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The role of extracellular signal-regulated kinases (ERK) in the regulation of mGlu5 receptors in neurons

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Abstract

The metabotropic glutamate (mGlu) receptor 5 is a G protein-coupled receptor and is densely expressed in the mammalian brain. Like other glutamate receptors, mGlu5 receptors are tightly regulated by posttranslational modifications such as phosphorylation, although underlying mechanisms are incompletely investigated. In this study, we investigated the role of a prime kinase, extracellular signal-regulated kinase 1 (ERK1), in the phosphorylation and regulation of mGlu5 receptors *in vitro* and in striatal neurons. We found that recombinant ERK1 proteins directly bound to the C-terminal (CT) tail of mGlu5 receptors *in vitro*. Endogenous ERK1 also interacted with mGlu5 receptor proteins in adult rat striatal neurons *in vivo*. The kinase showed the ability to phosphorylate mGlu5 receptors. A serine residue in the distal region of mGlu5 CT was found to be a primary phosphorylation site sensitive to ERK1. In functional studies, we found that pharmacological inhibition of ERK with an inhibitor U0126 reduced the efficacy of mGlu5 receptors in stimulating production of cytoplasmic inositol-1,4,5-triphosphate, a major downstream conventional signaling event, in striatal neurons under normal conditions. These results identify mGlu5 as a new biochemical substrate of ERK1. The kinase can interact with and phosphorylate an intracellular domain of mGlu5 receptors in striatal neurons and thereby control its signaling efficacy.

Keywords

MAPK; ERK; mGlu5; IP₃; G protein-coupled receptor, striatum; DHPG; U0126

Introduction

Group I metabotropic glutamate (mGlu) receptors (mGlu1 and mGlu5) are widely distributed in mammalian brains. In the striatum, in particular, mGlu5 receptors are densely

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Conflict of interest

The authors declare that they have no conflict of interest.

expressed (Shigemoto et al., 1993; Testa et al., 1994; 1995; Tallaksen-Greene et al., 1998). This receptor is noticeably expressed peri- and post-synaptically (Lujan et al., 1996; Kuwajima et al., 2004) and is linked to synaptic transmission and plasticity and to the pathogenesis of various cognitive and psychiatric disorders (Niswender and Conn, 2010; Traynelis et al., 2010; Nicoletti et al., 2011). At the post-receptor level, mGlu5 receptors are coupled to phospholipase C β 1 through G α q proteins. Activation of mGlu5 receptors thus elevates phosphoinositide hydrolysis, yielding diacylglycerol and inositol-1,4,5-triphosphate (IP₃). The latter triggers intracellular Ca²⁺ release and thereby regulates a variety of Ca²⁺-sensitive downstream effectors. As a synaptic receptor, mGlu5 receptors are also subjected to the regulation by basal or changing synaptic input. One of the regulation mechanisms is carried out by protein kinases. That is, an individual submembranous protein kinase can directly interact with an intracellular domain of mGlu5 to phosphorylate specific amino acids, which thereby robustly modulates signaling efficacy of modified mGlu5 receptors (Enz, 2012; Fagni, 2012). Of note, the intracellular C-terminal tail (CT) of mGlu5 receptors is large, which provides a sufficient space for mGlu5-associated regulators to interact with. In fact, the most mGlu5-interacting proteins identified so far bind to this region to exert their roles in regulating the receptor (Kim et al., 2008; Mao et al., 2011).

Mitogen-activated protein kinases (MAPK) are a family of protein kinases densely expressed in neurons of adult rat brains. Abundant MAPKs are located in the cytoplasm where they upon activation translocate to the nucleus to phosphorylate transcription factors and regulate gene expression, thereby transcriptionally controlling synaptic transmission and plasticity (Sweatt, 2004; Thomas and Huganir, 2004; Wang et al., 2007). In addition to the substrates within the nucleus (Treisman, 1996; Kosako et al., 2009), MAPKs phosphorylate a subset of substrates outside the nucleus. For instance, the extracellular signal-regulated kinase (ERK), a prototypical MAPK member, targets non-nuclear substrates, such as protein kinase C (PKC) α (Debata et al., 2010) and Kv4.2 potassium channels (Adams et al., 2000; Schrader et al., 2006). Some synapse-enriched proteins also serve as preferred substrates of ERK, including synapsin I (Jovanovic et al., 1996), δ -catenin (Edbauer et al., 2009), PSD-93 (Guo et al., 2012), and PSD-95 (Sabio et al., 2004). Apparently, ERK functions in both nuclear and non-nuclear domains, although studies on non-nuclear substrates are limited and little is known about the role of ERKs in phosphorylating and regulating glutamate receptors, such as group I mGlu receptors.

We thus carried out this study to explore whether ERK acts as a pivotal regulator of mGlu5 receptors. We first explored the interaction of ERK, specifically the ERK1 isoform, with mGlu5 receptors *in vitro* and in striatal neurons *in vivo*. We then investigated whether ERK1 phosphorylates mGlu5 receptors and mapped accurate phosphorylation sites in a confined intracellular domain of the receptor. We finally examined whether ERK modulates the efficacy of the receptor in activating its key downstream effector in striatal neurons.

Materials and Methods

Animals

We used adult male Wistar rats (200–350 g) from Charles River (New York, NY) in this study. Animals were housed in pairs at 23°C and humidity of 50 $\pm$ 10% with food and water

available *ad libitum*. The animal room was on a 12/12 h light/dark cycle with lights on at 0700. All animal use procedures were in strict accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee.

Construction and synthesis of recombinant proteins

Glutathione-S-transferase (GST)-fusion proteins were designed and synthesized as described previously (Jin et al., 2013a; 2013b). These proteins contained truncated proteins of interest. Briefly, the cDNA fragments encoding the mGlu5-intracellular loop 1 (IL1, I602-E615), mGlu5-IL2 (R667-Q692), mGlu5-IL3 (K759-K771), mGlu5-CT1 (K827-L964), mGlu5-CT2 (Y965-L1171), or mGlu5-CT (K827-L1171) were generated by PCR amplification from cDNA clones of rat mGlu5a (NCBI accession #: NP_058708.1). We subcloned these cDNAs into BamHI-EcoRI sites of the pGEX4T-3 plasmid (GE Healthcare, Piscataway, NJ). Constructs were sequenced to confirm appropriate splice fusion. After GST-fusion proteins were expressed in *E. coli* BL21 cells, they were purified as described by the manufacturer.

Western blot

According to the method described previously (Jin et al., 2013a), proteins were separated on sodium dodecyl sulfate (SDS) NuPAGE Bis-Tris 4–12% gels (Invitrogen, Carlsbad, CA). Separated proteins were then transferred to polyvinylidene fluoride membranes. Membranes were incubated with primary antibodies overnight at 4°C and followed by incubation with a secondary anti-rabbit or anti-mouse antibody. We used an enhanced chemiluminescence reagent (GE Healthcare) to develop immunoblots.

In vitro binding assay

We performed this assay as described previously (Jin et al., 2013a; 2013b). Briefly, His-tagged inactive ERK1 (20 ng; Millipore, Billerica, MA) was equilibrated to binding buffer which contains 200 mM NaCl, 0.2% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), and 50 mM Tris, pH, 7.5. After binding reactions were initiated by adding GST-fusion proteins, the reaction was allowed for 2–3 h at 4°C. We then used 10% glutathione sepharose 4B beads (100 µl) to precipitate GST-fusion proteins. The precipitate was washed three times. Bound proteins were eluted with 4X lithium dodecyl sulfate (LDS) loading buffer, resolved by SDS-PAGE, and immunoblotted with antibodies indicated.

Pull-down assay

We performed pull-down (affinity purification) assays with solubilized rat striatal lysates according to the procedures described previously (Liu et al., 2009; Jin et al., 2013b).

Phosphorylation and dephosphorylation reactions

To test phosphorylation of mGlu5 fragments *in vitro*, we incubated GST or GST-fusion proteins with active ERK1 (Millipore) for 30 min at 30°C in a reaction buffer containing 10 mM HEPES pH 7.4, 10 mM MgCl₂, 1 mM Na₃VO₄, 1 mM DTT, 0.1 mg/ml BSA, and 50 µM ATP. An amount of 2.5 µCi/tube [γ -³²P]ATP (~3000 Ci/mmol, PerkinElmer, Waltham, MA) was used for autoradiography. Elk-1 (GST-tagged, Cell Science, Canton, MA; ~81

kDa) was used as a positive control as Elk-1 is known to be phosphorylated by ERK at multiples sites involving SP, TP, PXSP phosphomotifs (Gille et al., 1995; Yang et al., 1998; Cruzalegui et al., 1999). Phosphorylation reactions were stopped by adding LDS sample buffer. After boiling sample for 3 min, phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. To assess dephosphorylation, GST-fusion proteins after phosphorylation reactions were precipitated and the supernatant containing ERK1 was removed. Precipitates were washed twice. They were then suspended in a solution containing 50 mM Tris-HCl, pH 8.5, 1 mM MgCl₂, 0.1 mM ZnCl₂ and calf intestine alkaline phosphatase (CIP, 100 units/ml; Roche, Indianapolis, IN) and incubated for 1 h at 37°C. Dephosphorylation reactions were stopped by adding a LDS sample buffer. Samples were subjected to standard gel electrophoresis and autoradiography.

Coimmunoprecipitation

We carried out coimmunoprecipitation as described previously (Liu et al., 2009; Jin et al., 2013a; 2013b). Briefly, rats were decapitated after anesthesia and brains were removed. The striatum was dissected and homogenized in isotonic sucrose homogenization buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, and a protease/phosphatase inhibitor cocktail (Thermo Scientific, Rochester, NY). Homogenates were centrifuged (800 *g*, 10 min) at 4°C. The supernatant was centrifuged again (10,000 *g*, 15 min, 4°C). The pellet was resuspended in the sucrose homogenization buffer containing Triton X-100 (0.5%, v/v). The suspension was incubated with gentle rotation for 20 min at 4°C and centrifuged (32,000 *g*, 20 min). The pellet was solubilized in sucrose-Triton buffer containing 1% sodium deoxycholate and a protease/phosphatase inhibitor cocktail for 1 h at 4°C. Solubilized proteins were used in coimmunoprecipitation. These proteins were incubated with a rabbit antibody against ERK1/2 or mGlu5. The immuno-complex was precipitated with 50% protein A or G agarose/sepharose bead slurry (GE Healthcare). Proteins were separated on Novex 4–12% gels, followed by blotting with a primary mouse antibody against ERK1 or mGlu5.

Striatal slice preparation

Striatal slices from young adult rats were prepared according to our previous procedures (Liu et al., 2009; Jin et al., 2013b). Once slices were ready, drugs were added and incubated at 30°C. Slices were collected for the following neurochemical assays.

IP₃ assays

To measure intracellular IP₃ levels, we used a HitHunter IP₃ Fluorescence Polarization Assay Kit from DiscoverX (Fremont, CA) by following manufacturer's instructions (Jin et al., 2013b; Tabatadze et al., 2015; Cansev et al., 2015).

Antibodies, pharmacological agents and peptides

Antibodies used in this study include rabbit antibodies against mGlu5 (Millipore), ERK1/2 (Cell Signaling Technology, Danvers, MA), pERK1/2 (phosphorylation at T202/Y204 for pERK1; Cell Signaling), GST (Sigma-Aldrich, St. Louis, MO), pS/TP (Abcam), or PXpSP (Cell Signaling), phosphoserine (Invitrogen), or mouse antibodies against mGlu5 (Abcam,

Cambridge, MA), ERK1 (Abcam), ERK1/2 (Cell Signaling), or pTP (Cell Signaling). Pharmacological agents, including (*RS*)-3,5-dihydroxyphenylglycine (DHPG), 3-methyl-aminothiophene dicarboxylic acid (3-MATIDA) and U0126, were purchased from Tocris Cookson Inc. (Ballwin, MO). All drugs were freshly prepared at the day of experiments.

Statistics

The results in this study are presented as means $\pm$ SEM. We evaluated data using a Student's t-test or 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.

Results

Binding between ERK1 and mGlu5 receptors

We first investigated possible binding between ERK1 and mGlu5 receptors in an *in vitro* binding system. A set of GST-fusion proteins containing different intracellular domains of mGlu5 were synthesized (Fig. 1A). These GST-fusion proteins were then used as immobilized molecules to precipitate purified ERK1 proteins added into the system. We found an mGlu5 fragment, CT1, effectively precipitated ERK1 (Fig. 1B). Other fragments, including IL1, IL2, IL3 and CT2, and GST alone did not. Elk-1, a transcription factor and a known binding partner of ERK1, precipitated ERK1 as expected, which served as a positive control. These data reveal that ERK1 binds to mGlu5 at a CT1 region. This binding activity was further supported by results from pull-down assays. As shown in Fig. 1C, GST-mGlu5-CT1 but not GST-mGlu5-CT2 or GST itself pulled down endogenous ERK1 proteins from the adult rat striatal lysates as detected by pull-down assays followed by Western blot with an anti-ERK1 antibody. Using an anti-ERK1/2 antibody, we found that a primary isoform of ERK that was pulled down by GST-mGlu5-CT1 was ERK1 (Fig. 1D).

Phosphorylation of mGlu5 receptors by ERK1

The binding of ERK1 to mGlu5 implies a likelihood that the kinase may phosphorylate mGlu5 receptors. To test this, we assayed the incorporation of ^{32}P into mGlu5 fragment proteins by active ERK1. ERK1 did not phosphorylate IL1, IL2, and IL3 fragments (data not shown). ERK1 however phosphorylated GST-mGlu5-CT but not GST (Fig. 2A). Elk-1, a known prototypic substrate of ERK (Gille et al., 1995; Cruzalegui et al., 1999), was also phosphorylated by ERK1 (Fig. 2A). Phosphorylation of mGlu5-CT and Elk-1 occurred only in the presence of the phosphate donor ATP (Fig. 2B). Adding CIP to a duplicate reaction dephosphorylated the ERK1-induced phosphorylation of mGlu5-CT (Fig. 2C). These results demonstrate that mGlu5 is a sufficient phosphorylation substrate of ERK1 and is phosphorylated at its CT region.

Accurate phosphorylate sites in mGlu5 receptors

ERK is known to phosphorylate serine and threonine at a consensus phosphorylation motif, i.e., serine/threonine-proline (S/TP) motif (Songyang et al., 1996). There are a total of eleven S/TP motifs in the entire mGlu5-CT segment (Fig. 3A). To identify accurate phosphorylation site(s) among them, we tested the ERK1-mediated phosphorylation of CT

using three phospho- and motif-specific antibodies. The ERK1-mediated phosphorylation was detected in CT when using an antibody against a phospho-serine/threonine-proline (pS/TP) motif (Fig. 3B). No phosphorylation was seen in CT with an antibody against a phospho-threonine-proline (pTP) motif (Fig. 3C). With an antibody against a phospho-PXSP (PXpSP) motif, where X represents any amino acid, we observed evident CT phosphorylation (Fig. 3D). All three motif antibodies were able to detect phosphorylation of Elk-1 which is known to contain SP, TP, and PXSP motifs sensitive to ERK-mediated phosphorylation (Gille et al., 1995; Cruzalegui et al., 1999). Thus, ERK1 seems to phosphorylate serine rather than threonine residues in the CT region.

To substantiate the above finding, we synthesized site-directed CT mutants (Fig. 4A) and compared these mutants with wild type (WT) CT in phosphorylation responses to ERK1. Mutations of all six serine sites (S982/984/1098/1126/1141/1153) to alanine (M1) abolished ^{32}P incorporation into CT, while mutations of all five threonine sites (T1049/1055/1103/1123/1138) to alanine (M2) did not (Fig. 4B). To identify accurate serine site(s) accepting phosphate, we synthesized additional mutants (Fig. 4C). Serine-to-alanine mutations of S982 (M3), S982/984 (M4), or S982/984/1098 (M5) had a minimal impact on CT phosphorylation (Fig. 4D). Remarkably, when the mutation was expanded to the fourth (S1126) or fifth (S1141) serine (M6 or M7, respectively), a complete loss of phosphorylation was seen. These results indicate that a serine residue in a distal CT location, i.e., S1126, is a primary phosphorylation site in response to ERK1. Of note, S1126 and surrounding amino acids (PPSP) align well with the ERK phosphorylation motif, PXpSP. We thus assessed CT phosphorylation using the anti-PXpSP motif antibody. The ERK1-mediated phosphorylation was in fact detected in WT CT and a mutant (M2) containing S1126 (Fig. 4E), but not in other mutants (M1, M6, and M7) lacking S1126 (Fig. 4F). These results support that S1126 is a primary phosphorylation site.

Interactions between ERK1 and mGlu5 receptors in neurons *in vivo*

We next determined whether endogenous ERK1 and mGlu5 interact with each other in striatal neurons which express a high level of mGlu5 receptors (Testa et al., 1994; 1995; Tallaksen-Greene et al., 1998). We examined the ERK1-mGlu5 interaction using coimmunoprecipitation with solubilized rat striatal lysates. In assays with an anti-mGlu5 antibody, we found a clear ERK1 band in mGlu5 precipitates as detected by an anti-ERK1 antibody (Fig. 5A). In reverse coimmunoprecipitation, mGlu5 immunoreactive proteins were present in ERK1/2 precipitates (Fig. 5B). Thus, native ERK1 interacts with mGlu5 receptors in striatal neurons *in vivo*. To determine whether mGlu5 receptors are serine-phosphorylated in striatal neurons, we immunoprecipitated mGlu5 proteins from striatal lysates. We then detected phosphorylation signals of precipitated mGlu5 receptors using an anti-phosphoserine or anti-PXpSP motif antibody. Both antibodies detected phosphorylation signals from precipitated mGlu5 receptors (Fig. 5C), indicating that striatal mGlu5 receptors exhibit constitutive phosphorylation likely at a PXpSP motif under normal conditions.

Inhibition of ERK attenuates mGlu5 signaling

G α_q -coupled mGlu5 receptors activate phospholipase C β 1 to enhance phosphoinositide hydrolysis, resulting in a rapid increase in cytoplasmic levels of IP $_3$ (Niswender and Conn,

2010; Traynelis et al., 2010; Nicoletti et al., 2011). To evaluate the functional role of ERK in modulating mGlu5 signaling, we examined the impact of inhibition of ERK on the mGlu5-mediated IP₃ production. In rat striatal slices, an mGlu1 selective antagonist 3-MATIDA (10 μ M) was added to block mGlu1 receptors. In the presence of 3-MATIDA, a group I mGlu agonist DHPG (50 μ M, 20 s) was added to analyze the mGlu5-mediated IP₃ formation. We found that DHPG produced a rapid rise in an amount of cytosolic IP₃ (Fig. 6A).

Pretreatment of striatal slices with U0126, a cell permeable and highly selective inhibitor of the ERK activator MEK (MAPK/ERK kinase), at 5 μ M (30 min prior to and during DHPG treatment) significantly reduced IP₃ responses to DHPG (Fig. 6A). These results indicate that ERK activity is essential for mGlu5 postreceptor signaling under basal conditions. In support of this notion, immunoprecipitated mGlu5 receptors showed basal phosphorylation at an S/TP motif (Fig. 6B). Adding U0126 (5 μ M, 30 min) to striatal slices significantly reduced S/TP phosphorylation of immunopurified mGlu5 receptors (Fig. 6C).

Discussion

In this study, we initiated an effort to investigate the putative relationship between ERK1 and mGlu5 receptors. We found that purified ERK1 proteins bound to mGlu5 receptors at a CT region *in vitro*. Native ERK1 was also found to form complexes with endogenous mGlu5 receptors in rat striatal neurons. The interaction of ERK1 with mGlu5 implies the ability of the kinase to phosphorylate the latter. In fact, active ERK1 catalyzed phosphorylation of mGlu5 receptors in the present study. The phosphorylation primarily occurred at a serine site located in the distal region of mGlu5 CT. Functionally, the MEK inhibitors reduced the response of the mGlu5-associated signaling pathway to ligand stimulation in striatal neurons under normal conditions. These results reveal a linkage from ERK1 to mGlu5 receptors in striatal neurons. ERK1 possesses the ability to phosphorylate mGlu5 and promote the receptor to operate efficiently.

ERK1 was found to bind to the CT domain of mGlu5 receptors. This is consistent with the notion that the CT region of the receptor harbors robust protein-protein interactions between the receptor and intracellular proteins. Indeed, the CT which is large in size interacts with most mGlu5 interacting partners discovered so far (Enz, 2012; Fagni, 2012). Within the CT region, ERK1 bound to the membrane proximal CT1 fraction. Given that ERK1 phosphorylated a serine site in the distal mGlu5 CT, ERK1 binds to and phosphorylates mGlu5 CT evidently at different sites. This separation between binding and phosphorylation sites seems to be the case for most ERK substrates (Songyang et al., 1996; Yang et al., 2017). In addition, constitutive phosphorylation of mGlu5 receptors by ERK has been found to take place under normal conditions since the MEK inhibitor U0126 reduced basal serine phosphorylation of immunoprecipitated mGlu5 receptors from cultured cortical neurons (Hu et al., 2012).

Glutamate receptors represent a group of membrane-bound receptors that are tightly regulated by phosphorylation (Mao et al., 2011; Lu and Roche, 2012). Like other glutamate receptors, mGlu5 receptors are subjected to the modulation by protein kinases in its expression and function (Dhami and Ferguson, 2006; Kim et al., 2008; Mao et al., 2008). Several early studies have defined a role of PKC in this event. Through phosphorylating a

single residue in the membrane proximal region of mGlu5a CT, PKC determines the pattern of Ca^{2+} responses in mGlu5a-expressing cells (Kawabata et al., 1996; Kim et al., 2005). PKC can also through phosphorylating multiple serine/threonine sites promote the rapid desensitization of mGlu5 receptors in response to a brief period of ligand stimulation (Gereau and Heinemann, 1998). In addition to PKC, we found that ERK1 is another kinase phosphorylating and regulating mGlu5 receptors in this study. ERK1 can directly interact with mGlu5 CT and phosphorylate a residue (S1126) in the distal CT region. Importantly, ERK is critical for mGlu5 signaling. Pharmacological inhibition of ERK reduced the efficacy of the receptor in stimulating its downstream effector in striatal neurons.

Cyclin-dependent kinase 5 (CDK5) is another kinase phosphorylating serine and threonine in a proline-directed motif, S/TP. In rat brain tissue, CDK5 normally and dynamically phosphorylated endogenous mGlu5 CT at S1167, i.e., S1126 in this study (Orlando et al., 2009). Knockdown of CDK5 with siRNAs reduced basal levels of S1167 phosphorylation in cultured rat cortical neurons (Orlando et al., 2009). However, S1167 phosphorylation was not completely absent in brain tissue from CDK5 knockout mice, indicating that other proline-directed kinases may target this site (Orlando et al., 2009). In fact, transfection of constitutively active MEK, which activates ERK, increased mGlu5 S1126 phosphorylation in HEK293T cells (Hu et al., 2012). In mouse cultured cortical neurons, U0126 significantly reduced S1126 phosphorylation of immunoprecipitated mGlu5 receptors (Hu et al., 2012). Thus, ERK, like CDK5, can phosphorylate mGlu5 receptors at S1126. Our results solidify this phosphorylation model by providing additional evidence through a different approach. Furthermore, we identified ERK1 as a responsible isoform. Regarding the consequence of S1126 phosphorylation, S1126 is noted to reside within the region of the receptor that binds to the scaffolding protein Homer (Tu et al., 1998). Thus, S1126 phosphorylation is reasoned to have an impact on Homer binding. This was demonstrated by the finding that S1126 phosphorylation by CDK5 or ERK increased the Homer EVH1 binding to mGlu5 receptors (Orlando et al., 2009; Hu et al., 2012). This increased Homer binding activity has functional consequences. Coexpression of Preso1, a multidomain scaffolding protein that binds to mGlu5 receptors, Homer and ERK, enhanced mGlu5 S1126 phosphorylation and the Homer binding to mGlu5 receptors in HEK293T cells (Hu et al., 2012). Expression of Preso1 also inhibited the mGlu5 signaling in suppressing the mGlu5 coupling to voltage-sensitive Ca^{2+} channels in superior cervical ganglia neurons. The Preso1 effect was abolished by blocking S1126 phosphorylation and the Homer binding to mGlu5 receptors through point mutation. However, interestingly, in another study (Park et al., 2013), dopamine D1 receptors activated MAPKs to phosphorylate mGlu5 receptors at S1126, which in turn created a binding site for Pin1, a prolyl isomerase. Pin1, by catalyzing isomerization of S1126-phosphorylated mGlu5 receptors, potentiated mGlu5-dependent N-methyl-D-aspartate receptor-mediated currents in cultured striatal neurons. Of note, this role of Pin1 is conditional upon the presence of Homer1a. Non-crosslinking Homer1a is expressed at a minimal level, whereas crosslinking Homer1b/c are abundant and preclude Pin1 interactions with mGlu5 receptors, under normal conditions. Homer1a was induced in the adult mouse striatum by dopamine stimulation (cocaine) after 1 h or in striatal cultures by 50 mM KCl at 30 min to a level sufficient to compete with Homer1b/c to enable Pin1-mGlu5(pS1126) interactions (Park et al., 2013). Apparently, Pin1-mGlu5(pS1126) interactions are activity- and protein synthesis-dependent

and play a relatively delayed functional role in constructing synaptic plasticity. This Homer1-sensitive Pin1-mGlu5(pS1126) mechanism may occur in a defined subset of synapses and underscore the complex of the role of Homer1a in synaptic plasticity as Homer1a was also found to mediate global homeostatic scaling down of synaptic strength (Hu et al., 2010) and to facilitate the mGlu5 inhibition of NMDA receptors (Moutin et al., 2012).

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Figure 1. Binding assays between ERK1 and mGlu5 receptors.

(A) GST-fusion mGlu5 fragments. (B) *In vitro* binding assays with immobilized GST-fusion proteins containing distinct intracellular domains of mGlu5 receptors. A membrane staining with Coomassie Brilliant Blue (CBB) is present below to show protein loading. (C) Pull-down assays with immobilized CT1 and CT2 fragments of mGlu5 receptors. Note that GST-mGlu5-CT1 but not other GST-fusion proteins or GST alone precipitated ERK1 in both *in vitro* binding (B) and pull-down (C) assays. (D) Pull-down assays with immobilized CT1 and CT2 fragments of mGlu5 receptors followed by immunoblots with an anti-ERK1/2 antibody. Purified ERK1 or rat striatal lysates were added into *in vitro* binding (B) and pull-down (C and D) assays, respectively. ERK1 bound to GST-fusion proteins was visualized with immunoblots (IB).

Figure 2. Phosphorylation of mGlu5 receptors by ERK1.

(A) An autoradiograph illustrating ERK1-mediated phosphorylation of GST-mGlu5-CT and Elk-1. Note that GST-mGlu5-CT and Elk-1 were evidently phosphorylated by ERK1 in the presence of active ERK1. (B) An autoradiograph illustrating phosphorylation of GST-mGlu5-CT and Elk-1 in the presence of ATP. (C) An autoradiograph illustrating phosphorylation of GST-mGlu5-CT in the absence but not presence of CIP. Phosphorylation reactions were carried out with GST-mGlu5-CT or the positive control Elk-1 in the presence of [γ - 32 P]ATP and active ERK1. Reactions were subjected to gel electrophoresis followed by autoradiography. Solid and open arrows indicate phosphorylated Elk-1 and GST-mGlu5-CT, respectively.

Figure 3. Serine phosphorylation of mGlu5 receptors by ERK1.

(A) Amino acid sequence analysis of the entire mGlu5 CT (K827-L1171). Note that there are a total of eleven ERK phosphorylation motifs (S/TP) in the CT region. These motifs are bold and underlined. (B) An immunoblot showing ERK1-mediated phosphorylation of GST-mGlu5-CT at S/TP motifs. (C) An immunoblot showing ERK1-mediated phosphorylation of GST-mGlu5-CT at TP motifs. (D) An immunoblot showing ERK1-mediated phosphorylation of GST-mGlu5-CT at PXSP motifs. Phosphorylation reactions were carried out with GST-mGlu5-CT or positive control Elk-1 in the presence of ATP and active ERK1. Reactions were subjected to gel electrophoresis followed by immunoblot (IB) with motif antibodies.

Figure 4. Phosphorylation of mGlu5 receptors at a serine site.

(A) Mutations of all serine or threonine residues to alanine (S/T-to-A) in GST-mGlu5-CT. All serine and threonine residues were mutated to alanine in M1 and M2 mutants, respectively. (B) An autoradiograph illustrating ERK1-mediated phosphorylation of GST-mGlu5-CT WT and mutants (M1 and M2). Note that mutations of all serine sites (M1) abolished CT phosphorylation. (C) Mutations of individual serine sites to alanine in GST-mGlu5-CT. (D) An autoradiograph illustrating ERK1-mediated phosphorylation of GST-mGlu5-CT WT and mutants (M3-M7). (E) An immunoblot showing ERK1-mediated phosphorylation of GST-mGlu5-CT WT and mutants (M1 and M2) as detected by an anti-PXpSP motif antibody. (F) An immunoblot showing ERK1-mediated phosphorylation of GST-mGlu5-CT WT and mutants (M1, M6, and M7) as detected by an anti-PXpSP motif antibody. Phosphorylation reactions were carried out with GST-mGlu5-CT WT or mutants in the presence of active ERK1. Reactions were subjected to gel electrophoresis followed by autoradiography (B and D) or immunoblot (IB) with a motif antibody (E and F).

Figure 5. Interactions between ERK1 and mGlu5 receptors in striatal neurons.

(A) A coimmunoprecipitation (IP) assay of ERK1 and mGlu5 receptors with an anti-mGlu5 antibody (Ab). Note that ERK1 was present in mGlu5 precipitates (lane 5). (B) A reverse coimmunoprecipitation assays of ERK1 and mGlu5 receptors with an anti-ERK1/2 antibody. (C) Phosphorylation of immunopurified mGlu5 receptors as detected by an antibody against phosphoserine (pSer) or a PXpSP motif. Solubilized rat striatal lysates were used in IP assays. Lanes 3 and 4 in panels A and B showed no band due to the lack of a precipitating antibody (L3) and the use of an irrelevant IgG (L4). Precipitated proteins were visualized by immunoblots (IB) with indicated antibodies.

Figure 6. Effects of MEK inhibitors on mGlu5-mediated IP₃ production in striatal neurons.

(A) Effects of U0126 on the DHPG-stimulated IP₃ formation. Note that U0126 markedly reduced IP₃ responses to DHPG. (B) Phosphorylation of immunopurified mGlu5 receptors as detected by an antibody against a pS/TP motif. (C) Effects of U0126 on S/TP phosphorylation of immunopurified mGlu5 receptors. Note that U0126 significantly reduced S/TP phosphorylation of immunopurified mGlu5 receptors. Experiments were carried out in rat striatal slices in the presence of the mGlu1 antagonist 3-MATIDA (10 μ M). U0126 (5 μ M) was applied 30 min before and during 20-s incubation of DHPG (50 μ M) (A) or for 30 min alone (C). Data are presented as means $\pm$ SEM ($n = 4 - 6$ per group). Solubilized striatal lysates were used in immunoprecipitation (IP) assays (B and C), followed by immunoblots (IB) with indicated antibodies. * $P < 0.05$ versus vehicle (C) or vehicle + vehicle (A). + $P < 0.05$ versus vehicle + DHPG.