Neuronal Excitability

An Essential Role of Fyn in the Modulation of Metabotropic Glutamate Receptor 1 in Neurons

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Abstract

Fyn is a member of the Src family of nonreceptor tyrosine kinases and is broadly expressed in the CNS. As a synapse-enriched kinase, Fyn interacts with and phosphorylates local substrates to regulate synaptic transmission and plasticity, although our knowledge of specific targets of Fyn at synaptic sites remains incomplete and the accurate role of Fyn in regulating synaptic proteins is poorly understood. In this study, we initiated an effort to explore the interaction of Fyn with a metabotropic glutamate receptor (mGluR). We found that recombinant Fyn directly binds to mGluR1a at a consensus binding motif located in the intracellular C-terminus (CT) of mGluR1a in vitro. Similarly, endogenous Fyn interacts with mGluR1a in adult rat cerebellar neurons in vivo. Active Fyn phosphorylates mGluR1a at a conserved tyrosine residue in the CT region. In cerebellar neurons and transfected HEK293T cells, the Fyn-mediated tyrosine phosphorylation of mGluR1a is constitutively active and acts to facilitate the surface expression of mGluR1a and to potentiate the mGluR1a postreceptor signaling. These results support mGluR1a to be a novel substrate of Fyn. Fyn, by binding to and phosphorylating mGluR1a, potentiates surface expression and signaling of the receptors.

Key words: cerebellum; mGluR; phosphorylation; Src

Significance Statement

This work identified a novel signaling mechanism in cerebellar neurons. In these neurons, a nonreceptor tyrosine kinase, Fyn, binds to a glutamate receptor [i.e., metabotropic glutamate receptor 1 (mGluR1)] at synaptic sites. This binding enables the kinase to phosphorylate the receptor at a specific tyrosine site, thereby regulating surface expression/trafficking and mGluR1 signaling. These findings unravel a new mechanism underlying the regulation of glutamate receptors by tyrosine kinases in cerebellar neurons and advance the current knowledge on molecular neurobiology of glutamate receptors.

Introduction

L-Glutamate, a key transmitter in the brain, interacts with ionotropic and metabotropic glutamate receptors (mGluR) to achieve its action (Niswender and Conn, 2010; Traynelis et al., 2010). Among eight mGluR subtypes (mGluR1–8), mGluR1 has been an attractive target in recent studies. As a G-protein-coupled receptor (GPCR), mGluR1 activates G α q -coupled phospholipase C β 1 (PLC β 1).
leads to an increase in phosphoinositide hydrolysis, yielding diacylglycerol and inositol triphosphate (IP$_3$) to trigger protein kinase C (PKC) and Ca$^{2+}$-signaling pathways, respectively (Niswender and Conn, 2010). Noticeably, mGluR1 is enriched in the cerebellum (Martin et al., 1992) and is distributed mostly at perisynaptic and postsynaptic sites (Lujan et al., 1996; Kuwajima et al., 2004). Thus, mGluR1 plays a pivotal role in the regulation of synaptic transmission (Traynelis et al., 2010; Nicoletti et al., 2011). mGluR1 is regulated by a phosphorylation-dependent mechanism. Like many other membrane-bound GPCRs, mGluR1 has four intracellular domains, including three intracellular loops (ILs; IL1, IL2, and IL3) and a C-terminal tail. It is the C terminus (CT) that is large in size (359 aa in a long-form splice-variant mGluR1a) and is sufficient to provide a space for protein-protein interactions. In fact, a number of submembranous proteins have been identified to interact with mGluR1 CT (Enz, 2007; 2012; Fagni, 2012). One group of noticeable mGluR1 interacting partners is protein kinases. These kinases are thought to phosphorylate specific residues in mGluR1a CT and thereby modulate function of the modified receptors (Kim et al., 2008; Mao et al., 2011). However, responsible kinases and detailed mechanisms underlying their regulatory roles are poorly understood.

Proteins can be phosphorylated at tyrosine sites by receptor or nonreceptor tyrosine kinases. Src family kinases (SFKs), a subfamily of nonreceptor tyrosine kinases (Neet and Hunter, 1996), have been studied mostly for their roles in phosphorylating and regulating proteins. Five SFK members of a total of nine are expressed in the brain (Mao and Wang, 2016). Among these five SFK members, Fyn (isoform 1, also known as FynB), a 59 kDa protein, is of particular interest (Cooke and Perlmutter, 1989; Saito et al., 2010). This kinase is enriched at synaptic structures. Thus, Fyn is thought to function at synaptic sites and act as a key regulator in synaptic transmission and plasticity (Neet and Hunter, 1996). Indeed, Fyn tyrosine-phosphorylates ionotropic glutamate receptors and other synaptic proteins, thereby modulating the expression and function of these receptors/proteins and synaptic signaling (Ohnishi et al., 2011; Schenone et al., 2011). However, at present, whether and how Fyn regulates mGluRs remains elusive.

This study therefore explored possible Fyn–mGluR interactions. We found that mGluR1a is a novel substrate of Fyn. Recombinant Fyn directly binds to mGluR1a at its CT region in vitro. Endogenous Fyn forms complexes with mGluR1a in rat cerebellar neurons in vivo. Active Fyn phosphorylates the mGluR1a CT at a specific tyrosine site. This phosphorylation is functionally relevant as it significantly modulates surface expression and mGluR1a signaling in cerebellar neurons and HEK293T cells. Together, we have discovered a previously unrecognized Fyn–mGluR1a coupling that is involved in the regulation of mGluR1a.

### Materials and Methods

#### Animals

Adult male Wistar rats (weight, 200–300 g) were purchased from Charles River. Animals were individually housed at a temperature of 23°C and a humidity of 50 ± 10% with food and water available ad libitum. The animal room was on a 12 h light/dark cycle with lights on at 7:00 A.M. All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

### Glutathione S-transferase fusion protein synthesis

Glutathione S-transferase (GST) fusion proteins were synthesized. The cDNA fragments encode mGluR1a-CT1 (K841–T1000), mGluR1a-CT2 (P1001–L1199), mGluR1a-CT1a (K841–N885), mGluR1a-CT1b (A886–K931), mGluR1a-CT1c (N925–T1000), mGluR1a-IL1 (R618–E629), mGluR1a-IL2 (K678–Q706), mGluR1a-IL3 (K773–K785), GluA1-CT (E809–L889), and GluA2-CT (E834–I883). These cDNA fragments were generated by PCR amplification from full-length (FL) cDNA clones (rat mGluR1; UniProtKB accession #P23385) and subcloned into BamHI-EcoRI sites of the pGEX4T-3 plasmid (GE Healthcare). To confirm appropriate splice fusion, constructs were sequenced. GST fusion proteins were expressed in Escherichia coli BL21 cells and purified as described by the manufacturer.

#### Cell cultures and transfection

HEK293T cells were cultured in Eagle’s Minimum Essential Medium from American Type Culture Collection at 37°C with 5% CO$_2$. The medium contains 10% fetal bovine serum and 1% penicillin-streptomycin (Sigma-Aldrich). Transfections were conducted on an ~70–80% confluent monolayer using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer instructions. Experiments were performed 18 h after the transfection. Mammalian expression vectors for expressing proteins with a FLAG tag include FL rat mGluR1a and Fyn wild type (WT) in pcDNA3.1 + C(K)DYK vectors bearing the CMV promoter (GenScript) and the pcDNA3.1 + C(K)DYK empty vector control. Site-directed mutations were introduced into pcDNA3.1 (+) constructs containing mGluR1a or Fyn by a QuikChange site-directed mutagenesis kit (Stratagene).

#### Affinity purification (pull-down) assay

Pull-down assays were conducted with solubilized rat cerebellar lysates (50–100 µg) according to the procedures described previously (Liu et al., 2009; Guo et al., 2010). At least three experiments were performed for each analysis.

#### In vitro binding assay

Recombinant His-tagged active Fyn (FynB) with an FL of 537 aa (10 ng; Millipore), His-tagged panxillin (10 ng; RayBiotech), FLAG-tagged focal adhesion kinase (Fak; 10 ng) or FLAG-tagged Fyn mutant (Y531F or K299M) was equilibrated to binding buffer containing 200 mM NaCl, 0.2% Triton X-100, 0.1 mg/ml bovine serum albumin (BSA), and 50 mM Tris, pH 7.5. Binding reactions were initiated by adding purified GST fusion proteins and continued for 2–3 h at 4°C. We then used glutathione Sepharose 4B beads (10%, 100 µl) to precipitate GST fusion proteins.
proteins. After 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 the antibodies indicated.

**Phosphorylation assays in vitro**

GST fusion proteins or His-tagged paxillin (0.1—0.5 μM) were incubated with recombinant active Fyn (Sigma-Aldrich) or Fak (Creative Biomart) for 30 min (30°C) in a reaction buffer (25 μl) containing 10 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM Na₃VO₄, 1 mM dithiothreitol, 0.1 mg/ml BSA, and 50 μM ATP. An amount of 2.5 μCi/tube [γ-³²P]ATP (~3000 Ci/mmol; PerkinElmer) was added for autoradiography. Phosphorylation reactions were stopped by adding and boiling the LDS sample buffer (3 min). Phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography or immunoblotting. The amount of radioactivity that was incorporated into the substrate bands was assessed by liquid scintillation counting of the radioactive substrate bands excised from the gels. At least three experiments were performed for each analysis. Phosphorylation stoichiometry was determined by the amount of radioactive phosphate incorporated into substrate (mol phosphate/mol substrate).

**Dephosphorylation reactions**

GST fusion proteins that were phosphorylated by the above procedure were precipitated, and the supernatant containing Fyn was removed. Precipitates were washed twice and were 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).
The suspension was incubated for 1 h at 37°C. Dephosphorylation reactions were stopped by adding an LDS sample buffer. Samples were then subjected to standard gel electrophoresis and immunoblotting.

**Coimmunoprecipitation**

The rat cerebellum was removed after anesthesia and decapitation and was homogenized in cold isotonic homogenization buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific). Lysates were then processed at 4°C. After centrifugation (800 × g, 10 min), the supernatant was centrifuged again (10,000 × g, 15 min) to get P2 synaptosomal pellets. P2 pellets were solubilized in the buffer containing Triton X-100 (0.5%, v/v), 1% sodium deoxycholate, and a protease/phosphatase inhibitor cocktail for 1 h. After centrifugation, the solubilized supernatant was used for coimmunoprecipitation. HEK293T cells were lysed and solubilized in RIPA buffer (Sigma-Aldrich). Solubilized supernatant proteins after centrifugation were used for coimmunoprecipitation.

**Cerebellar slice preparation**

After rats were anesthetized and decapitated, rat brains were removed. The cerebellum was cut into coronal slices (300 μm) using a VT1200S vibratome (Leica). Slices were preincubated in artificial CSF (ACSF) containing the following (in mM): 10 glucose, 124 NaCl, 3 KCl, 1.25 KH2PO4, 26 NaHCO3, 2 MgSO4, and 2 CaCl2, bubbled with 95% O2/5% CO2, pH 7.4, in an incubation tube at 30°C under constant oxygenation with 95% O2/5% CO2 for 60 min. Additional preincubation after the solution was replaced with fresh ACSF was made for 10–20 min. Drugs were added and incubated at 30°C. Slices were collected after drug treatment for neurochemical assays.
Surface protein biotinylation

Surface protein biotinylation on brain slices was performed following established protocols (Kato et al., 2012; Knackstedt et al., 2013; Pabba et al., 2014). Briefly, rat cerebellar slices (300 μm) after drug treatments were incubated in ACSF containing 1 mg/ml EZ-LINK-Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific) for 45 min at 4°C. To biotinylate surface proteins of HEK293T cells, cells were incubated with EZ-LINK-Sulfo-NHS-SS-Biotin for 30 min at 4°C. Unreacted biotinylation reagent was removed by washing and was quenched by 100 mM glycine. Slices and HEK293T cells were homogenized by sonication in an HEPES-Triton-SDS lysis buffer containing the following (in mM): 25 HEPES, 150 NaCl, 1% Triton X-100, 0.5% SDS, and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific). After centrifugation (1000 × g, 10 min) at 4°C, the supernatant was collected and used as the total protein fraction. An equal aliquot of total proteins was incubated with neutrAvidin resin (Thermo Fisher Scientific) overnight at 4°C. Biotinylated proteins (i.e., surface proteins) were precipitated by centrifugation and were then eluted with an LDS sample buffer (boiling for 3 min). The abundance of proteins of interest in surface and total fractions was analyzed by immunoblotting.

Western blot
To separate proteins, SDS NuPAGE Bis-Tris 4–12% gels (Invitrogen) were used. Separated proteins were transferred to polyvinylidene fluoride membranes. We then incubated membranes with primary antibodies overnight at 4°C, which was followed by incubation with secondary antibodies. An enhanced chemiluminescence reagent (GE Healthcare) was used to develop immunoblots.

IP3 assays
Intracellular IP3 levels in rat cerebellar slices or HEK293T cells were measured using a HitHunter IP3 Fluorescence Polarization Assay Kit from DiscoveRx or a rat
IP3 ELISA kit from CUSABIO following the manufacturer instructions (Jin et al., 2013; Cansev et al., 2015; Tabat-adze et al., 2015).

**Antibodies and pharmacological agents**

The antibodies we used in this study include rabbit antibodies against mGluR1a (Millipore), Src with phosphorylated tyrosine 416 (pan-pY416, Cell Signaling Technology), Fyn (Cell Signaling Technology), Src (Cell Signaling Technology), phosphotyrosine (pY; Millipore), Fak (Cell Signaling Technology), paxillin (Cell Signaling Technology), FLAG (Cell Signaling Technology), or β-actin (Sigma-Aldrich), or mouse antibodies against mGluR1a (BD), Fyn (Santa Cruz Biotechnology), pY (PY20, BD), or transferrin receptors (TfRs; Thermo Fisher Scientific). The antibody against pY416 reacts with the following Src family members when autophosphorylated at a conserved activation residue: Y416 (chicken Src), Y419 (rat Src), and Y420 (rat Fyn). Pharmacological agents, including (S)-3,5-dihydroxyphenylglycine (DHPG), 3-methyl-aminothiophene dicarboxylic acid (3-MATIDA), 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-

d]pyrimidin-4-amine (PP2), 1-phenyl-1H-pyrazolo[3,4-
d]pyrimidin-4-amine (PP3), and oxotremorine-M, were purchased from Sigma-Aldrich. All drugs were freshly prepared on the day of the experiments.

**Statistics**

The results in this study are presented as the mean ± SEM. Data were evaluated using the Student’s t test or a one-way ANOVA followed by a Bonferroni (Dunn) comparison of groups using least-squares-adjusted means. Probability levels of <0.05 were considered to be statistically significant.

**Results**

Phosphorylation of mGluR1a by Fyn

Intracellular domains of mGluR1a include IL1, IL2, IL3, and CT. Notably, only the CT region contains tyrosine residues. To explore possible phosphorylation at any of these tyrosine residues, we synthesized two GST fusion recombinant proteins covering the different segments of CT [i.e., mGluR1a-CT1(K841-T1000) and mGluR1a-CT2(P1001-L1199)] in
addition to a GST protein (Fig. 1A). These three proteins were then used for testing their tyrosine phosphorylation in response to active Fyn in phosphorylation assays in vitro. Using an anti-phosphotyrosine antibody, we found phosphorylation signals in GST-mGluR1a-CT1 but not GST-mGluR1a-CT2 and GST alone (Fig. 1B). Similarly, through monitoring the incorporation rate of 32P into the proteins, we detected phosphorylation in CT1 but not CT2 (Fig. 1C). Stoichiometric ratios of 0.48 ± 0.02 and 0.89 ± 0.05 mol phosphate/mol CT1 were obtained after 2 and 30 min of incubation, respectively. Phosphorylation was also seen in GST-GluA2-CT (Fig. 1D).

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There are two tyrosine sites in mGluR1a-CT1: tyrosine 937 (Y937) and tyrosine 955 (Y955; Fig. 2A). We next tested the phosphorylation response of these two sites to Fyn to identify the accurate phosphorylation site. To this end, we synthesized CT1 proteins carrying site-directed mutations (Fig. 2B). We then compared these mutants with WT CT1 in phosphorylation reactions. Noticeably, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C). In contrast, the mutation of Y937 to phenylalanine (mutation 1, Y937F) abolished CT1 phosphorylation as detected by 32P incorporation (Fig. 2C).
responses were detected by an anti-pY antibody (Fig. 2D).

Evidently, Y937 is a primary site subjected to phosphorylation by Fyn, while Y955 is insensitive to Fyn. Amino acid alignment analysis reveals conservation of Y937 in the human, rat, and mouse mGluR1a.

Binding activity between Fyn and mGluR1a

Fyn binds to many of its known substrates (Knox and Jiang, 2015). To determine whether Fyn binds to mGluR1a, we conducted a series of protein–protein binding assays. In these assays, we used GST fusion proteins containing discrete intracellular domains of mGluR1a as immobilized molecules to precipitate a target binding partner [i.e., Fyn]. In pull-down assays, GST-mGluR1a-CT1 precipitated native Fyn from adult rat cerebellar lysates, while GST alone and other GST fusion proteins containing IL1, IL2, IL3, and CT2 did not (Fig. 3A). No GST fusion proteins precipitated Fak (Fig. 3A). Fyn is predominantly expressed in the cerebellum (Umemori et al., 1992; Bare et al., 1993), and meanwhile Fak is also expressed in this region (Burgaya et al., 1995; Megeney et al., 1999). To determine whether Fyn directly binds to mGluR1a, we performed in vitro binding assays with purified Fyn and mGluR1a proteins. GST-mGluR1a-CT1 bound to and precipitated Fyn (Fig. 3B). GST alone and other GST fusion proteins showed no precipitation of Fyn. Fak was not precipitated by any GST fusion proteins (Fig. 3C). GST-Fak precipitated its known substrate paxillin (Fig. 3D). These results support that Fyn binds to mGluR1a at the CT1 region of the receptor.

We next compared active and inactive Fyn for their binding activity to mGluR1a-CT1. As shown in Figure 3E, a constitutively active form of Fyn (i.e., Fyn-Y531F; Cheng et al., 1991), bound to CT1. So did an inactive form of Fyn [i.e., a kinase-dead and dominant-negative mutant of Fyn (K299M); Twamley et al., 1992; Twamley-Stein et al., 1993]. The inactive form of K299M was confirmed by the lack of phosphorylation at a conserved and activation-associated autophosphorylation site (Y416; Roskoski, 2005; Okada, 2012). To further narrow down the binding region, we tested different parts of CT1 in their binding activity to Fyn. CT1c (N925–T1000) precipitated Fyn (Fig. 3F). In contrast, CT1a (K841–N885) and CT1b (A886–K931) did not. Thus, CT1c is a core region harboring Fyn. Interestingly, the Fyn-mediated phosphorylation site Y937 resides within this region. Adjacent to Y937, there are three consensus proline-rich motifs (PXXP, where “X” represents any amino acid; Fig. 3G) where Fyn could bind via its Src-homology 3 (SH3) domain (Ren et al., 1993; Hauck et al., 2001), a highly conserved platform among SFKs for protein–protein interactions (Saïto et al., 2010).

As expected, CT1c, but not CT1a and CT1b fragments, was tyrosine phosphorylated by Fyn (Fig. 3H).

Interactions of Fyn with mGluR1a in cerebellar neurons

Our next attempt was to determine whether Fyn interacts with mGluR1a in neurons. This was examined in coimmunoprecipitation assays using the rat cerebellar tissue because mGluR1 but not mGluR5 is expressed in the cerebellum (Martin et al., 1992; Shigemoto et al., 1993) and Purkinje neurons, the principal neurons of cerebellar cortex, express mGluR1a as a predominant splice variant (Fotuhi et al., 1993). In a coimmunoprecipitation assay in which an anti-mGluR1a antibody was added into solubilized synaptosomal protein samples (P2), we observed strong mGluR1a immunoreactivity (Fig. 4A). Noticeably, Fyn immunoreactivity was also exhibited in mGluR1a precipitates, indicating that endogenous Fyn and mGluR1a form complexes in cerebellar neurons in vivo. This notion is supported by a reverse coimmunoprecipitation assay in which an anti-Fyn antibody was added to precipitate Fyn. mGluR1a immunoreactivity was seen in Fyn precipitates (Fig. 4B). To determine whether native mGluR1a in cerebellar neurons is tyrosine phosphorylated, we immunoprecipitated pY proteins from the cerebellum using an anti-pY antibody. In this defined pool of pY proteins, we found a detectable amount of mGluR1a (Fig. 4C). In immunoprecipitated mGluR1a proteins, we also saw pY signals (Fig. 4D). Thus, mGluR1a in cerebellar neurons is among a subset of proteins subjected to tyrosine phosphorylation under normal conditions.

Phosphorylation of cerebellar mGluR1a by SFKs

To determine whether tyrosine phosphorylation of mGluR1a in cerebellar neurons is mediated by SFKs, we investigated the impact of inhibition of SFKs on mGluR1a tyrosine phosphorylation. To inhibit SFKs, we used a widely used SFK inhibitor, PP2 (Hanke et al., 1996). We first evaluated the efficacy and potency of PP2 in inhibiting SFKs in a concentration–response study in rat cerebellar slices. Adding PP2 (0.01, 0.1, 1, and 10 μM, 30 min)
greatly reduced the phosphorylation of SFKs at Y416, while PP2 had no effect on a total amount of Fyn or Src (Fig. 5A). This effect was clearly concentration dependent. To test the effect of PP2 on specific Fyn, we immunoprecipitated Fyn proteins from the cerebellum after PP2 treatment (Fig. 5B). We then analyzed changes in pY416 signals in the immunoprecipitated fraction of Fyn proteins. PP2 (10 μM, 30 min) substantially reduced the pY416 level of Fyn (Fig. 5C). PP2, however, did not alter total Fyn expression. These results establish an effective concentration of PP2 in inhibiting Fyn in our brain slice model. We then used this concentration to examine the effect of PP2 on mGluR1a tyrosine phosphorylation.

Adding PP2 to cerebellar slices (10 μM, 30 min) considerably reduced tyrosine phosphorylation of mGluR1a. As shown in Figure 5D, mGluR1a immunoreactivity in pY precipitates was largely reduced in PP2-treated slices compared with vehicle control slices. In contrast, PP3, an inactive analog of PP2, did not alter mGluR1a levels in pY precipitates (Fig. 5E). Thus, a PP2-sensitive SFK acts as a central kinase responsible for maintaining constitutive tyrosine phosphorylation of mGluR1a in cerebellar neurons.

**Roles of SFKs in regulating surface expression of mGluR1a**

Tyrosine phosphorylation of mGluR1a by Fyn may have an impact on surface expression of the receptors. To evaluate this, we investigated whether PP2 alters surface levels of mGluR1a. A surface protein biotinylation protocol was used to monitor changes in the surface expression of mGluR1a (see Materials and Methods). In rat cerebellar slices, the application of PP2 (10 μM, 30 min) markedly reduced the amount of mGluR1a in the surface fraction (Fig. 6A). The total cellular levels of mGluR1a were not altered. A surface protein, the TfR, and an intracellular protein, β-actin, were measured as a surface and intracellular protein control, respectively. As shown in Figure 6B, TfRs were densely seen in surface and total fractions, whereas β-actin was not shown in surface fractions, demonstrating the selectivity of our biotinylation method in cross-linking surface proteins. No significant change in the surface levels of TfRs was seen following PP2 incubation (Fig. 6B). These results indicate a significant role of SFKs in determining the number of mGluR1a in the surface compartment of cerebellar neurons.
Roles of SFKs in regulating mGluR1-IP₃ signaling

To further explore the functional roles of tyrosine phosphorylation of mGluR1, we investigated the effect of PP2 on the mGluR1-associated signaling activity. Activation of mGluR1 increases phosphoinositide hydrolysis, yielding a key signaling molecule, IP₃ (Niswender and Conn, 2010; Traynelis et al., 2010). We thus measured the mGluR1-induced IP₃ yield as function of mGluR1. DHPG, an mGluR1/5 agonist, induced a typical increase in cytosolic IP₃ levels after it was added to cerebellar slices (50 μM, 20 s; Fig. 7A). This increase was completely blocked by an mGluR1-selective antagonist, 3-MATIDA (10 μM; data not shown), verifying the role of mGluR1 in mediating the DHPG-stimulated IP₃ production. Pretreatment with PP2 (1 μM, 30 min before DHPG) significantly reduced IP₃ responses to DHPG. A greater reduction in IP₃ responses was seen following pretreatment with PP2 at a higher concentration (10 μM). In contrast to PP2, PP3 at two concentrations (1 and 10 μM) had no significant influence over IP₃ responses to DHPG (Fig. 7B). Neither PP2 nor PP3 altered basal levels of IP₃. These results indicate that the inhibition of SFKs leads to a reduction in mGluR1-mediated IP₃ production.

Muscarinic acetylcholine receptors (mAChRs) are expressed in the cerebellum (Whitham et al., 1991; Tayebati et al., 2001; Billups et al., 2006). Of the five mAChR subtypes, M₁, M₃, and M₅ subtypes are Gq-coupled receptors and are linked to the activation of PLCβ1 (Felder, 1995; Wess, 1996). The activation of Gq-coupled mAChRs, predominantly M₃ receptors, present on cerebellar Purkinje neurons with a mAChR agonist oxotremorine-M blocked long-term potentiation at cerebellar parallel fiber–Purkinje cell synapses (Rinaldo and Hansel, 2013). In this study, we found that oxotremorine-M (10 μM, 20 s) elevated IP₃ levels in cerebellar slices (Fig. 7C). This elevation was not affected by PP2. Thus, SFK activity is not involved in the regulation of IP₃ responses to mAChR activation.

We next synthesized a cell-permeable, Tat-fusion peptide that contains the proline-rich motifs (PXXP) in the CT₁c fragment (Fig. 7D). This peptide was designed to interfere with the Fyn–mGluR1a interaction and was thus named as an interaction-dead (Tat-mGluR1a-id) peptide. In fact, the application of Tat-mGluR1a-id (10 μM, 45 min) significantly reduced the interaction of Fyn with mGluR1a in cerebellar slices, while a sequence-scrambled control peptide (Tat-scramble) did not (Fig. 7E). This establishes the importance of these proline-rich motifs for the binding of Fyn to mGluR1a. Of note, Tat-mGluR1a-id reduced the IP₃ response to DHPG, while Tat-scramble had no effect (Fig. 7F). Thus, the Fyn–mGluR1a interaction is critical for the mGluR1a–IP₃ signaling.

Fyn regulates mGluR1a in HEK293T cells

The role of Fyn in phosphorylating and regulating mGluR1a was further analyzed in HEK293T cells. FLAG-tagged mGluR1a (WT or Y937F, a phosphorylation-deficient mutant due to the mutation of tyrosine 937 to phenylalanine) and Fyn (Y531F or K299M) were readily transfected in HEK293T cells (Fig. 8A). The amount of total mGluR1a protein (WT or Y937F) seemed to remain...
stable after cotransfected with either the constitutively active Fyn (Y531F) or inactive Fyn (K299M). The active and inactive states of Fyn-Y531F and Fyn-K299M, respectively, were confirmed by the detection of pY416 signals in Fyn-Y531F but not K299M. B, DHPG-stimulated IP₃ production in HEK293T cells cotransfected with mGluR1a. C, DHPG-stimulated IP₃ production in HEK293T cells cotransfected with mGluR1a and active and inactive Fyn. Note that the DHPG-stimulated IP₃ production was enhanced by cotransfection with Y531F but not K299M. D, Effects of the site-directed mutation at mGluR1a-Y937 on the Fyn-induced surface expression of mGluR1a. mGluR1a WT or Y937 phosphorylation-deficient mutant (Y937F) was cotransfected with active Fyn (Y531F). Note that the mutation of Y937 abolished the active Fyn-induced increase in surface expression of mGluR1a. E, Effects of the site-directed mutation at mGluR1a-Y937 on the Fyn-induced increase in IP₃ responses to DHPG. Note that cotransfection with active Fyn (Y531F) augmented IP₃ responses to DHPG in HEK293T cells cotransfected with WT but not Y937F mGluR1a. Representative immunoblots are shown to the left of the quantified data (A, D). Proteins were visualized by immunoblots (IB). In IP₃ assays, DHPG (50 μM) was added and cells were collected 20 s after DHPG incubation. Data are presented as the mean ± SEM (n = 3-6/group) and were analyzed by one-way ANOVA. *p < 0.05 vs vector (A, B, D) or vector plus vehicle (C, E). +p < 0.05 vs vector plus DHPG (C, E).

Figure 9. Surface expression of mGluR1a and agonist-stimulated IP₃ production in HEK293T cells. A, Effects of cotransfection of Fyn on the surface expression of mGluR1a. mGluR1a was cotransfected with active (Y531F) or inactive (K299M) Fyn. Note that surface expression of mGluR1a was elevated by cotransfection with Y531F but not K299M. B, DHPG-stimulated IP₃ production in HEK293T cells transfected with mGluR1a. C, DHPG-stimulated IP₃ production in HEK293T cells cotransfected with mGluR1a and active and inactive Fyn. Note that the DHPG-stimulated IP₃ production was enhanced by cotransfection with Y531F but not K299M. D, Effects of the site-directed mutation at mGluR1a-Y937 on the Fyn-induced surface expression of mGluR1a. mGluR1a WT or Y937 phosphorylation-deficient mutant (Y937F) was cotransfected with active Fyn (Y531F). Note that the mutation of Y937 abolished the active Fyn-induced increase in surface expression of mGluR1a. E, Effects of the site-directed mutation at mGluR1a-Y937 on the Fyn-induced increase in IP₃ responses to DHPG. Note that cotransfection with active Fyn (Y531F) augmented IP₃ responses to DHPG in HEK293T cells cotransfected with WT but not Y937F mGluR1a. Representative immunoblots are shown to the left of the quantified data (A, D). Proteins were visualized by immunoblots (IB). In IP₃ assays, DHPG (50 μM) was added and cells were collected 20 s after DHPG incubation. Data are presented as the mean ± SEM (n = 3-6/group) and were analyzed by one-way ANOVA. *p < 0.05 vs vector (A, B, D) or vector plus vehicle (C, E). +p < 0.05 vs vector plus DHPG (C, E).
and mGluR1a-Y937F exhibited a similar level of surface and total expression (Fig. 9D). However, active Fyn-induced increases in surface expression of mGluR1a were seen only in WT, but not mutant mGluR1a (Y937F), indicating that Y937 phosphorylation is critical for this event. In IP3 assays, the Y937F mutation completely abolished an augmentation of IP3 responses to DHPG induced by the cotransfection of active Fyn (Fig. 9E). These results support that the phosphorylation of mGluR1a at Y937 is essential for Fyn to enhance mGluR1a signaling.

Discussion

This study was conducted to explore a new substrate of Fyn. We found that an mGluR subtype, mGluR1a, is a target of Fyn. Fyn directly bound to mGluR1a CT in vitro. Similarly, endogenous and synapse-enriched Fyn interacted with mGluR1a in rat cerebellar neurons. Active Fyn phosphorylated mGluR1a at a conserved tyrosine site (Y937) in the CT region. This phosphorylation was constitutively active. The phosphorylation at Y937 was an important step for promoting surface expression of mGluR1a. By regulating surface expression of the receptor, Fyn controlled the mGluR1a-associated signaling transduction. In sum, the results obtained in vitro and in vivo support mGluR1a as a novel synaptic substrate of Fyn. Through directly binding to and phosphorylating mGluR1a, Fyn regulates mGluR1a in surface expression and postreceptor signaling.

The Fyn–mGluR1a interaction is a new model discovered in this study. This interaction is characterized by occurring in mGluR1a CT, a region thought to be a major protein–protein interaction area. Indeed, mGluR1a CT is relatively large and is a binding domain for most mGluR1a-interacting partners so far identified (Enz, 2012; Fagni, 2012). As to the specific binding site in mGluR1a CT, proline-rich PXXP motifs that are preferentially recognized by a protein–protein interaction module (SH3 domain) of Fyn (Ren et al., 1993) exist in the CT1c region. Consistent with this, CT1c but not CT1a and CT1b bound to Fyn. Thus, a PXXP-containing zone in CT1c likely harbors a binding site to Fyn. This is supported by the finding that a PXXP-containing peptide derived from mGluR1a-CT1c reduced the association between Fyn and mGluR1a in cerebellar neurons (this study). In addition to the binding site in the CT region, tyrosine phosphorylation of mGluR1a induced by Fyn occurred at a residue (Y937) within the CT1c. Thus, the CT1c is a major region that Fyn interacts with to tyrosine phosphorylate and regulate the receptor.

Another important characteristic of the Fyn–mGluR1a coupling is its constitutively active nature. Following the demonstration of the binding between recombinant Fyn and mGluR1a proteins and tyrosine phosphorylation of mGluR1a in vitro, we investigated the interaction between two native proteins in neurons. In cerebellar neurons expressing Fyn (Uemori et al., 1992; Bare et al., 1993; Cioni et al., 2013) and mGluR1a (Martin et al., 1992; Shigemoto et al., 1993), the Fyn–mGluR1a complex was detected under basal conditions. In parallel with the basal Fyn–mGluR1a interaction, the Fyn-mediated tyrosine phosphorylation of mGluR1a occurred significantly under normal conditions. More importantly, the constitutively active phosphorylation is functionally relevant and contributes to the regulation of surface expression and signaling of mGluR1a under normal conditions (see below).

Fyn is enriched at synaptic sites and is conceived to play a critical role in regulating local synaptic proteins (Ohnishi et al., 2011; Schenone et al., 2011). Several substrates and binding partners of Fyn have been previously discovered at synaptic sites. Central among them is the NMDA glutamate receptor (Suzuki and Okumura-Noji, 1995; Köhr and Seeburg, 1996; Trepanier et al., 2012; Knox and Jiang, 2015). In addition, two prominent synaptic scaffold proteins, PSD-95 and PSD-93, are substrates of Fyn (Nada et al., 2003; Du et al., 2009). This study adds mGluR1a as another synaptic substrate of Fyn. The results from a series of biochemical and functional studies conducted in the present work are consistent with a notion that mGluR1a is subjected to the tyrosine phosphorylation and regulation by Fyn.

Phosphorylation is an important mechanism for the regulation of GPCRs. As a typical GPCR, mGluR1 is subjected to the regulation by a phosphorylation-dependent mechanism. Several common protein kinases have been implicated in this event (Dhami and Ferguson, 2006; Kim et al., 2008; Mao et al., 2008). For example, PKC is involved in the phosphorylation and regulation of mGluR1 based on a number of early studies (Manzoni et al., 1990; Catania et al., 1991; Thomsen et al., 1993; Alaluf et al., 1995; Medier and Bruch, 1999; Fratticci and Duvoisin, 2000). Calcium/calmodulin-dependent protein kinase II (CaMKII) is another kinase that contributes to the regulation of mGluR1a. By phosphorylating threonine 871 near the G-protein-coupling domain of mGluR1a (Dhami and Ferguson, 2006), CaMKII serves as an important element in forming a feedback loop that facilitates the agonist-induced desensitization of mGluR1a in striatal neurons (Jin et al., 2013). However, both PKC and CaMKII catalyze phosphorylation at serine and threonine residues. Whether a kinase that phosphorylates proteins at a tyrosine site is involved in the regulation of mGluR1 is unclear. This study provides evidence supporting a new tyrosine kinase–GPCR model that a synapse-enriched tyrosine kinase Fyn acts as a key regulator of mGluR1. However, unlike the activity-dependent and inhibitory nature of the regulation of mGluR1 by PKC and CaMKII, Fyn functions significantly under basal conditions and facilitates the receptors in their expression and postreceptor signaling. More specifically, in cerebellar neurons where mGluR1a and Fyn are predominantly expressed (Uemori et al., 1992; Bare et al., 1993; Fotuhi et al., 1993), Fyn constitutively binds to the largest intracellular domain of mGluR1a, which may serve to accumulate the kinase at synaptic sites under basal conditions. Synaptic Fyn then phosphorylates a tyrosine residue in mGluR1a to promote steady-state surface expression of the receptors and thereby maintain the activity level of mGluR1a signaling. Fyn may regulate trafficking, endocytosis, dimerization, or other steps important for surface expression of mGluR1a to modulate the number of the receptors in the
surface compartment, although exact underlying mechanisms are unclear at present. Future studies need to clarify accurate mechanisms underlying the impact of the Fyn phosphorylation of mGlurR1a on the surface expression of the receptors.

References

Alauf S, Muhlvihi ER, McIlhinney RA (1995) Rapid agonist mediated phosphorylation of the metabotropic glutamate receptor 1α by protein kinase C in permanently transfected BHK cells. FEBS Lett 367:301–305. CrossRef
Bare DJ, Lauder JM, Wilkie MB, Maness PF (1993) p59fyn in rat brain is localized in developing axonal tracts and subpopulations of adult neurons and glia. Oncogene 8:1429–1436. Medline
Bilups D, Bilups B, Challiss RA, Nahorski SR (2006) Modulation of Gq-protein-coupled inositol trisphosphate and Ca²⁺ signaling by the membrane potential. J Neurosci 26:9983–9995. CrossRef Medline
Burgaya F, Menegon A, Menegoz M, Valtorta F, Girault JA (1995) Special phosphomotif antibody. Mol Cell Proteomics 8:681–695. CrossRef Medline
Cheng SH, Espino PC, Marshall J, Harvey R, Merrill J, Smith AE (1991) Structural elements that regulate pp59tyc catalytic activity, transforming potential, and ability to associate with polyoma virus middle-T antigen. J Virol 65:170–179. Medline
Cioni JM, Tellely L, Saywell V, Cadilhac C, Jourdan C, Huber AB, Huang JZ, Jahnnaault-Taligiani C, Ano F (2013) SEMA3A signaling controls layer-specific interneuron branching in the cerebel. Curr Biol 23:850–861. CrossRef Medline
Cooko MP, Perlmutter RM (1989) Expression of a novel form of the p59fyn proto-oncogene in hematopoietic cells. New Biol 1:66–74. Medline
Dhami GK, Ferguson SS (2006) Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis. Pharmacol Ther 111:260–271. CrossRef Medline
Du CP, Gao J, Tai JM, Liu Y, Qi J, Wang W, Hou XY (2009) Increased phosphorylation of the metabotropic glutamate receptor 1 by calcium/calmodulin-dependent protein kinase II. J Neurosci 33:3402–3412. CrossRef Medline
Enz R (2007) The trick of the tail: protein-protein interactions of the Src family. J Physiol 492:445–452. CrossRef Medline
Fyn in neurodevelopment and ischemic brain injury. Dev Neurosci 37:311–320. CrossRef Medline
Enz R, Neuman BA, Wightman RM, Seldin MF (2012) Glutamate receptor iGluRs in the monkey subthalamic nucleus. J Comp Neurol 45:445–452. CrossRef Medline
Enz R (2012) Metabotropic glutamate receptors and interacting proteins: evolving drug targets.Curr Drug Targets 13:145–156. Medline
Fagni L (2012) Diversity of metabotropic glutamate receptor-interacting proteins and pathophysiological functions. Adv Exp Med Biol 970:63–79. CrossRef Medline
Felder CC (1995) Muscarinic acetylcholine receptors: signal transduction through multiple effectors. FASEB J 9:619–625. Medline
Futuhi M, Sharp AH, Glatt CE, Hwang PM, von Krosigk M, Snyder SH, Dawson TM (1993) Differential localization of phosphoinositide-linked metabotropic glutamate receptor (mGlur1) and the inositol 1,4,5-trisphosphate receptor in rat brain. J Neurosci 13:2001–2012. CrossRef Medline
Francesconi A, Duvoisin RM (2000) Opposing effects of protein kinase C and protein kinase A on metabotropic glutamate receptor signaling: selective desensitization of the inositol trisphosphate/ Ca²⁺ pathway by phosphorylation of the receptor-G protein-coupling domain. Proc Natl Acad Sci U S A 97:6185–6190. CrossRef Medline
Guo ML, Fibuch EE, Liu XY, Choe ES, Buch S, Mao LM, Wang JQ (2010) CaMKIIα interacts with M4 muscarinic receptors to control receptor and psychomotor function. EMBO J 29:2070–2081. CrossRef Medline
Hanke JH, Gardener JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 271:695–701. Medline
Hauck CR, Hunter T, Schlaepfer DD (2001) The v-Src SH3 domain facilitates a cell adhesion-independent association with focal adhesion kinase. J Biol Chem 276:17653–17662. CrossRef Medline
Haussay T, Huganir RL (2004) Tyrosine phosphorylation and regulation of the AMPA receptor by Src family tyrosine kinases. J Neurosci 24:6152–6160. CrossRef Medline
Ino D, Guo ML, Xue B, Fibuch EE, Choe ES, Mao LM, Wang JQ (2013) Phosphorylation and feedback regulation of metabotropic glutamate receptor 1 by calcium/calmodulin-dependent protein kinase II. J Neurosci 33:3402–3412. CrossRef Medline
Kato AS, Knierman MD, Sluda ER, Isaac JT, Nisenbaum ES, Bredt DS (2012) Glutamate receptor iGluRs and protein kinase C and protein kinase A on metabotropic glutamate receptor 1 and 2. J Neurosci 32:15296–15308. CrossRef Medline
Kim CH, Lee J, Lee JY, Roche KW (2008) Metabotropic glutamate receptors: phosphorylation and receptor signaling. J Neurosci Res 86:1–10. CrossRef Medline
Knickstedt LA, Trantham-Davidson HL, Schwendt M (2013) The role of ventral and dorsal striatum mGluRs in relapse to cocaine-seeking and extinction learning. Addict Biol 18:97–101. CrossRef Medline
Lujan R, Jiang X (2015) Fyn in neurodevelopment and ischemic brain injury. Dev Neurosci 37:311–320. CrossRef Medline
Körögh G, Seeburg PH (1996) Subtype-specific regulation of recombinant NMDA receptor-channels by protein tyrosine kinases of the src family. J Physiol 492:445–452. CrossRef Medline
Kuwajima H, Hall RA, Aiba A, Smith Y (2004) Subcellular and sub-synaptic localization of group I metabotropic glutamate receptors in the monkey subthalamic nucleus. J Comp Neurol 474:589–602. CrossRef Medline
Liu XY, Mao LM, Zhang GC, Papasian CJ, Fibuch EE, Lan LX, Zhou HF, Xu M, Wang JQ (2009) Activity-dependent modulation of limbic dopamine D3 receptors by CaMII. Neurosci 61:425–458. CrossRef Medline
Lujan R, Nusser Z, Roberts JD, Shimogori R, Somogyi P (1996) Perisynaptic localization of metabotropic glutamate receptors mGlur1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. Eur J Neurosci 8:1488–1500. Medline
Manzoni QJ, Finiels-Marlier F, Sassetti I, Blockaert J, le Peuch C, Sladecek FA (1990) The glutamate receptor of the Qp-type activates protein kinase C and is regulated by protein kinase C. Neurosci Lett 109:146–151. Medline
Mao LM, Wang JQ (2016) Tyrosine phosphorylation of glutamate receptors by non-receptor tyrosine kinases: roles in depression-like behavior. Neurotransmitter in vivo. Neuropharmacol 55:403–408. CrossRef Medline
Mao LM, Guo ML, Jin DZ, Fibuch EE, Choe ES, Mao LM, Wang JQ (2011) Posttranslational modification biology of glutamate receptors and drug addiction. Front Neuroanat 5:19. CrossRef Medline
Martin LJ, Blackstone CD, Huganir RL, Price DL (1992) Cellular localization of a metabotropic glutamate receptor in rat brain. Neuron 9:259–270. Medline
Medler KF, Bruch RC (1999) Protein kinase Cbeta and delta selectively phosphorylate odorant and metabotropic glutamate receptors. Chem Senses 24:295–299. Medline

Menegon A, Burgaya F, Baudot P, Dunlap DD, Girault JA, Valtorta F (1999) FAK and PYK2/CAKβ, two related tyrosine kinases highly expressed in the central nervous system: similarities and differences in the expression pattern. Eur J Neurosci 11:3777–3788. CrossRef

Nada S, Shima T, Yanai H, Husi H, Grant SG, Okada M, Akiyama T (2003) Identification of PSD-93 as a substrate of the Src family tyrosine kinase Fyn. J Biol Chem 278:47610–47621. CrossRef Medline

Neet K, Hunter T (1996) Vertebrate non-receptor protein-tyrosine kinase families. Genes Cell 1:147–169. Medline

Nicoletti F, Bockaert J, Collingridge GL, Conn PJ, Ferraguti F, Schoepp DD, Wroblewski JT, Pin JP (2011) Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 60:1017–1041. CrossRef Medline

Niswender CM, Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol 50:295–322. CrossRef Medline

Ohnishi H, Murata Y, Okazawa Y, Matozaki T (2011) Src family kinases: modulators of neurotransmitter receptor function and behavior. Trends Neurosci 34:629–637. CrossRef

Okada M (2012) Regulation of the Src family kinase by Csk. Int J Biol Sci 8:1385–1397. CrossRef Medline

Orlando LR, Ayala R, Kett LR, Curley AA, Duffner J, Bragg DC, Tsai LH, Dunah AW, Young AB (2009) Phosphorylation of the homobinding domain of group I metabotropic glutamate receptors by cyclin-dependent kinase 5. J Neurochem 110:557–569. CrossRef

Pabba M, Wong AYC, Ahlskog N, Hristova E, Biscaro D, Nassrallah W, Ngsae JK, Snyder M, Beique JC, Bergeron R (2014) NMDA receptors are upregulated and trafficked to the plasma membrane after sigma-1 receptor activation in the rat hippocampus. J Neurosci 34:11325–11338. CrossRef Medline

Panetti TS (2002) Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration. Front Biosci 7:d143–d150. Medline

Ren R, Mayer BJ, Cicchetti P, Baltimore D (1993) Identification of a ten-amino acid proline-rich SH3 binding site. Science 259:1157–1161. Medline

Rinaldo L, Hansel C (2013) Muscarinic acetylcholine receptor activation blocks long-term potentiation at cerebellar parallel fiber-Purkinje cells synapses via cannabinoid signaling. Proc Natl Acad Sci U S A 110:11181–11186. CrossRef Medline

Roskoski R Jr (2005) Src kinase regulation by phosphorylation and dephosphorylation. Biochem Biophys Res Commun 331:1–14. CrossRef Medline

Saito YD, Jensen AR, Salgia R, Posadas EM (2010) Fyn: a novel molecular target in cancer. Cancer 116:1629–1637. CrossRef Medline

Schenone S, Brullo C, Musumeci F, Biava M, Falchi F, Botta M (2011) Fyn kinase in brain diseases and cancer: the search for inhibitors. Curr Med Chem 18:2921–2942. Medline

Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, Mizuno N (1993) Immunohistochemical localization of a metabotropic glutamate receptor, mGlur5, in the rat brain. Neurosci Lett 163:53–57. Medline

Suzuki T, Okumura-Noji K (1995) NMDA receptor subunits epsilon 1 (NR2A) and epsilon 2 (NR2B) are substrates for Fyn in the post-synaptic density fraction isolated from the rat brain. Biochem Biophys Res Commun 216:582–588. Medline

Tabatake N, Huang G, May RM, Jain A, Woolley CS (2015) Sex differences in molecular signaling at inhibitory synapses in the hippocampus. J Neurosci 35:11252–11265. Medline

Tayebati SK, Vitali D, Scordella S, Amenta F (2001) Muscarinic cholinergic receptors subtypes in rat cerebellar cortex: light microscope autoradiography of age-related changes. Brain Res 889:256–259. Medline

Thomsen C, Mulvihill ER, Haldeman B, Pickering DS, Hampson DR, Suzdak PD (1993) A pharmacological characterization of the mGlur1α subtype of the metabotropic glutamate receptor expressed in a cloned baby hamster kidney cell line. Brain Res 619:22–28. CrossRef

Traynelis SF, Wollmith LP, McBain CJ, Menniti ES, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62:405–496. CrossRef Medline

Trepanier CH, Jackson MF, MacDonald JF (2012) Regulation of NMDA receptors by the tyrosine kinase Fyn. FEBS J 279:12–19. CrossRef Medline

Twamley GM, Kytta RM, Hall B, Courtenide SA (1992) Association of Fyn with the activated platelet-derived growth factor receptor: requirements for binding and phosphorylation. Oncogene 7:1893–1901. Medline

Twamley-Stein GM, Pepperkok R, Ansoerg W, Courtenide SA (1993) The Src family tyrosine kinases are required for platelet-derived growth factor-mediated signal transduction in NIH 3T3 cells. Proc Natl Acad Sci U S A 90:7696–7700. Medline

Umehori H, Wanaka A, Kato H, Takeuchi M, Toyahama Y, Yamamoto T (1992) Specific expression of Fyn and Lyn, lymphocyte antigen receptor-associated tyrosine, in the central nervous system. Mol Brain Res 16:303–310. Medline

Wess J (1996) Molecular biology of muscarinic acetylcholine receptors. Crit Rev Neurobiol 10:69–99. Medline

Whitham EM, Challiss RA, Nahorski SR (1991) M3 muscarinic cholinceptors are linked to phosphoinositide metabolism in rat cerebellar granule cells. Eur J Pharmacol 206:181–189. Medline