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Linkage of non-receptor tyrosine kinase Fyn to mGlu5 receptors in striatal neurons in a depression model

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Abstract

The Src family kinase (SFK) is a subfamily of non-receptor tyrosine kinases. The SFK member Fyn is enriched at synaptic sites in the limbic reward circuit and plays a pivotal role in the regulation of glutamate receptors. In this study, we investigated changes in phosphorylation and function of the two key SFK members (Fyn and Src) and SFK interactions with a metabotropic glutamate (mGlu) receptor in the limbic striatum of adult rats in response to chronic passive stress, i.e., prolonged social isolation which is a pre-validated animal paradigm modeling depression in adulthood. In rats that showed typical anhedonic/depression-like behavior after chronic social isolation, phosphorylation of SFKs at a conserved and activation-associated autophosphorylation site (Y416) was not altered in the two subdivisions of the striatum, the nucleus accumbens and caudate putamen. The total level of phosphorylation and kinase activity of individual Fyn and Src immunopurified from the striatum also remained stable after social isolation. Noticeably, Fyn and Src were found to interact with a $G_{\alpha q}$-coupled mGlu5 receptor in striatal neurons. The interaction of Fyn with mGlu5 receptors was selectively elevated in socially isolated rats. Moreover, social isolation induced an increase in surface expression of striatal mGlu5 receptors, which was reduced by an SFK inhibitor. These results indicate that Fyn interacts with mGlu5 receptors in striatal neurons. Adulthood social isolation in rats enhances the Fyn-mGlu5 interaction, which appears to be critical for the upregulation of surface mGlu5 receptor expression in striatal neurons.

Keywords

Caudate putamen; nucleus accumbens; Fyn; Src; mGlu5; metabotropic glutamate receptor; anhedonia; antidepressant; social isolation

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Introduction

Depression (major depressive disorder or clinical depression) is a mood disorder. Based on distinct categories of signs and symptoms, depression is classified into multiple subtypes. Anhedonia is one of the main symptoms of depression and is characterized by the loss of interest in various rewarding activities. The limbic reward circuit in the central nervous system participates in controlling natural rewarding activities. The striatum containing the ventral nucleus accumbens (NAc) and the dorsal caudate putamen (CPu) is a key structure within the limbic reward circuit and is thought to play a role in anhedonic depression (Zacharko and Anisman, 1991; Nestler and Carlezon, 2006). In fact, a transcription factor (cAMP response-element binding protein) in NAc neurons is sensitive to depression and its local activity is associated with depression-like behavior and antidepressant activity in a number of animal experiments (Pliakas et al., 2001; Barrot et al., 2002; Newton et al., 2002; Carlezon et al., 2005; Dinieri et al., 2009; Wallace et al., 2009; Green et al., 2010). More recently, metabotropic glutamate (mGlu) receptors in striatal neurons are discovered as a sensitive substrate of depression. In adult rats subjected to a prolonged period (10–12 weeks) of social isolation, a pre-validated animal paradigm modeling depression in adulthood (Wallace et al., 2009), mGlu5 receptor protein levels were elevated in the striatum (Mao and Wang, 2018). Thus, the limbic mGlu5 receptor represents a critical element in the pathophysiology and/or symptomatology of depression (Pilc et al., 2002; 2008; Kato et al., 2015), although intracellular signaling pathways linking depression to the striatal mGlu5 receptor upregulation are unclear.

Protein phosphorylation is an important posttranslational modification that regulates expression, trafficking, and function of proteins after protein synthesis. The Src family kinase (SFK) is a subfamily of non-receptor tyrosine kinases. By phosphorylating specific tyrosine site(s), SFKs regulate distribution and function of modified proteins (Kalia et al., 2004; Ohnishi et al., 2011). Among nine known members of SFKs, five members are expressed in the brain. Fyn and Src, two key SFK members that have been most extensively studied and have drawn the most attention, are enriched at synaptic sites and actively modulate excitatory synaptic transmission and plasticity (Kalia et al., 2004; Ohnishi et al., 2011; Schenone et al., 2011; Mao et al., 2017). Of note, Src and especially Fyn are abundant in the striatum (Pascoli et al., 2011). Thus, Fyn and/or Src may serve as a signaling molecule linking extracellular signals to synaptic receptor expression in striatal neurons.

Fyn and Src are autophosphorylated at a conserved residue, tyrosine 416 (Y416), in the activation loop. An increase in phosphorylation at this site is expected to increase the kinase activity (Cooper and MacAuley, 1988; Okada, 2012). Noticeably, the level of phosphorylation at Y416 is sensitive to changing synaptic input and SFK activity is linked to the pathogenesis of various neuropsychiatric and neurological disorders (Schenone et al., 2011; Nygaard, 2018). However, to date, little is known about the responsivity of striatal SFKs to chronic depressive stress. In this study, we explored adaptive changes in phosphorylation, expression, and kinase activity of Fyn and Src in the NAc and CPu in an animal model of depression, i.e., prolonged social isolation in adult rats. In addition, we investigated whether Fyn and Src interact with mGlu5 receptors in striatal neurons and

whether SFKs play a role in the upregulation of mGlu5 receptor expression in the striatum after chronic social isolation.

Experimental procedures

Animals

Wistar male rats (Charles River, New York, NY; RRID:RGD_2312511; catalog #: 2312511) arrived at 7–8 weeks of age (200–225 g) and were used in this study that was not pre-registered. Animals were housed at 23°C and humidity of 50 ± 10% with water and food available *ad libitum* and a 12-h/12-h light/dark cycle. Animal use was kept in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (University of Missouri-Kansas City, reference #: 1006–4). The Animal Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines have been followed.

Prolonged adulthood social isolation

This was conducted as described previously (Wallace et al., 2009; Mao and Wang, 2018). Briefly, rats were randomly divided into two groups (Fig. 1A). One group of rats were housed in home cages individually (one per cage) for 10–12 weeks as socially isolated rats. The other group of rats were housed two animals per cage for the same period of time. This group of rats served as controls. After 10–12 weeks of social isolation, we used these rats for behavioral assessments. The next day, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital at a dose of 55–60 mg/kg and were sacrificed for following neurochemical assays. We chose sodium pentobarbital to ensure deep anesthesia prior to decapitation. A computer-generated randomization table (GraphPad software/QuickCalcs, La Jolla, CA) was used to randomly divide animals into different biochemical experimental groups. After this division, the group of socially isolated rats showed a significant decrease in sucrose intake as compared to control rats. We determined sample size by the sample size calculation with alpha = 0.05 and beta = 0.2 (80% power). Between the beginning and end of the experiments, there were no sample size differences. The criteria for inclusion/exclusion were based on the animal health state. The healthy animals with no sign of illness as evaluated by the body weight and visual observations were used in the analysis. A total of 24 rats were used in socially isolated and control groups (n = 12 per group) in the first study. Among these rats, 12 rats (n = 6 per group) were used in a study investigating the effect of social isolation on SFK phosphorylation in the CPu and NAc, while other 12 rats (n = 6 per group) were used to test the effect of social isolation on Y416 phosphorylation and kinase activity of immunopurified Fyn and Src and on SFK-mGlu5 interactions in the striatum. In a separate study, the effect of the SFK inhibitor on responses of mGlu5 receptors to social isolation was examined in 24 rats (n = 6 per group).

Sucrose preference test

This test was carried out to measure an operational index of anhedonia (reduced responsiveness to a pleasurable stimulus). We performed a modified two-bottle-choice paradigm as described previously (Wallace et al., 2009; Mao and Wang, 2018). Briefly, after rats were initially habituated to two bottles of water for 5 days, animals were allowed

unlimited access to two bottles, one containing tap water and another one containing 1% (w/v) sucrose, for 24 h. The amounts of water and sucrose solutions consumed were measured. Preference for sucrose was calculated as the percentage of the volume of sucrose consumed (ml per 24 h) divided by the total fluid (sucrose + water) intake (ml per 24 h).

Western blot analysis

Rats were anesthetized and sacrificed by decapitation. Brains were removed and were cut into coronal slices. The entire striatum or different striatal subdivisions (NAc and CPu) were dissected. Dissected tissue was homogenized in a sucrose homogenization buffer containing 0.32 M sucrose, 10 mM HEPES, pH 7.4, 2 mM EDTA, and a protease/phosphatase inhibitor cocktail (ThermoFisher). Homogenates were centrifuged (800 *g*, 10 min) at 4°C. The supernatant was collected and was solubilized in the buffer containing 0.5% Triton X-100 (v/v) and 1% sodium deoxycholate. Protein concentrations were determined. Samples were stored at −80°C until use.

Western blots were performed as described previously (Jin et al., 2013). Briefly, proteins (24 μg/well) were separated on 4–12% NuPAGE gels (Invitrogen, Carlsbad, CA) and were then transferred to polyvinylidene fluoride membranes. Membranes were incubated with a primary antibody overnight at 4°C. After 1-h incubation with a goat anti-mouse or anti-rabbit secondary antibody, immunoblots were developed with the enhanced chemiluminescence reagent (GE Healthcare Life Sciences, Piscataway, NJ). The density of blots was measured using the NIH ImageJ (Bethesda, MD). Samples were normalized to β-actin levels. For mGlu5 receptor immunoblots, no reducing agents, such as dithiothreitol, and antioxidants were used.

Immunoprecipitation

As described previously (Jin et al. 2019), homogenized and solubilized striatal proteins (300 μg) were incubated with a mouse antibody against Src, Fyn, or mGlu5 receptors. We then precipitated protein-antibody complexes with 50% protein A and G agarose/sepharose bead slurry (Amersham). Immunoblots were performed with a rabbit antibody against Src, Fyn, phosphorylated Y416 (pY416), or mGlu5 receptors.

Coimmunoprecipitation

Coimmunoprecipitation was conducted by following a previously published procedure (Jin et al., 2013). Solubilized striatal proteins were incubated with a rabbit antibody (150 μg lysate proteins for an anti-Fyn antibody and 300 μg lysate proteins for an anti-mGlu5 antibody). The complex was precipitated with 50% protein A and G agarose/sepharose bead slurry (Amersham). Proteins were separated and detected in immunoblots with a mouse antibody.

Striatal slice preparation

Rat striatal slices were prepared for pharmacological studies (Jin et al., 2013). Briefly, rats were anesthetized. After decapitation, brains were removed and cut using a Leica VT1200S vibratome. Slices were preincubated at 30°C in artificial cerebrospinal fluid (ACSF) containing (in mM) 10 glucose, 124 NaCl, 3 KCl, 1.25 KH_2PO_4, 26 $NaHCO_3$, 2 $MgSO_4$,

and 2 $CaCl_2$, bubbled with 95% O_2-5% CO_2, pH 7.4. The solution was replaced for an additional preincubation (10–20 min). The SFK inhibitor, 3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (PP2), was added and incubated at 30°C.

Surface protein biotinylation

Surface protein biotinylation was performed on striatal slices (Jin et al., 2017). Briefly, rat striatal slices (300 μm) after drug treatments were incubated in ice-cold ACSF containing 1 mg/ml EZ-LINK-Sulfo-NHS-SS-Biotin (ThermoFisher) for 45 min. After slices were washed and were quenched by glycine (100 mM), slices were homogenized by sonication in an HEPES-Triton-SDS lysis buffer containing (in mM) 25 HEPES, 150 NaCl, 1% Triton X-100, 0.5% SDS, and a protease/phosphatase inhibitor cocktail (ThermoFisher). After centrifugation at 800 *g* (10 min, 4°C), the supernatant was collected and used as the total protein fraction. An equal aliquot of total proteins was incubated with neutrAvidin resin (ThermoFisher) overnight. Biotinylated proteins (i.e., surface proteins) were precipitated by centrifugation and were then eluted with a lithium dodecyl sulfate sample buffer. Protein levels in surface and total fractions were analyzed by immunoblot.

Tyrosine kinase activity assay

A Takara Universal Tyrosine Kinase Assay Kit (Clontech Laboratory, Inc., Mountain View, CA) was used to assess Fyn and Src kinase activity as described previously (Jin et al., 2019). Briefly, the dissected striatum was homogenized. From homogenates, a mouse antibody against Fyn or Src was used to immunoprecipitate Fyn or Src, respectively. Immunopurified Fyn or Src together with ATP were added to microplate wells covered with an immobilized tyrosine kinase substrate poly(Glu-Tyr) for 30 min. Wells were then washed, blocked, and incubated with a horseradish peroxidase-conjugated antibody against phosphotyrosine. Phosphorylated substrates in absorbance were measured at 450 nm.

Antibodies

Rabbit primary antibodies used in this study include those against Fyn (RRID:AB_631528; Santa Cruz Biotechnology, Santa Cruz, CA), Src (RRID:AB_2106047; Cell Signaling Technology, Danvers, MA), mGlu5 (RRID:AB_2295173; MilliporeSigma, Burlington, MA), and actin (RRID:AB_476693; MilliporeSigma). A rabbit antibody against pY416 (RRID:AB_10013641; Cell Signaling) reacts with the SFK members when phosphorylated at a conserved activation residue, a pan Y416 site. Mouse antibodies include those against Fyn (RRID:AB_627642; Santa Cruz), Src (RRID:AB_2106058; Cell Signaling), mGlu5 (RRID:AB_1523943; Abcam, Cambridge, MA), and transferrin receptors (TfR, RRID:AB_2533029; ThermoFisher). Validation data for antibodies are available from the companies.

Statistics

Data were statistically evaluated using GraphPad Prism 6 (RRID:SCR_002798; GraphPad software, La Jolla, CA) after the normality of data was tested. We did not conduct test for outliers on the data obtained in the study and no rats were excluded from the analysis. We

used two-way analysis of variance (ANOVA) followed by a *post hoc* test for comparing multiple groups or two-tailed unpaired Student's *t*-test for comparing two groups. Box and whisker plots were used for graphical representations with median and quartiles indicated. Whiskers indicate minimum and maximum data values. A value of $P < 0.05$ was considered as a statistically significant level. No blinding was performed.

Results

Depression-like behavior in socially isolated animals

Rats showed depression-like behavior after prolonged social isolation in adulthood (Wallace et al., 2009; Mao and Wang, 2018). For instance, a decrease in natural reward behavior, e.g., a deficit in sucrose intake, consistently occurred as a core symptom of anhedonic depression in socially isolated rats (Wallace et al., 2009; Mao and Wang, 2018). Since sucrose intake can be measured objectively in rodents, we monitored changes in this anhedonic behavior in isolated versus control (double-housed) rats as the first step of the current study to validate the model of social isolation. Using a two-bottle test, we found that rats after chronic social isolation (10–12 weeks) exhibited a marked decline in sucrose intake as compared to control rats (Fig. 1B). As a result, sucrose preference calculated was lower in socially isolated rats than that in control rats (Fig. 1C). These behavioral results provide evidence for the prolonged adulthood social isolation paradigm serving as an animal model with the depression-like phenotype.

Effects of social isolation on phosphorylation and expression of striatal Fyn and Src

To determine the effect of social isolation on SFK phosphorylation and expression in the striatum, rats were sacrificed after 10–12 weeks of social isolation. The two subdivisions of the striatum (CPu and NAc) were dissected for immunoblot analysis of changes in SFK phosphorylation and expression. No significant change in total pY416 levels in the CPu was found in socially isolated rats compared to double-housed control rats (Fig. 2A). The amount of total Fyn or Src proteins in the CPu remained stable between the two groups of rats. In the NAc, similar results were found following chronic social isolation. Levels of pY416, Fyn, and Src in the NAc of isolated rats were not different from that in the NAc of control rats (Fig. 2B). These results demonstrate that prolonged social isolation in adulthood has an insignificant impact on basal levels of phosphorylation and expression of Fyn and Src in the striatum.

Effects of social isolation on phosphorylation and kinase activity of immunopurified Fyn and Src

The autophosphorylation site Y416 is conserved among SFK members (Okada, 2012). To determine the impact of social isolation on individual Fyn and Src in terms of Y416 phosphorylation, we immunopurified Fyn and Src proteins from the striatum. We then tested pY416 signals of immunopurified Fyn and Src in Western blot. In a pool of Fyn immunoprecipitates, pY416 levels were not altered in socially isolated rats relative to control rats (Fig. 3A). Similarly, pY416 levels in Src immunoprecipitates remained stable between isolated and control rats (Fig. 3B). These data indicate that social isolation does not alter the phosphorylation level of either Fyn or Src in the striatum.

To determine the effect of social isolation on Fyn and Src kinase activity, we assessed changes in kinase activity levels of Fyn and Src proteins immunopurified from the striatum using a kinase assay kit. An insignificant change in Fyn kinase activity in the striatum was observed in socially isolated rats relative to control rats (Fig. 4A). Like Fyn, striatal Src kinase activity levels were not altered following social isolation (Fig. 4B). Thus, social isolation has no effect on striatal Fyn and Src kinase activity when assessed in an immunopurified protein pool.

Interactions of Fyn and Src with mGlu5 receptors in striatal neurons

Fyn interacted with mGlu5 receptors in transfected HEK293T cells as detected by coimmunoprecipitation (Um et al., 2013). Src also coimmunoprecipitated with mGlu5 receptors in cultured rat striatal neurons (Mao et al., 2005). To determine whether Fyn and Src interact with mGlu5 receptors in striatal neurons *in vivo*, we performed coimmunoprecipitation assays with solubilized lysates from the adult rat striatum. In samples precipitated with an anti-mGlu5 antibody, we observed a strong Fyn-immunoreactive band (Fig. 5A). A Src band was also seen in mGlu5 precipitates. These data indicate that Fyn and Src form complexes with mGlu5 receptors in striatal neurons *in vivo*. This notion is further supported by a result from a reverse coimmunoprecipitation assay. In a protein pool precipitated with an anti-Fyn antibody, an mGlu5 receptor band was shown, while there was no Src immunoreactivity in this pool of precipitates (Fig. 5B).

Effects of social isolation on Fyn-mGlu5 interactions

We next wanted to investigate the effect of social isolation on the interaction of Fyn and Src with mGlu5 receptors in the rat striatum. As shown in Fig. 6A, social isolation significantly enhanced the Fyn-mGlu5 interaction as evidenced by the finding that the amount of Fyn proteins in mGlu5 precipitates was increased in socially isolated rats compared to control rats. This indicates that more Fyn proteins were recruited to mGlu5 receptors after social isolation. The amount of Y416-phosphorylated SFKs that coimmunoprecipitated with mGlu5 receptors was also elevated after social isolation (Fig. 6B), indicating that a principal SFK component that was recruited to mGlu5 receptors by social isolation is the phosphorylated species (active form) of SFKs. In contrast to Fyn, Src exhibited a stable interaction level with mGlu5 receptors between two groups of rats (Fig. 6C). Thus, unlike Fyn, Src is less sensitive in its interaction with mGlu5 receptors in response to social isolation.

Effects of SFK inhibition on responses of mGlu5 receptors to social isolation

Surface expression of mGlu5 receptors in the rat striatum was elevated after chronic social isolation (Mao and Wang, 2018). To determine the role of Fyn in this event, we investigated the effect of inhibition of Fyn with an SFK inhibitor PP2 on the social isolation-induced increase in surface expression of mGlu5 receptors in the striatum. In striatal slices prepared from socially isolated rats and control rats, the mGlu5 receptor level in surface membranes as detected by surface biotinylation was elevated in socially isolated rats as compared to control rats (Fig. 7A). Adding PP2 (5 μM, 45 min) significantly lowered the increase in surface mGlu5 receptor expression in isolated rats. PP2 also reduced the increase in total mGlu5 protein levels in the striatum induced by social isolation. In addition to mGlu5

receptors, a surface marker (TfR) was assayed as a surface protein control. No significant changes in surface and total expression of TfRs were found in the striatum of socially isolated rats relative to control rats (Fig. 7B). These results indicate that a PP2-sensitive SFK, likely Fyn, participates in mediating the effect of social isolation on mGlu5 receptor expression in striatal neurons.

Discussion

Studies on the responsivity of brain Fyn and Src to depression are limited. We thus set forth to investigate changes in phosphorylation (activation), expression, and function of Fyn and Src in the striatum in response to a chronic stressor. We utilized a unique animal model of depression, i.e., prolonged social isolation in adult rats, to test Fyn/Src responses in adulthood. Of note, this model has been shown to characteristically induce anhedonia/depression-like behavior in species (rodents) that have important social interactions (Wallace et al., 2009; Mao and Wang, 2018, this study). Moreover, this chronic model is particularly useful for exploring a long-lasting adaptive event likely implicated in enduring depression-related behavior (Krishnan and Nestler, 2011). We found that there was no significant change in Y416 phosphorylation levels in the NAc and CPu lysates or in immunopurified Fyn and Src proteins in rats showing depression-like behavior after chronic social isolation. Expression of total Fyn and Src proteins in the NAc and CPu also remained stable in socially isolated rats relative to control rats. Functionally, social isolation did not alter kinase activity of Fyn and Src in the striatum. Thus, social isolation has a minimal impact on global expression and function of Fyn and Src in the striatum.

Fyn and Src may participate in the ionotropic glutamate receptor plasticity critical for depression-related behavior (Mao and Wang, 2016). Chronic unpredictable stress for six weeks reduced the sucrose preference in mice, establishing a behavioral endpoint of anhedonia (Lopes et al., 2016). The same chronic stress also elevated the amount of Fyn in the postsynaptic density (PSD) of hippocampal neurons. Given that Fyn positively modulates the function of the GluN2B-containing *N*-methyl-D-aspartate (NMDA) receptors by phosphorylating a primary site of Y1472 (Nakazawa et al., 2001; Salter and Kalia, 2004), levels of Y1472-phosphorylated GluN2B and total GluN2B receptors were concurrently elevated in the hippocampal PSD microdomain. Since mice lacking the cytoskeletal protein Tau did not exhibit both anhedonic behavior and an increase in Fyn/GluN2B expression in the PSD, Tau is considered as a mediator of these stress-induced behavioral and molecular events. In addition to the GluN2B subunit, the GluN2A subunit is also tyrosine-phosphorylated. Y1325 is one of the major sites phosphorylated by Fyn and Src (Taniquchi et al., 2009). Y1325 phosphorylation is required for Src to induce potentiation of the NMDA receptor channel in the mouse striatum (Taniguchi et al., 2009). Noticeably, mice expressing mutant GluN2A with a Y1325F mutation to prevent the phosphorylation at this site *in vivo* showed antidepressant-like behavior (Taniguchi et al., 2009). Thus, GluN2A Y1325 phosphorylation is implicated in depression-related behavior. Consistent with this notion, GluN2A knockout mice showed antidepressant-like profiles (Boyce-Rustay and Holmes, 2006) and NMDA receptor antagonists are generally of antidepressant activity (Paul and Skolnick, 2003; Hashimoto, 2011; Tokita et al., 2012; Dutta et al., 2015; Aleksandrova et al., 2017; Jaso et al., 2017).

In addition to NMDA receptors, mGlu receptors draw increasing attention in depression and antidepressant activity (Pilc et al., 2008). The mGlu5 receptor is a $G_{\alpha q}$-coupled receptor (Niswender and Conn, 2010). Stimulating mGlu5 receptors activates phospholipase Cβ1 and thereby hydrolyzes phosphoinositide into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. As a principally postsynaptic receptor, mGlu5 is particularly enriched in medium spiny projection neurons of the striatum (Testa et al., 1994; Tallaksen-Greene et al., 1998). Accumulating evidence shows that mGlu5 antagonists and negative allosteric modulators consistently produced antidepressant effects in various animal models of depression (Tatarczynska et al., 2001; Pilc et al., 2002; Wieronska et al., 2002; Palucha et al., 2005; Li et al., 2006; Molina-Hernandez et al., 2006; Belozertseva et al., 2007; Pomierny-Chamiolo et al., 2010; Liu et al., 2012; Kato et al., 2015). Similarly, mGlu5 knockout mice showed an antidepressant feature (Li et al., 2006). Thus, the mGlu5 receptor is considered to be a significant regulator in glutamatergic synaptic transmission and plasticity in relation to depression.

Molecular mechanisms underlying the role of mGlu5 receptors in the social isolation-induced depression are poorly understood. In a recent study, an increase in mGlu5 receptor expression was found in the striatum of adult rats following prolonged social isolation (Mao and Wang, 2018). This increase could be seen in surface membranes at synaptic sites. In parallel, the mGlu5-IP_3 signaling was enhanced in the striatum of isolated rats. Thus, mGlu5 receptors in striatal neurons undergo the adaptive upregulation after social isolation. How this upregulation occurs is unclear. An early study showed that mGlu5 receptors in rat striatal neurons are subjected to tyrosine phosphorylation (Orlando et al., 2002). Evidence from the present study supports that the upregulation of striatal mGlu5 receptors may be partially mediated via a signaling mechanism involving Fyn. In details, Fyn and mGlu5 receptors interact with each other in striatal neurons, similar to the Fyn-mGlu5 interaction observed in transfected HEK293T cells (Um et al., 2013). Social isolation selectively increases the Fyn-mGlu5 interaction. This increase seems to contribute to the upregulation of surface expression of mGlu5 receptors in isolated rats since the SFK inhibitor PP2 reduced the social isolation-induced increase in surface mGlu5 expression in striatal neurons. Together, results from this and a previous study (Mao and Wang, 2018) provide initial evidence for a possible molecular mechanism underlying the role of mGlu5 receptors in the social isolation-induced depression. That is, chronic social isolation upregulates mGlu5 receptor activity in the limbic reward region via a Fyn-dependent pathway. Upregulated mGlu5 receptors could then act in concert with NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors to constitute the adaptive remodeling of excitatory synaptic transmission and plasticity, leading to anhedonic behavior. In support of this scenario, mGlu5 receptors potentiated NMDA receptor activity (Huang and van den Pol, 2007; Rosenbrock et al., 2010) and stimulation of group I mGlu receptors reduced surface and synaptic AMPA receptor expression and dampened the AMPA receptor-mediated glutamatergic transmission in striatal and hippocampal neurons (Snyder et al., 2001; Xiao et al., 2001; Zho et al., 2002; Mangiavacchi and Wolf, 2004). As such, the antidepressant-like activity is usually observed after using the mGlu5 antagonists (see above), the NMDA receptor antagonists (see above), and the positive modulators of AMPA

receptors (Paul and Skolnick, 2003; Bleakman et al., 2007; Hashimoto, 2011; Tokita et al., 2012; Aleksandrova et al., 2017; Jaso et al., 2017).

Of note, prolonged administration of antidepressant agents increased binding of [^{3}H]MPEP to mGlu5 receptors in the rat hippocampus and cortex (Nowak et al., 2014). Moreover, different changes in mGlu5 receptor protein expression were seen in animal studies, depending on stress models used, symptoms analyzed, and brain regions surveyed (Nowak et al., 2014; Mao and Wang, 2018). These differences underscore the complexity of responses and roles of mGlu5 receptors in depression. Within the striatum, two equally populated projection neurons (striatonigral versus striatopallidal neurons) are noteworthy as they exhibit distinct phenotypes and work cooperatively to control the basal ganglia output. Future work will clarify the cell type of projection neurons and the subset of synapses within the striatum that are sensitive to stress and are responsible for defined depressive symptoms and antidepressant effects.

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Abbreviations:

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
CPu	caudate putamen
IP_3	inositol-1,4,5-triphosphate
mGlu	metabotropic glutamate
NAc	nucleus accumbens
NMDA	*N*-methyl-D-aspartate
PP2	3-(4-chlorophenyl) 1-(1,1-dimethylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine
PSD	postsynaptic density
PTK2	protein tyrosine kinase 2
SDS	sodium dodecyl sulfate
SFK	Src family kinase
SI	social isolation
TfR	transferrin receptors

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- Non-receptor tyrosine kinase Fyn interacts with metabotropic glutamate (mGlu) receptor 5 in striatal neurons *in vivo*.
- Social isolation in adult rats induced depression-like behavior and elevated striatal Fyn-mGlu5 interactions.
- Inhibition of Fyn reversed the isolation-induced increase in surface mGlu5 expression.
- Thus, social isolation upregulates surface mGlu5 expression likely via a signaling mechanism involving Fyn.

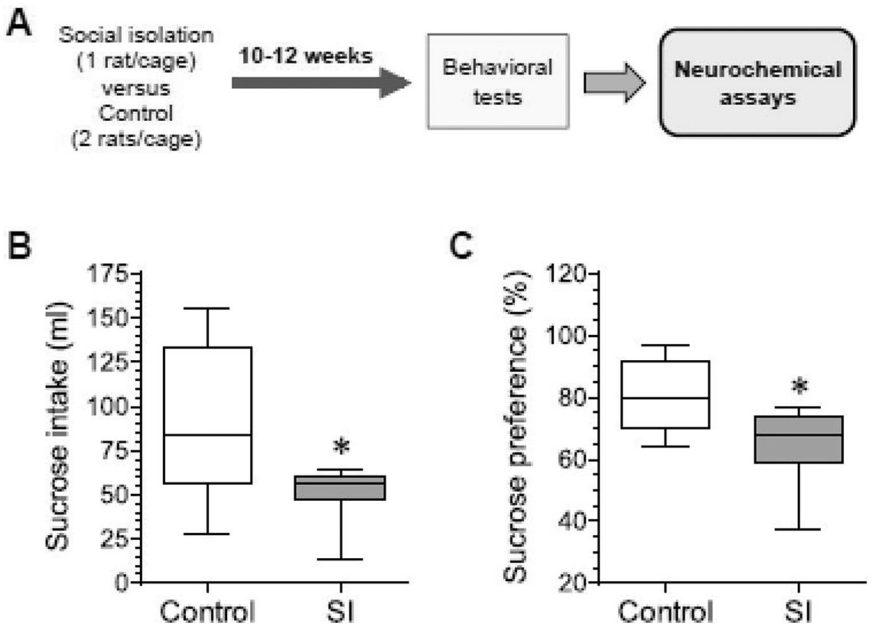

Figure 1. Depression-like behavior induced by chronic social isolation in adult rats.

(A) Timeframe illustrating social isolation followed by behavioral and neurochemical assessments. **(B)** Effects of chronic social isolation on sucrose intake. **(C)** Effects of chronic social isolation on sucrose preference. Following 10–12 weeks of prolonged social isolation (SI), rats underwent the sucrose intake test prior to striatal tissue collection for neurochemical assays. Note that social isolation reduced the sucrose intake (B) and sucrose preference (C) during a period of 24-h test. Data are presented as median ± interquartile range (n = 12 per group) with 'n' equal to the number of animals. *$P < 0.05$ versus double-housed control rats (Student's *t*-test). P values = 0.003 (B) and 0.002 (C).

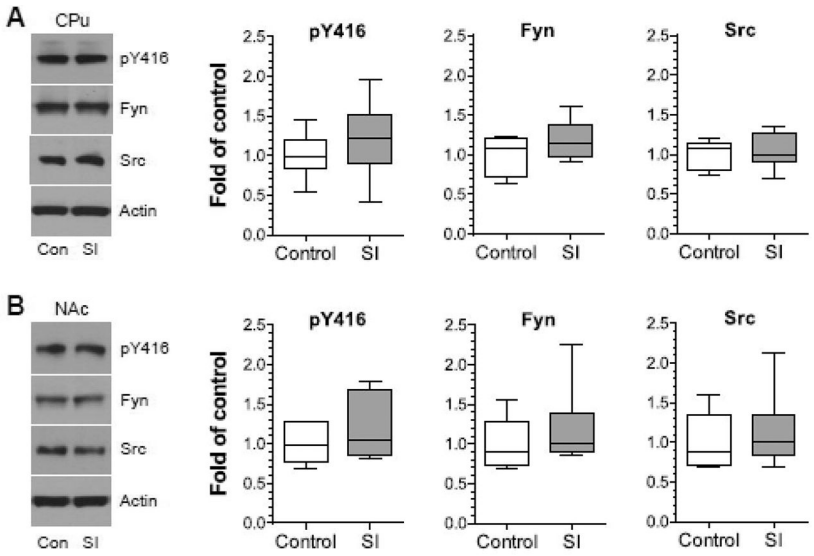

Figure 2. Effects of chronic social isolation on phosphorylation and expression of Fyn and Src in the rat striatum.

(A) Effects of social isolation on pY416, Fyn, and Src levels in the CPu. **(B)** Effects of social isolation on pY416, Fyn, and Src levels in the NAc. Note that social isolation (SI) had no significant effect on phosphorylation and expression of Fyn and Src in the CPu (A) and NAc (B) as compared to control (Con) rats. Representative immunoblots are shown left to the quantified data. Data were statistically analyzed using Student's *t*-test (n = 6 per group) with 'n' equal to the number of animals.

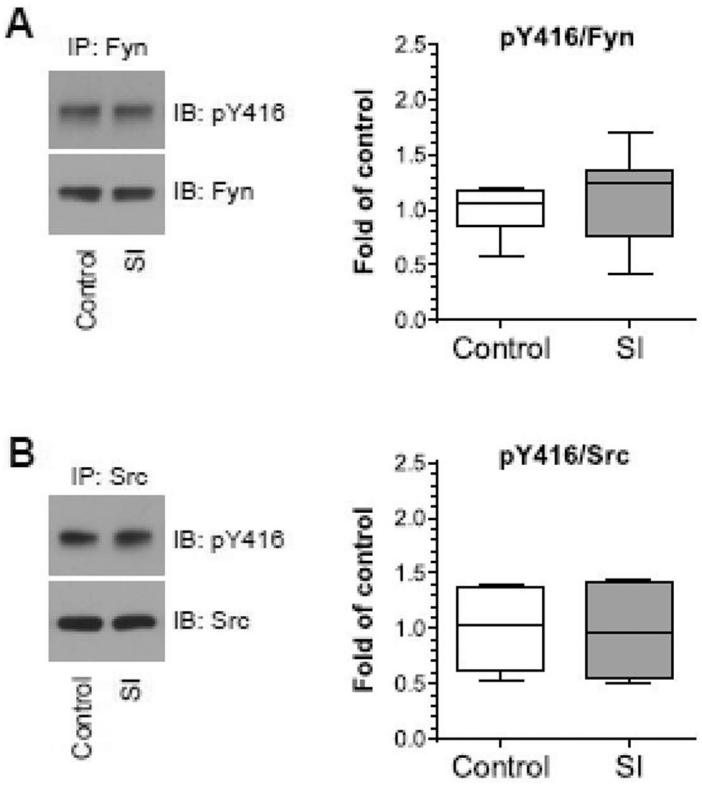

Figure 3. Effects of social isolation on phosphorylation of immunopurified Fyn and Src in the rat striatum.

(A) Effects of social isolation on Y416 phosphorylation of immunoprecipitated Fyn proteins. **(B)** Effects of social isolation on Y416 phosphorylation of immunoprecipitated Src proteins. Fyn and Src were precipitated from the striatum of socially isolated (SI) rats and control rats by immunoprecipitation (IP). Immunoprecipitated proteins were visualized by immunoblots (IB) with indicated antibodies. Representative immunoblots are shown left to the quantified data. Data were statistically analyzed using Student's *t*-test (n = 6 per group) with 'n' equal to the number of animals.

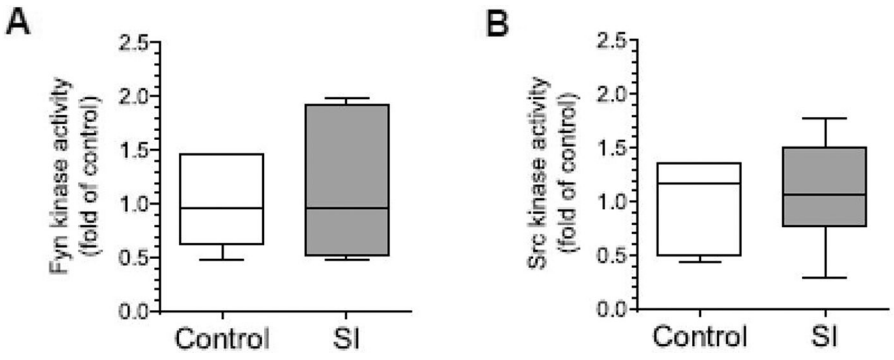

Figure 4. Effects of social isolation on Fyn and Src kinase activity in the rat striatum.

(A) Effects of social isolation on Fyn kinase activity. **(B)** Effects of social isolation on Src kinase activity. Fyn and Src were immunoprecipitated from the striatum of socially isolated (SI) rats and control rats. Data were statistically analyzed using Student's *t*-test (n = 6 per group) with 'n' equal to the number of animals.

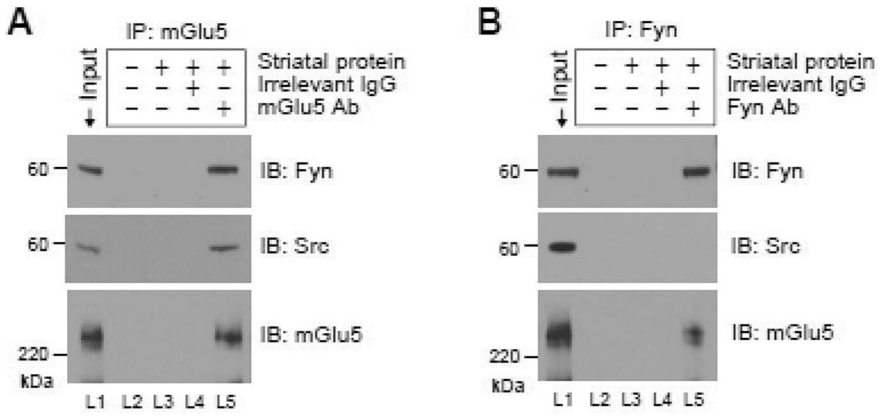

Figure 5. Interactions of non-receptor tyrosine kinases with mGlu5 receptors in the rat striatum.

(A) Coimmunoprecipitation (IP) of Fyn/Src and mGlu5 receptors in striatal neurons as detected with an anti-mGlu5 antibody (Ab). **(B)** Reverse coimmunoprecipitation of Fyn and mGlu5 receptors in striatal neurons as detected with an anti-Fyn antibody. Note that Fyn, Src, and mGlu5 receptors were seen in mGlu5 precipitates in lane 5 (L5) (A). No specific bands were shown in lanes 3 and 4 due to the absence of a precipitating antibody (L3) and the presence of an irrelevant IgG (L4). Solubilized rat striatal lysates were used in IP assays. Immunoprecipitated proteins were visualized by immunoblots (IB) with indicated antibodies.

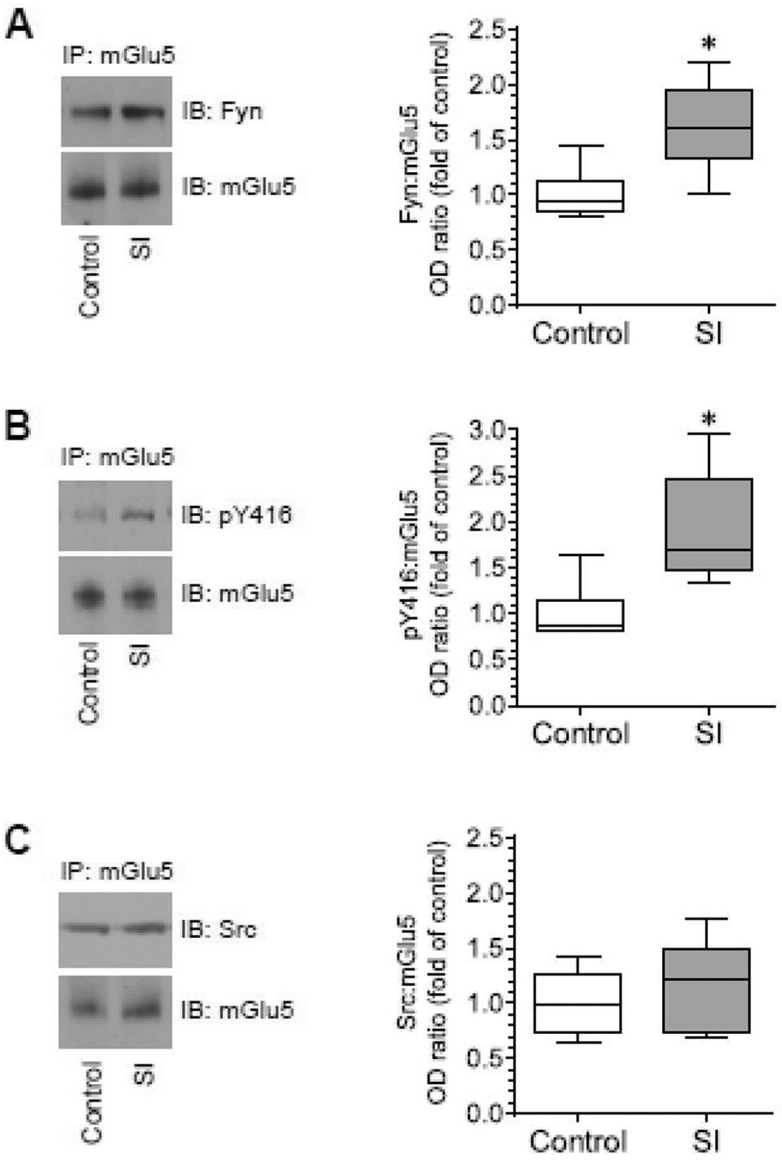

Figure 6. Effects of social isolation on the interaction of Fyn and Src with mGlu5 receptors in the rat striatum.

(A) Effects of social isolation (SI) on the Fyn-mGlu5 interaction. **(B)** Effects of social isolation on SFK Y416 phosphorylation in mGlu5 precipitates. **(C)** Effects of social isolation on the Src-mGlu5 interaction. Note that social isolation elevated the Fyn-mGlu5 interaction (A), although the Src-mGlu5 interaction was not significantly altered (C). Representative immunoblots are shown left to the quantified data. Solubilized rat striatal lysates were used in immunoprecipitation (IP). Immunoprecipitated proteins were visualized by immunoblots (IB) with indicated antibodies. Data were statistically analyzed using Student's *t*-test (n = 6 per group). $*P < 0.05$ versus double-housed control animals. *P* values = 0.009 (A), 0.008 (B), and 0.406 (C).

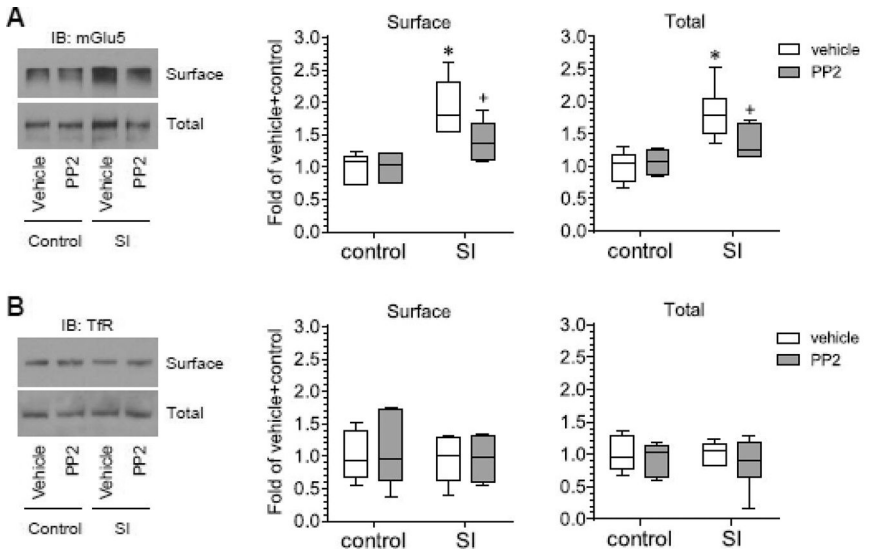

Figure 7. Effects of the SFK inhibitor on responses of striatal mGlu5 receptors to social isolation.

(A) Effects of PP2 on the social isolation (SI)-induced increase in surface and total expression of mGlu5 receptors in the striatum. **(B)** Changes in surface and total expression of TfRs in the striatum of socially isolated rats and control rats in the presence and absence of PP2. PP2 (5 μM) was applied to striatal slices prepared from socially isolated rats and control rats. Slices were collected 45 min after PP2 incubation. Surface proteins were isolated by biotinylation and analyzed by immunoblots (IB) with indicated antibodies. Representative immunoblots are shown left to the quantified data. Data were analyzed by two-way ANOVA followed by a *post hoc* test (n = 6 per group): surface mGlu5: vehicle versus PP2, $F(1,20) = 4.590$, $P = 0.044$, control versus SI, $F(1,20) = 28.90$, $P < 0.001$, and interaction, $F(1,20) = 4.412$, $P = 0.048$; total mGlu5: vehicle versus PP2, $F(1,20) = 3.109$, $P = 0.093$, control versus SI, $F(1,20) = 23.71$, $P < 0.001$, and interaction, $F(1,20) = 5.188$, $P = 0.034$. *$P < 0.05$ versus vehicle in double-housed control animals. + $P < 0.05$ versus vehicle in socially isolated animals.