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Protein kinase A directly phosphorylates metabotropic glutamate receptor 5 to modulate its function

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Abbreviation: mGluR5, metabotropic glutamate receptor 5; PKA, protein kinase A; PKC, protein kinase C; ERK, extracellular signal-regulated kinase.

Abstract

Metabotropic glutamate receptor 5 (mGluR5) regulates excitatory postsynaptic signaling in the central nervous system (CNS) and is implicated in various CNS disorders. Protein kinase A (PKA) signaling is known to play a critical role in neuropsychiatric disorders such as Parkinson's disease, schizophrenia and addiction. Dopamine signaling is known to modulate the properties of mGluR5 in a cAMP- and PKA-dependent manner, suggesting that mGluR5 may be a direct target for PKA. Our study identifies mGluR5 at Ser870 as a direct substrate for PKA phosphorylation and demonstrates that this phosphorylation plays a critical role in the PKA-mediated modulation of mGluR5 functions such as extracellular signal-regulated kinase (ERK) phosphorylation and intracellular Ca^{2+} oscillations. The identification of the molecular mechanism by which PKA signaling modulates mGluR5-mediated cellular responses contributes to the understanding of the interaction between dopaminergic and glutamatergic neuronal signaling.

Introduction

Glutamate is the major excitatory neurotransmitter in the CNS, and regulates the excitability of neurons by activating ionotropic and metabotropic (mGluRs) glutamate receptors (Monaghan *et al.* 1989). mGluRs are G protein-coupled receptors, and are divided into three groups (group I, II and III) on the basis of sequence homology, G protein-effector coupling (Schoepp *et al.* 1990) and agonist

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pharmacology (Tanabe *et al.* 1992). Group I mGluRs (mGluR1 and mGluR5), especially mGluR5, play an important role in the regulation of neuronal excitability and synaptic plasticity (Niswender & Conn 2010). mGluR5 is involved in the pathophysiology of various CNS disorders, including anxiety disorders (Swanson *et al.* 2005), schizophrenia (Conn *et al.* 2009), Alzheimer's disease (Malter *et al.* 2010), Parkinson's disease (Johnson *et al.* 2009), addiction (Olive 2010), and Fragile X syndrome (Catania *et al.* 2007).

Group I mGluRs are coupled to G_q-proteins, and stimulate the activity of phospholipase C (PLC) (Hermans & Challiss 2001) and synthesis of inositol-1,4,5-triphosphoate (IP_3) and diacylglycerol, leading to an increase in intracellular Ca^{2+} and protein kinase C (PKC) activity (Kawabata *et al.* 1996). In addition, group I mGluRs bind to scaffold Homer proteins, which are linked to IP_3 receptors and Shank, which itself is associated with the NMDA receptor/PSD95 complex (Sheng & Kim 2002). mGluR5 is reported to induce the phosphorylation of extracellular signal-regulated kinase (ERK), via mechanisms mediated by the Homer1b/c and the IP_3/intracellular Ca^{2+} signaling pathways (Mao *et al.* 2005b), and the inhibition of protein phosphatase 2A (PP-2A) activity by Src-dependent tyrosine phosphorylation of the PP-2A catalytic subunit (Mao *et al.* 2005a). In addition, mGluR5 interacts with adenosine A_{2A} receptors (Kachroo *et al.* 2005) and enhances adenosine A_{2A} receptor-mediated PKA signaling via ERK-dependent mechanisms in the striatum (Nishi *et al.* 2003, Nishi *et al.* 2005).

Group I mGluRs are subject to the regulation by protein phosphorylation (Kim *et al.* 2008). The phosphorylation of mGluR5 at Ser839 by PKC is required for the generation of Ca^{2+}oscillations (Kawabata et al. 1996), and the phosphorylation at several other sites by PKC [Thr681 in the G protein-coupling region of the second intracellular loop (Francesconi & Duvoisin 2000), Ser901 in the calmodulin binding region (Lee *et al.* 2008), and potential sites (Thr606, Ser613, Thr665, Ser881 and Ser890) in the first and second intracellular loops and the C terminus (Gereau & Heinemann 1998)] plays a role in desensitization of mGluR5. Cdk5 is reported to phosphorylate mGluR5 in the Homer-binding domain (Orlando *et al.* 2009), suggesting that the interaction of mGluR5 with binding proteins is also regulated by phosphorylation. Furthermore, the phosphorylation state of mGluR5 is

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regulated by other protein kinases (e.g. Ca^{2+}/calmodulin-dependent kinase II (CaMKII), G protein-coupled receptor kinases, and tyrosine kinases) and protein phosphatases (Mao *et al.* 2008). PKA has also been shown to regulate mGluR5 activity (Poisik *et al.* 2007), but no evidence of direct phosphorylation of mGluR5 by PKA has been obtained.

cAMP/PKA signaling is one of the major intracellular signaling pathways in the CNS, and is regulated by dopamine D1 and D2 receptors. We hypothesized that mGluR5 and PKA signaling are mutually interactive, and that PKA may modulate the function of mGluR5 by its direct phosphorylation. Given that mGluR5 dysregulation has been implicated in various neuropsychiatric disease states, and that PKA is highly expressed in brain areas linked to neuropsychiatric diseases, the mechanism of mGluR5 regulation by PKA is an important question. In this study, we have identified serine 870 in the C-terminal tail of mGluR5 as a target of PKA phosphorylation and have shown that the phosphorylation of this residue affects the ability of mGluR5 to induce ERK activation and Ca^{2+} oscillations.

Materials and methods

Cloning of mGluR5b constructs and expression of the mGluR5b C-terminal fusion protein

The mouse mGluR5b coding sequence (1203 amino acid residues, Gene bank XM-149971), along with a Kozak sequence, was amplified by PCR using the following primers: 5'-atggtccttctgttgatcctgtcagtcctacttctgaaa-3' (forward) and 5'-caacgatgaagaactctgcgtgtaatctctgatgatgag-3' (reverse). The amplified products were subcloned into the pcDNA3.1/myc-His (Invitrogen, Rockville, MA) and pEGFP-N3 (Clontech) vectors. The mCherry construct was amplified by PCR and inserted in the place of GFP in the pEGFP-N3 mGluR5b vector. Site point mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). For the generation of an mGluR5 C-terminal construct, the sequence encoding residues 827-1203 was amplified by PCR using the following primers: 5'- gagagaattcaaaccggagagaaatgtgcg-3' (forward) and 5'–gcgtctcaagaagtagcaaccagctgctct-3' (reverse). The amplified fragment was subcloned into the pGEX 4T1 vector (GE Healthcare, Piscataway, NJ), and appropriate point mutations were

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introduced. The mGluR5 C-terminal fusion protein was expressed in *E. coli* and purified with Glutathione Sepharose 4b (GE Healthcare).

Cell culture and transfection

HEK293T cells were cultured in DMEM containing 10% (v/v) FBS. Cells were cultured at 37°C in a humidified 95% O_2/5% CO_2 atmosphere. Cells were transiently transfected by the calcium phosphate method with an mGluR5b plasmid DNA in Ca^{2+}-containing BES buffer. For intracellular Ca^{2+} measurements, HEK293A cells (Qbiogene, Carlsbad, CA) were cultured on glass coverslips in DMEM supplemented with 10% FBS. One day after plating, cells were transfected with the wild-type or mutant Cherry- or GFP-tagged mGluR5b constructs using the ExGene 500 *in vitro* Transfection Reagent (Fermentas, Thermo Fisher Scientific, St. Leon-Rot, Germany). Experiments were performed 24 h after transfection.

Cell harvesting and protein purification

Transfected cells were homogenized in RIPA buffer containing protease and phosphatase inhibitors. Homogenized samples were centrifuged at 100,000 x *g* for 1 h and supernatants were injected into His-nickel columns for FPLC system purification (GE Healthcare). Samples were immunoprecipitated with an c-Myc 9E10 monoclonal antibody agarose conjugate (Santa Cruz Biotechnology Cat# sc-40, RRID:AB_627268). Immunopurified samples were separated on 3-8% Tris-acetate gels (Bio-Rad, Hercules, CA), which were either stained by Coomassie G-250 (Pierce, Rockford, IL) or processed for Western blot analysis using an anti-mGluR5 antibody (Millipore Cat# AB5675, RRID:AB_2295173).

Mass spectrometry analysis

Single-stage and tandem mass spectra were collected on an LC-ESI-ion trap mass spectrometer (LCQ Deca, Thermo Electron, San Jose) and a MALDI-ion trap mass spectrometer (MALDI-LTQ XL,

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Thermo Electron, San Jose). The MALDI matrix used was 4-hydroxy-a-cyano-cinnamic acid. Tandem mass spectra were collected for 0.5-2 min using injection times of 100-1000 ms, activation times of 300-500 ms, and relative collision energies of 30-40%. Standard methods were used to identify phosphopeptides and sites of phosphorylation (McLachlin & Chait 2001).

In vitro phosphorylation reactions

Reactions for the phosphorylation of the mGluR5 C-terminus and DARPP-32 (10 µM) by the catalytic subunit of PKA (2500 units/µl) were performed in 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, and 50 µM ATP in the presence of 10 µCi [g-^{32}P]-ATP at 30 ^{o}C. The phosphorylation reactions were stopped by the addition of SDS-polyacrylamide gel electrophoresis (Page *et al.*) sample buffer, after which the samples were boiled for 5 min. The phosphorylated proteins were separated by SDS-PAGE and analyzed by autoradiography (Kodak, Rochester, NY), followed by normalization of ^{32}P incorporation to the amount of protein used.

Phosphopeptide mapping

After autoradiography, gel pieces containing ^{32}P-labeled mGluR5 C-termini were destained (50% methanol/10% acetic acid) and washed (50% methanol). Gel pieces were incubated with 10 µg/mL TPCK-Trypsin (Roche) in 50 mM NH_4HCO_3, pH 8.1 for 15 h at 37 ^{o}C. Supernatants were lyophilized, and the extraction efficiency was quantified by Cerenkov counting. Two-dimensional phosphopeptide mapping was performed as previously described (Nairn & Greengard 1987). Electrophoretic separation was performed at pH 3.5 for 150 min at 400 V, and ascending chromatography was performed in pyridine/n-butanol/acetic acid/water (15:10:3:12). The pattern of trypsin-digested phosphopeptides was examined by autoradiography**.**

Generation of a phosphorylation state-specific antibody

A rabbit polyclonal phosphorylation state-specific antibody against phospho-Ser870 of mGluR5 was raised using a synthetic peptide corresponding to amino acids 866-874 of mGluR5, with a

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phosphoserine at the Ser-870 position (KRRGpSSGET). The peptide contained cysteine and was conjugated to keyhole limpet hemocyanin. Affinity purification was performed using dephospho- and phosphopeptides coupled to a sulfo-link coupling gel (Pierce). A recombinant human monoclonal antibody Fab fragment against phospho-Ser870 peptide (AbD09842.2) was obtained by selection from the libraries of antibody genes with the HuCAL® technology (MorphoSys AG, Planegg, Germany).

mGluR5 phosphorylation in neostriatal slices

Neostriatal slices were prepared from male C57BL/6 mice (6–10 weeks of age) and mGluR5 knockout mice (The Jackson Laboratory, Bar Harbor, Maine; Stock# 003121). The mice were handled in accordance with the Institutional Animal Care and Use Committee of Kurume University School of Medicine, using approved protocols. All efforts were made to minimize animal suffering and to reduce the number of animals used. Slices were treated with drugs as specified in each experiment and processed as previously described (Uematsu *et al.* 2005). Immunoblotting was carried out by using phospho-Ser870 mGluR5 antibodies and a total mGluR5 antibody. None of the experimental manipulations used in the present study altered the total amount of mGluR5. By comparing the signals of phospho-Ser870 and total mGluR5 standards, the stoichiometry of mGluR5 phosphorylation at Ser870 in neostriatal slices was estimated.

Phosphorylation of ERK1/2

HEK293T cells were transfected with mGluR5 wild-type and S870A mutant cDNAs. Cells were treated with DHPG (100 μM) for various time intervals, and reactions were terminated by placing the culture dishes on ice. Cells were harvested using ice-cold RIPA buffer. The samples were resolved by SDS-PAGE and subjected to Western blot analysis with a phospho-p44/42 MAP kinase antibody (Cell Signaling Technology Cat# 9101S, RRID:AB_331646) and a p44/42 MAP kinase antibody (Cell Signaling Technology Cat# 9102, RRID:AB_330744). Antibody binding was detected using the ECL

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immunoblotting detection system (Amersham, Arlington Heights, IL) and the Odyssey infrared imaging system (LI-COR, Lincoln, NE).

Intracellular Ca^{2+} imaging

Cells were incubated with 1 µM of Fura-2 AM (Molecular Probes) for 20 min at 37°C. Imaging was performed at 37°C using an upright microscope (Axioskop 2 FS, Zeiss) equipped with a CCD camera (ORCA-ER, Hamamatsu Photonics, Japan). The cells were excited at 340 nm and 380 nm, emitted light was detected using a band-pass filter at 510/30 nm. Images were acquired every 1 s. After 1 min of baseline recordings in PBS (in mM: 137 NaCl, 0.9 $CaCl_2$, 0.49 $MgCl_2$, 2.7 KCl, 1.5 KH_2PO_4, and 8.1 Na_2HPO_4, pH 7.4), the cells were perfused with PBS containing 10 µM DHPG (Sigma-Aldrich), and recording was continued for 12-15 min. Image acquisition and analysis was performed using MetaFluor software (Molecular Devices, Sunnyvale, CA).

Subcellular distribution of mGluR5

The subcellular distribution of GFP-tagged mGluR5 was examined using two methods, confocal and total internal reflection fluorescence (TIRF) microscopy. In the confocal imaging experiments, live mGluR5b-transfected cells were mounted on the stage of an inverted laser scanning microscope (LSM-410, Zeiss). Images of the cells (excitation at 488 nm and emission at 515-525 nm) were collected before and after application of DHPG. In TIRF experiments, live cells were mounted on a microscope (Axiovert 200M, Zeiss) equipped with a TIRF slider and an AxioCam HR camera. Images were recorded with exposure time of 650 ms every 10 s for 3 min after DHPG application. All images were analyzed using the ImageJ software (RRID:nif-0000-30467).

Statistics

Data analysis was performed by using GraphPad Prism 5.0 (GraphPad software, La Jolla, CA). Data are expressed as means ± SEM. Parametric (unpaired *t*-test for Fig 4B and one-way ANOVA test for

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Fig 2D, Fig 3B) and non-parametric (Mann-Whitney test for Fig 4E and Friedman test for Fig 4A) tests were used. A P value of < 0.05 was considered significant.

Results

To examine whether mGluR5 is a substrate for PKA, HEK293T cells were transfected with a histidine- and MYC-tagged mGluR5 construct, cells were treated with forskolin (an activator of adenylyl cyclase and thus of PKA), and histidine and MYC tandem purifications were performed from the cell lysates. Purified protein samples were digested and subjected to single-stage and tandem mass spectroscopy on a MALDI-QqTOF mass spectrometer and MALDI-ion trap mass spectrometer, respectively. Three phosphopeptides that in total contained 13 serine and threonine residues were identified as putative PKA phosphorylation sites, all within the mGluR5 C-terminal domain (Fig. 1A). In addition to the 13 residues, two serine residues predicted from the PKA phosphorylation motifs (R-X-S/T, R-R/K-X-S/T) in the mGluR5 C-terminal domain were included for further analysis (Fig. 1A).

From our mass spectrometry analysis, all the putative PKA-sites in the full-length mGluR5 were located in the intracellular C-terminal region of the protein. Therefore, we generated an mGluR5b C-terminal fragment consisting of amino acids 826-1203 fused to glutathione S-transferase for further studies. Two-dimensional phosphopeptide maps of this fragment phosphorylated by PKA *in vitro* revealed the presence of four major trypsin-digested phosphopeptides (Fig. 1B, wild-type; labeled 1-4). To identify the sites of PKA phosphorylation, we compared the phosphopeptide map of the wild-type C-terminal fragment with those of C-terminal fragments with alanine mutations at each of the putative serine or threonine phosphorylation sites (Fig. 1C). Mutation of serine 870 to alanine (S870A) led to a selective disappearance of phosphopeptides 3 and 4 (Fig. 1C, S870A). Mutations of the other putative phosphorylation sites (Ser834, Thr837, Thr838, Ser839, Thr840, Ser859, Ser871, Thr874, Ser1045, The1197, Ser1199, Ser1200, Ser1201, Ser1202) did not affect the pattern of the

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phosphopeptide map (data not shown). The stoichiometry of mGluR5b C-terminal phosphorylation by PKA was estimated as 1.4 mol/mol (Fig. 1D).

Having identified the PKA phosphorylation site in mGluR5, we next generated two phosphorylation state-specific antibodies against an mGluR5 peptide containing this phospho-Ser870. We generated a rabbit polyclonal phospho-Ser870 mGluR5 antibody selectively recognizing the mGluR5 C-terminus phosphorylated by PKA (Fig. 2A). Another antibody, the monoclonal phospho-Ser870 mGluR5 antibody (AbD09842.2), detected signals of wild-type, but not S870A mutant, mGluR5 in the dimer form expressed in HEK293T cells (Fig. 2B). In mouse neostriatal slices, where mGluR5 is highly expressed (Spooren *et al.* 2001), Ser870 phosphorylation was detected under basal conditions by the rabbit polyclonal phospho-Ser870 mGluR5 antibody (Fig. 2C, D). The specificity of the rabbit polyclonal phospho-Ser870 mGluR5 antibody (Fig. 2C) and the monoclonal phospho-Ser870 mGluR5 antibody (data not shown) for mGluR5 was verified by the lack of signal for mGluR5 monomer and dimer forms in striatal tissue lysates prepared from mGluR5 knockout mice (even minor mGluR5-related species, which most likely represent mGluR5 species with posttranslational modifications and/or degradation products, also disappeared in tissues from mGluR5 knockout mice). The stoichiometry of mGluR5 phosphorylation at Ser870 was estimated to be ~40% and 10-20% in the dimer and monomer forms, respectively. Treatment of neostriatal slices with forskolin (10 μM) increased the levels of phospho-Ser870 mGluR5 approximately 1.5-fold (Fig. 2D, E). These data indicate that mGluR5 phosphorylation at Ser870 is regulated by changes in cAMP level in neostriatal neurons. The regulation of Ser870 phosphorylation by a dopamine D1 receptor agonist (SKF81297, 1 μM for 10 min) or protein phosphatase inhibitors (okadaic acid and calcineurin) in neostriatal slices was examined, but significant changes in the level of phospho-Ser870 mGluR5 were not detected (data not shown).

Activation of mGluR5 has been shown to increase ERK phosphorylation *in vitro* (Thandi et al. 2002) and *in vivo* (Hu *et al.* 2007) through PKC and Src-dependent pathways (Peavy *et al.* 2001), and thus ERK phosphorylation serves as an integrative measure of mGluR5 activity. We examined whether the mutation of Ser870 to a phosphomimetic aspartic acid or nonphosphorylatable alanine might influence the ability of mGluR5 to activate ERK in HEK293T cells. In cells transfected with wild-type mGluR5, treatment with a group I mGluR agonist, DHPG (100 μM), increased the

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phosphorylation of ERK1 and ERK2 after 3 to 15 min of incubation, with maximal activity at 3-5 min (Fig. 3A, B, E, F). Similarly to the wild-type mGluR5, treatment with DHPG (100 μM) increased the phosphorylation of ERK2 after 3 and 5 min of incubation in cells transfected with S870D mutant mGluR5 (Fig. 3E, F). However, DHPG failed to increase ERK1 and ERK2 phosphorylation in cells transfected with S870A mutant mGluR5 (Fig. 3C, D, E, F), despite similar expression of the wild-type and mutant mGluR5 proteins (Fig. 3A, C). Subcellular distribution, as evaluated with confocal and total internal reflection fluorescence (TIRF) microscopy (Fig. 4A, B), was similar for all constructs tested. These results indicate that PKA phosphorylation of mGluR5 at Ser870 is required for activation of ERK signaling.

Since agonist stimulation of mGluR5 triggers oscillatory changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), a phenomenon known to be regulated by PKC phosphorylation, we tested whether Ser870 phosphorylation is also necessary for mGluR5-triggered $[Ca^{2+}]_i$ oscillations. The average number of $[Ca^{2+}]_i$ peaks in agonist-responding cells within a 10 min recording window was significantly higher in cells expressing wild-type compared to S870A mutant mGluR5 (Fig. 4C, D, E). Out of all cells expressing wild-type mGluR5, 21% of cells responded to DHPG with characteristic $[Ca^{2+}]_i$ oscillations, producing three or more $[Ca^{2+}]_i$ peaks. However, only 12% of cells expressing S870A mutant mGluR5 demonstrated $[Ca^{2+}]_i$ oscillations (Fig. 4C, D, F). Previously only one pair of residues, Ser839/Thr840, which is located in the G-protein coupling region of mGluR5, has been implicated in the regulation of $[Ca^{2+}]_i$ oscillations (by PKC) (Kim *et al.* 2005). Our data demonstrate that mGluR5 phosphorylation at S870, which is located near the Ser839/Thr840 pair, is also involved in agonist-triggered $[Ca^{2+}]_i$ oscillations.

Discussion

We have demonstrated that PKA regulates the function of mGluR5 by direct phosphorylation at serine 870 (Ser870), an amino acid residue located within the calmodulin binding *site I*Δ (Minakami *et al.* 1997) and close to the PKC site (S839) needed for generating Ca^{2+} oscillations (Kim et al. 2005, Mao

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et al. 2008). We have shown that in neostriatal neurons, mGluR5 is phosphorylated at Ser870 under basal conditions, but that Ser870 phosphorylation is also increased following an elevation of intracellular cAMP levels. Our analysis of cells expressing wild-type or mutant mGluR5 revealed that the phosphorylation at Ser870 plays a critical role in the ability of mGluR5 to induce activation of both ERK signaling and intracellular Ca^{2+} oscillations. Thus our findings demonstrate a direct mechanism by which PKA signaling modulates mGluR5-mediated cellular responses.

Activation of mGluR5 induces phosphorylation of ERK in cell lines (Thandi *et al.* 2002), neurons (Mao et al. 2005a), and glial cells (Peavy & Conn 1998). Activation of wild-type, but not S870A mutant, mGluR5 increased the phosphorylation of ERK1/2, suggesting a critical role of Ser870 in mGluR5-induced ERK activation. The coupling of mGluR5 with $G_{q/11}$-protein is likely the essential step for activation of downstream ERK signaling. Ser870, located within the calmodulin binding region, is also close to the $G_{q/11}$-protein-coupling region in the C-terminus, Lys827-Lys850 (Mao et al. 2008) and thus the phosphorylation at Ser870 may directly affect the efficiency of $G_{q/11}$-protein coupling.

The phosphomimetic S870D mutant form of mGluR5, but not the unphosphorylatable S870A mutant form of mGluR5, had the ability to induce ERK phosphorylation, although the efficiency of the S870D mutant mGluR5 did not exceed that of the wild-type mGluR5. These results suggest that phosphorylated Ser870 is required for normal ERK activation by mGluR5. The increase in Ser870 phosphorylation upon strong PKA activation with forskolin was relatively small (~1.5 fold), and a physiological stimulus such as dopamine D1 receptor activation or protein phosphatase inhibitor treatment failed to modulate Ser870 phosphorylation in neostriatal neurons. The reason for the limited modulation of Ser870 phosphorylation is currently unknown, but may be due to a high stoichiometry of Ser870 phosphorylation (~40%) under basal conditions.

Activation of mGluR5 triggers high-frequency Ca^{2+} oscillations (Kawabata *et al.* 1998). Kim et al. (Kim et al. 2005) reported that this unique feature of mGluR5 is dependent on the phosphorylation of Ser839 by PKC in the $G_{q/11}$-protein-coupling region of the mGluR5 C-terminus. In

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this study, we demonstrate that Ser870 is also important for the induction of Ca^{2+} oscillations by mGluR5. The attenuation of Ca^{2+} oscillations induced by the S870A mutant form of mGluR5 was unlikely due to an increased internalization, because the subcellular distribution of mGluR5 was not affected by the S870A mutation. Since Ser870 is located within the calmodulin binding region, the modulation of Ca^{2+}/calmodulin signaling by Ser870 (PKA-site) and $G_{q/11}$-protein-coupling by Ser839 (PKC-site) may coordinately influence Ca^{2+} oscillations. Further studies are required to clarify the role of the PKA vs the PKC phosphorylation site in generating Ca^{2+} oscillations, including potential interactions between the two sites.

mGluR5 has been implicated in the pathophysiology of schizophrenia (Conn et al. 2009), Parkinson's disease (Johnson et al. 2009) and addiction (Olive 2010). PKA has also been implicated as a key signaling molecule in these disorders. Studies using mGluR5 knockout mice revealed that mGluR5 plays a critical role in sensorimotor gating (Brody *et al.* 2004) and in psychostimulant-induced locomotor activity (Park *et al.* 2013), in which dopamine/PKA signaling serves as the main signaling cascade. Altered activity of dopamine/PKA signaling under pathophysiological conditions may affect the phosphorylation state of mGluR5 at Ser870, leading to the changes in mGluR5-mediated cellular and behavioral responses. Thus, we predict that the PKA phosphorylation site of mGluR5 integrates dopaminergic and glutamatergic signaling at the synapse, and that the enhancement of mGluR5 signaling pathways by direct PKA phosphorylation may have therapeutic value for CNS disorders.

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Figure legends

Fig. 1 Phosphorylation of mGluR5 by PKA *in vitro*

(**A**) Sequence of the mGluR5b C-terminus showing potential PKA phosphorylation sites (serine and threonine residues) predicted by mass spectrometry (red with underline), as well as phosphorylation motif analysis (red). (**B, C**) Tryptic phosphopeptide maps of wild-type and S870A mutant mGluR5 C-termini, phosphorylated with PKA in the presence of [g-^{32}P]ATP *in vitro*. This experiment was repeated two times. (**D**) *In vitro* phosphorylation of mGluR5 C-terminus by PKA in comparison with that of DARPP-32.

Fig. 2 Regulation of mGluR5 Ser870 phosphorylation in the neostriatum

(**A**) A polyclonal phospho-Ser870 mGluR5 antibody selectively detected the mGluR5 C-terminus phosphorylated by PKA *in vitro*. This experiment was repeated two times. (**B**) Phospho-Ser870 signals for the mGluR5 dimer form were detected with a monoclonal phospho-Ser870 mGluR5 antibody (AbD09842.2) for wild-type, but not S870A mutant mGluR5 expressed in HEK293T cells (full length forms expressed in all cases). Left lane, mock transfection (mock); middle lane, wild-type mGluR5 (WT); right lane, S870A mutant mGluR5 (S➡A). (**C**) Phospho-Ser870 signals for the mGluR5 dimer and monomer forms were detected with a polyclonal phospho-Ser870 mGluR5 antibody in striatal tissues from wild-type mice (WT), but not from mGluR5 knockout mice (KO). Samples for wild-type and S870A mutant mGluR5 (**B**) and for wild-type and mGluR5 knockout mice (**C**) were processed, loaded onto gels and immunoblotted in parallel. (**D**, **E**) Phosphorylation of mGluR5 in mouse neostriatal slices treated with forskolin (10 µM) for the indicated times. Typical immunoblots for detection of phospho-Ser870 mGluR5 with the polyclonal antibody are shown (**D**, upper panel). The amount of phospho-Ser870 mGluR5 in the dimer form was quantified by densitometry (**E**). Values represent means ± SEM, n = 9 mice. *$P < 0.05$, **$P < 0.01$ compared with untreated slices; one-way ANOVA followed by Newman-Keuls test.

Fig. 3 ERK phosphorylation induced by activation of wild-type, S870A mutant or S870D mutant mGluR5 in HEK293T cells

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(**A-D**) HEK293T cells expressing wild-type (**A**, **B**) or S870A mutant (**C**, **D**) mGluR5 were incubatedwith a group I mGluR agonist, DHPG (100 μM), for the indicated times. Immunoblots used for detection of phospho-ERK1/2, total ERK1/2 and mGluR5 in HEK293T cells are shown (**A**, **C**). The amount of phosphorylated ERK2 was quantified by densitometry, and data were normalized to values obtained with untreated cells (**B**, **D**). Values are means ± SEM from six independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with untreated cells; one-way ANOVA followed by Newman-Keuls test. (**E, F**) Cells expressing wild-type, S870D mutant or S870A mutant mGluR5 were incubated with DHPG (100 μM) for 3 or 5 min. The quantified data were normalized to values obtained with untreated cells (**F**). DHPG treatment increased ERK2 phosphorylation in cells expressing wild-type and a phosphomimetic S870D point mutant, but not an un-phosphorylatable S870A point mutant. Values are means ± SEM from 12 experiments. *$P < 0.05$, **$P < 0.01$ compared with untreated cells; one-way ANOVA followed by Newman-Keuls test.

Fig. 4 mGluR5 subcellular distribution and intracellular Ca^{2+} oscillations in HEK293A cells transfected with wild-type or S870A mutant mGluR5

(**A**) Confocal images show the distribution of GFP-tagged wild-type and S870A mutant mGluR5 before and after DHPG (10 μM) application. The ratio of GFP signal in the membrane to that in the adjacent cytosol was quantified (right panels). Values are means ± SEM, Friedman's two-way ANOVA. In each group, 17 pairs of membrane regions and corresponding adjacent cytoplasm regions from 8 cells were analyzed in two independent experiments. (**B**) Total internal reflection fluorescence (TIRF) images showing the distribution of GFP signal in the basal plasma membrane of the cells. The intensity of the signal decreased with time after application of 10 μM DHPG with a similar rate in wild-type and S870A mutant mGluR5b. Values are means ± SEM, unpaired *t*-test. In each group, n = 9 cells from six (wild-type) and five (S870A) measurements, in two independent experiments. (**C, D**) Intracellular Ca^{2+} responses to DHPG (10 μM) in cells that express wild-type (**C**) or S870A mutant (**D**) mGluR5. Single cell traces are shown from two consecutive experiments. Insets: confocal images of cells transfected with the respective mGluR5 tagged with mCherry. (**E**) The average number of $[Ca^{2+}]_i$ peaks in cells responding to DHPG with at least one $[Ca^{2+}]_i$ peak. Values are means ± SEM, n = 51 cells from 8 coverslips (wild-type) and 46 cells from 10 coverslips (S870A) from five

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independent experiments. *$P < 0.05$ compared with wild-type; Mann-Whitney test. (**F**) The number of oscillating cells (responding to DHPG with at least three $[Ca^{2+}]_i$ peaks) expressed in % of transfected cells. Data were from 85 cells on 8 coverslips (wild-type) and 103 cells on 10 coverslips (S870A) from five independent experiments.

Fig. 5 Role of Ser870 in mGluR5-mediated signaling pathways

We have identified Serine 870 in the mGluR5 C-terminus as a PKA phosphorylation site. (a) Activation of mGluR5 (wild-type) by DHPG normally induces ERK phosphorylation and Ca^{2+} oscillations, which is likely mediated through activation of the PLC/IP_3 signaling pathways and release of Ca^{2+} from the endoplasmic reticulum (ER). Together with Ser839 (a PKC site), which is located within the $G_{q/11}$-protein-coupling region, Ser870, which is located within the calmodulin binding region, may coordinately influence ERK phosphorylation and Ca^{2+} oscillations. (b) When S870 is mutated to alanine (S870A), mGluR5-dependent ERK phosphorylation and Ca^{2+} oscillations are largely attenuated. Thus, Ser870 and its phosphorylation by PKA play a key role in the mGluR5-dependent regulation of ERK and intracellular Ca^{2+} signaling.

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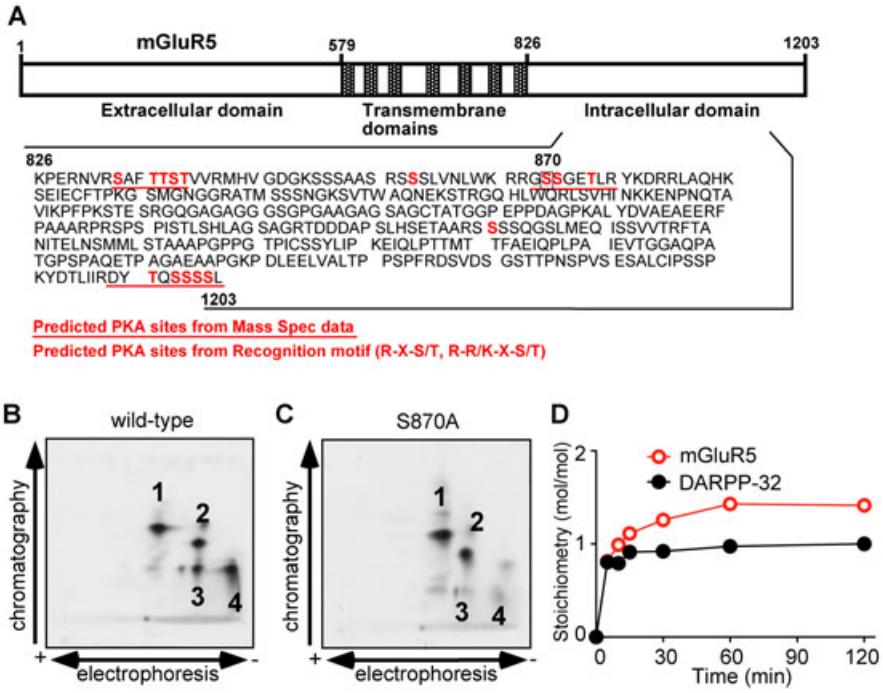
Fig 1



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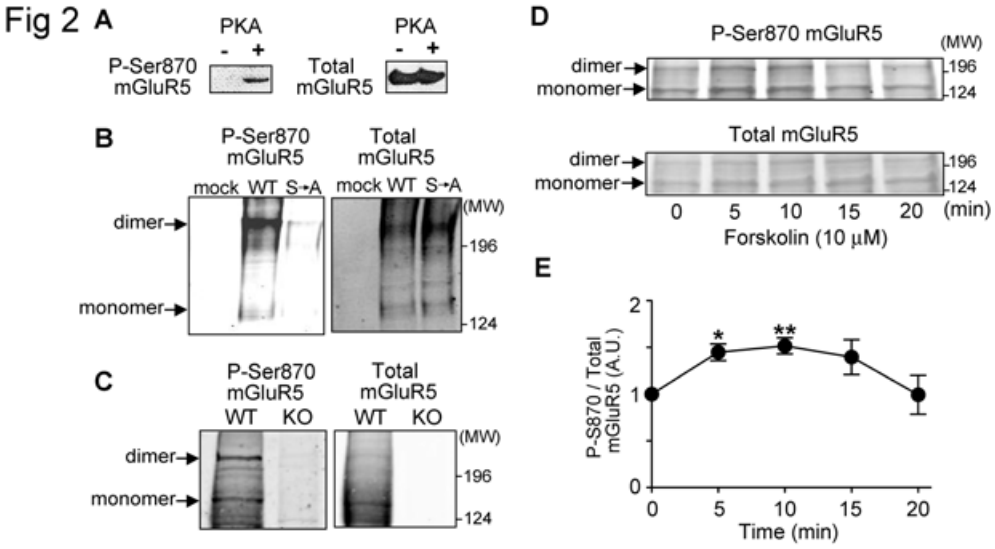

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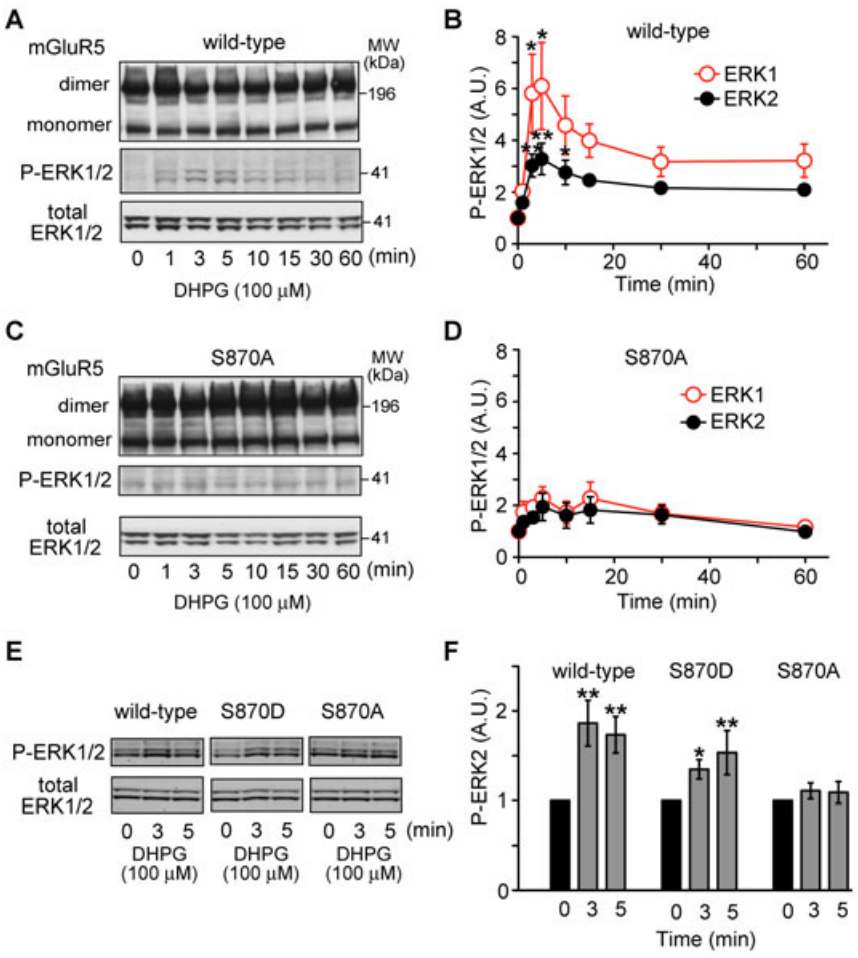
Fig 3



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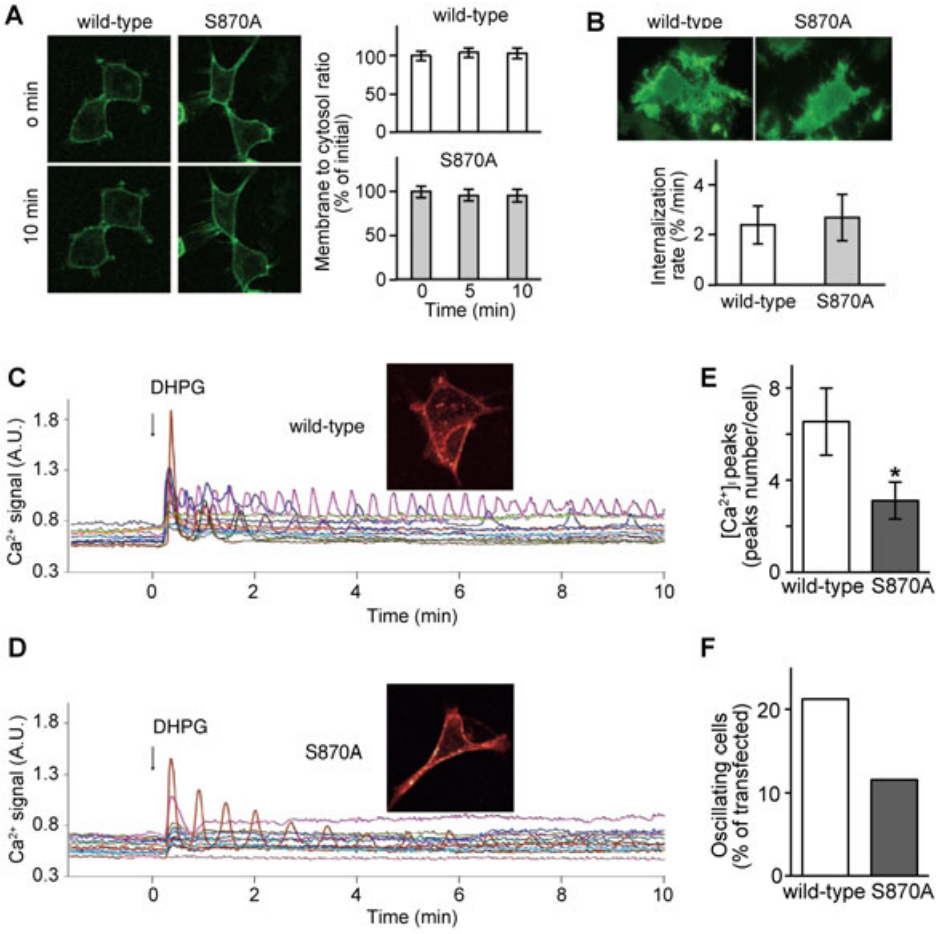

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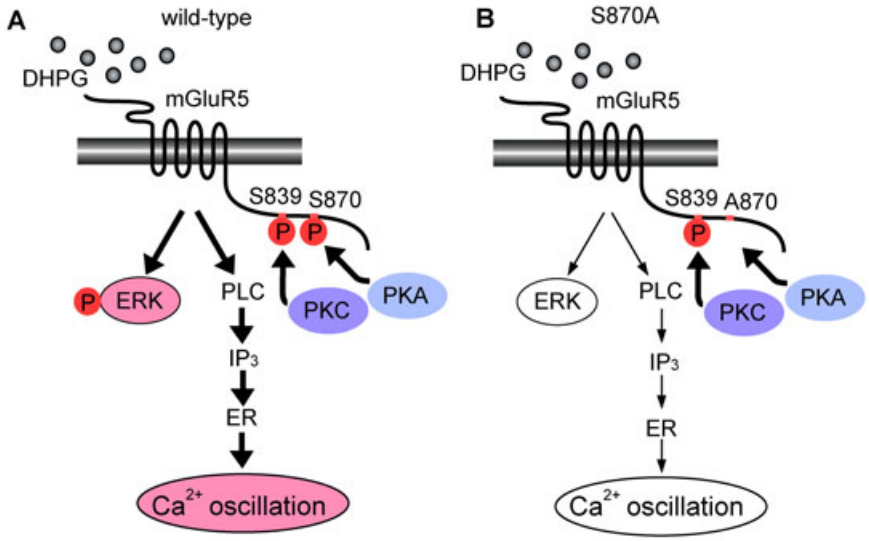

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