of AMPH.

## 2. Materials and methods

### 2.1. Animals

Adult male Wistar rats weighing 275—350 g (Charles River, New York, NY) were individually 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

Rats were treated with an intraperitoneal (i.p.) injection of d-amphetamine sulfate (Sigma-Aldrich, St. Louis, MO). AMPH was injected at three doses (0.5, 5, and 10 mg/kg). These doses were calculated as the salt and were chosen to include a dose of 5 mg/kg, an effective dose that caused typical motor stimulation and a marked increase in immediate early gene and opioid peptide gene expression in the rat striatum (Wang and McGinty, 1995). Age-matched rats received an acute injection of saline (1 ml/kg, i.p.) and served as controls. Rats were sacrificed 15 min after AMPH injection for the subsequent neurochemical analysis. We selected the time point of 15 min based on the finding that acute cocaine injection at 20 mg/kg increased GluA1-S845 phosphorylation in the mouse striatum at this time point (Snyder et al., 2000).

### 2.3. Fractionation of synaptic and extrasynaptic membranes

Enrichment of synaptic and extrasynaptic membranes was performed according to a method described previously (Mao et al., 2013). The method was developed based on the insolubility of the postsynaptic density and synaptic junctions in Triton X-100 (Davies et al., 2007, 2008; Goebel-Goody et al., 2009; Ferrario et al., 2011b). Briefly, rats were anesthetized with equithesin (5 ml/kg, i.p.) and decapitated. Brains were quickly removed and the striatum, including the dorsal caudate putamen and ventral nucleus accumbens, and mPFC, including the anterior cingulate, prelimbic, and infralimbic cortex, were dissected. These tissues were homogenized on ice in isotonic sucrose homogenization buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, and a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Thermo Scientific, Rochester, NY) in a glass grinding vessel with a motor driven Teflon pestle (clearance = 0.125 mm) at 700 rpm (8 strokes). The homogenate was centrifuged at 760*g* for 10 min at 4 °C to generate the supernatant 1 (S1) and the pellet 1 (P1). P1 was resuspended, homogenized, and centrifuged at 760*g* for 10 min at 4 °C to generate the S1′ and P1′ fractions. The S1′ fraction was added to the S1 fraction. S1 was then centrifuged at 10,000*g* for 30 min at 4 °C to generate the supernatant 2 (S2) containing cytosol proteins and the pellet 2 (P2) containing crude synaptosomal plasma membranes. P2 was washed once and centrifuged at 10,000*g* for 30 min at 4 °C. A large portion of washed P2 was resuspended in the sucrose homogenization buffer containing Triton X-100 (0.5%, v/v) using a motorized pestle. The suspension was incubated (20 min) at 4 °C, 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. The extrasynaptic fraction was concentrated by acetone precipitation (−20° overnight and then centrifugation at 3000*g*). The concentrated extrasynaptic pellet and the synaptic fraction were solubilized in sucrose—Triton buffer containing 1% 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 in Western blot.

### 2.4. Western blot analysis

As described previously (Mao et al., 2013), the equal amount of protein was separated on SDS NuPAGE Novex 4—12% gels (Invitrogen, Carsbad, CA). Proteins were transferred to the polyvinylidene fluoride membrane (Invitrogen) and blocked in a blocking buffer (5% nonfat dry milk in phosphate-buffered saline and 0.1% Tween 20) for 1 h. The blots were washed and incubated in the blocking buffer containing a primary antibody usually at 1:1000 overnight at 4 °C. This was followed by 1 h incubation in a horseradish peroxidase-linked secondary antibody against rabbit (Santa Cruz) at 1:5000. Immunoblots were developed with the enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Kaleidoscope-prestained standards (Bio-Rad, Hercules, CA) and 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 pGluA1-S831 (PhosphoSolutions, Aurora, CO), pGluA1-S845 (PhosphoSolutions), or C-terminus of GluA1 (Millipore).

### 2.5. Statistics

The results are presented as means ± S.E.M., and were evaluated using a one-way analysis of variance followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means or two-tailed unpaired Student's *t*-test. Probability levels of $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Subsynaptic distribution of pGluA1 and GluA1 in the striatum

The efficiency of our fractionation method in enriching synaptic and extrasynaptic proteins has been demonstrated in details in our early report (Mao et al., 2013). In this study, this same method was used again to define the distribution pattern of total and phosphorylated GluA1 proteins in synaptic versus extrasynaptic compartments