SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
MOLECULAR NEUROSCIENCE
NEUROSCIENCE
CELLULAR NEUROSCIENCE
SYNAPTIC TRANSMISSION

Received
28 September 2012

Accepted
16 November 2012

Published
30 January 2013

Correspondence and requests for materials should be addressed to C.T. (catherine.trepanier@utoronto.ca)

Group II metabotropic glutamate receptors modify N-methyl-D-aspartate receptors via Src kinase

Catherine Trepanier[1], Gang Lei[2], Yu-Feng Xie[2] & John F. MacDonald[1,2,3]

[1]Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada M5S 1A8, [2]Robarts Research Institute, 100 Perth Drive, London, Ontario, Canada N6A 5K8, [3]Department of Physiology, University of Toronto, Toronto Ontario, Canada M5S 1A8.

Group II metabotropic glutamate receptors (mGluR2/3) have emerged as important targets for the treatment of schizophrenia. Since hypofunction of N-methyl-D-aspartate receptors (NMDARs) has also been implicated in the etiology of schizophrenia, we examined whether postsynaptic mGluR2/3 regulate NMDAR function. Activation of mGluR2/3 significantly decreased the ratio of AMPA-to-NMDA excitatory postsynaptic currents at Schaffer Collateral-CA1 synapses and enhanced the peak of NMDA-evoked currents in acutely isolated CA1 neurons. The mGluR2/3-mediated potentiation of NMDAR currents was selective for GluN2A-containing NMDARs and was mediated by the Src family kinase Src. Activation of mGluR2/3 inhibited the adenylyl cyclase-cAMP-PKA pathway and thereby activated Src by inhibiting its regulatory C-terminal Src kinase (Csk). We suggest a novel model of regulation of NMDARs by Gαi/o-coupled receptors whereby inhibition of the cAMP-PKA pathway via mGluR2/3 activates Src kinase and potentiates GluN2A-containing NMDAR currents. This represents a potentially novel mechanism to correct the hypoglutamatergic state found in schizophrenia.

Glutamate is the primary excitatory neurotransmitter in the brain and binds both ligand-gated ion channels such as the N-methyl-D-aspartate receptor (NMDAR) and G protein-coupled metabotropic receptors (mGluRs). Hypofunction of the NMDAR (a hypoglutamatergic state) represents a major hypothetical mechanism explaining the etiology of schizophrenia[1–3]. This hypothesis is based on initial observations that non-competitive NMDAR antagonists induce a transient psychosis, disrupt affect and impair cognitive function in healthy humans, and can exacerbate preexisting symptoms in patients with schizophrenia[4,5]. Furthermore, NMDAR-mediated signaling and GluN2A tyrosine phosphorylation is significantly reduced in postmortem brains from schizophrenia subjects[3]. The NMDAR pathway is associated with several candidate genes for schizophrenia, including neuregulin-1, dysbindin, disrupted-in-schizophrenia 1 and metabotropic glutamate receptor 3 (mGluR3)[6]; thus, NMDAR-mediated signaling is proposed to act as a point of convergence for various candidate pathways in this disorder.

Accumulating evidence suggests that the group II family of mGluRs ($mGlu_2$ and $mGlu_3$; mGluR2/3) may represent an important target in the treatment of schizophrenia[7,8]. In NMDAR hypofunction models of psychosis, agonists of mGluR2/3 reduce phencyclidine- and dizocilpine-induced locomotor behaviours[9–11]. A recent phase II clinical trial showed that LY 214 0023, an oral prodrug of the selective mGluR2/3 agonist LY 404039, was effective in treating both positive and negative symptoms of schizophrenia after only 4 weeks of treatment[8]. Furthermore, group II mGluRs are highly expressed in regions of the brain associated with schizophrenia such as the prefrontal cortex and hippocampus[12,13]. Although there is ample evidence that mGluR2/3 agonists are effective in schizophrenia, the exact mechanisms for their antipsychotic effects are unclear.

Group II mGluRs are predominantly expressed on presynaptic terminals where they inhibit release of glutamate and GABA[14]. Activation of postsynaptic mGluR2/3 negatively modulates neuronal excitability and plasticity[15,16]. Given that mGluR2/3 mainly act presynaptically to inhibit glutamate release, it seems counter-intuitive that activation of these receptors would ameliorate the hypoglutamatergic state found in schizophrenia. Therefore we examined the effects of mGluR2/3 activation on NMDA-evoked currents in identified CA1 pyramidal neurons of the hippocampus, a brain region implicated in the pathophysiology of schizophrenia[17,18].

Results

We initially examined the effects of mGluR2/3 activation on NMDAR-mediated field EPSPs ($fEPSP_{NMDA}$) in the CA1 region. Application of the selective mGluR2/3 agonist LY 379268 (30 nM) did not affect the amplitude of $fEPSP_{NMDA}$ (Fig. 1a); however, there was an increase in the variance of the $fEPSP_{NMDA}$ response, suggesting that postsynaptic NMDAR function may be enhanced in spite of a reduction in presynaptic release of glutamate. To determine whether mGluR2/3 activation enhances postsynaptic NMDAR function, we examined the AMPA/NMDA ratio of excitatory postsynaptic currents (EPSCs) at Schaffer Collateral-CA1 synapses. Application of LY 379268 (30 nM) resulted in a significant decrease of AMPA/NMDA EPSC ratio ($p<0.05$, Fig. 1b), suggesting an enhancement of postsynaptic NMDAR function. To more directly examine the effects of mGluR2/3 activation on NMDARs, we examined the actions of LY 379268 on acutely isolated CA1 pyramidal neurons.

We previously demonstrated that Gαq- and Gαs-coupled receptors potentiate NMDAR currents in isolated CA1 neurons via activation Src kinase and PKA, respectively[19–23]. Given that mGluR2/3 couple to Gαi/o and inhibit adenylyl cyclase activity[14], we anticipated that these receptors would inhibit NMDAR-mediated currents. To examine the direct effects of group II mGluRs on NMDARs, we determined the actions of LY 379268 on acutely isolated CA1 pyramidal neurons. These isolated neurons have a population of both extrasynaptic and synaptic NMDARs. Surprisingly, application of LY 379268 (10 nM) to acutely isolated CA1 neurons potentiated NMDA-evoked currents, with a significant potentiation occurring after washout of LY 379268 (Fig. 2). The concentration of LY 379268 employed was chosen based on the 50% percent inhibitory concentration (IC_{50}) value of LY 379268 to displace [^{3}H]LY341495, a group II selective antagonist radioligand, from native rat brain homogenates and recombinant human mGlu2 and mGlu3 receptor subtypes[24]. The LY 379268-induced enhancement of NMDAR-mediated currents was blocked by co-application of the selective mGluR2/3 antagonist LY 341 495 (10 nM) (Fig. 3). Conversely, co-application of the selective group I mGluR antagonist, MPEP hydrochloride, did not prevent the enhancement of NMDAR currents by LY 379268 (Fig. 3). These results confirm that the LY 379268-mediated potentiation of NMDAR currents was indeed mediated by mGlu2/3 receptors. Given that PKA promotes Ca^{2+} permeation through NMDARs and increases the amplitude of NMDA-evoked currents[25], it seems counterintuitive that inhibition of the PKA pathway via group II mGluRs would mediate the enhancement of NMDAR currents. Instead, we hypothesized that stimulation of group II mGluRs results in a promiscuous activation of Gαq with a subsequent activation of PKC and/or Src leading to an enhancement of NMDAR currents[19,20,22,23]. To test this hypothesis, we employed the Src(40–58) peptide which mimics the unique domain of Src and prevents its interaction with the NADH dehydrogenase subunit 2 domain in the NMDAR complex[26]. Thus, Src(40–58) acts as an interfering peptide to prevent the regulation of NMDARs by endogenous Src[26]. Including Src(40–58) in the patch pipette prevented the enhancement of NMDAR currents by LY 379268 (Fig. 4a). The LY 379268-induced enhancement of NMDAR currents was not, however, prevented by application of the PKC inhibitor, bisindolylmaleimide I (0.5 μM, data not shown) suggesting that Src might be activated by an alternative mechanism. Using an approach similar to Gingrich *et al.*[26], we synthesized the Fyn interfering peptide, Fyn (39–57) that corresponds to a region of the unique domain of Fyn. We previously showed that Fyn(39–57) selectively blocks the potentiation of NMDAR currents by recombinant Fyn kinase but not by recombinant Src kinase[23]. Application of Fyn(39–57) inside the patch pipette failed to prevent the enhancement of NMDAR currents by LY 379268 (Fig. 4a) indicating that LY 379268 enhances Src but not Fyn activity to regulate NMDARs. To confirm these

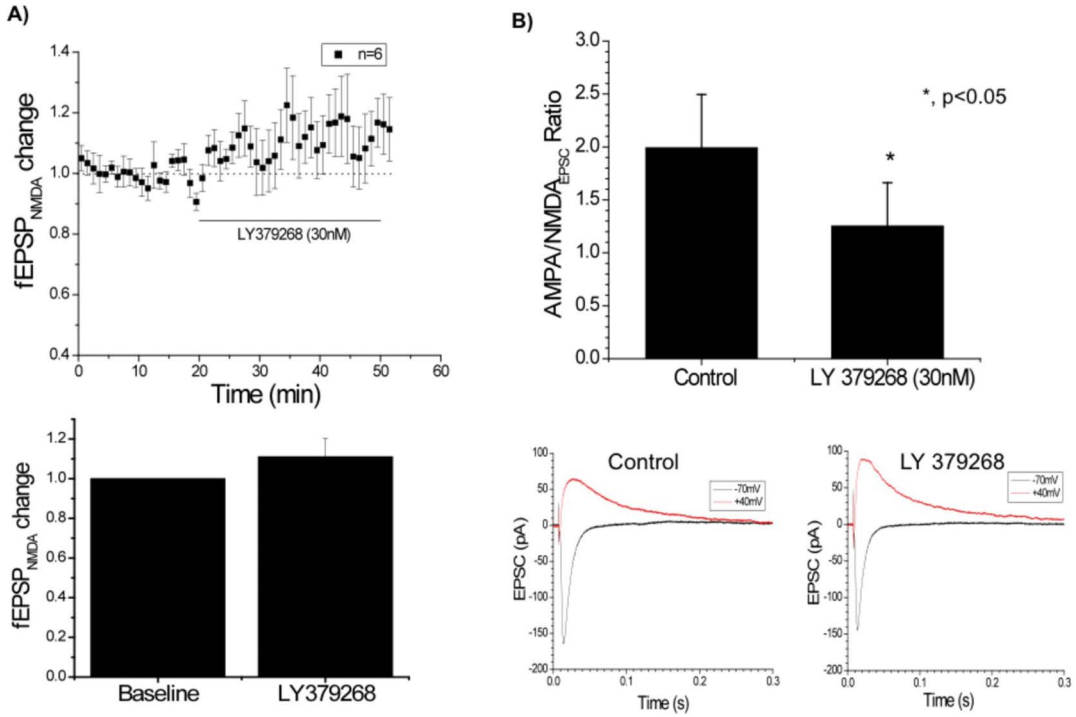

Figure 1 | Activation of group II mGluRs enhances postsynaptic NMDAR function. A) Bath application of LY 379268 (30 nM) to hippocampal slices does not change the amplitude of NMDAR-mediated fEPSPs relative to baseline (N=6). Below, quantification of NMDA-fEPSP change before and after application of LY 379268. B) Stimulation of mGluR2/3 with LY 379268 (30 nM) significantly decreased the AMPA-NMDA EPSC ratio (N=11) relative to baseline ($p<0.05$). Below, example traces showing EPSCs at +40 mV and −70 mV from control and LY 379268-treated slices, respectively; AMPA EPSC amplitude was measured at −70 mV and NMDA EPSC amplitude was measured at +40 mV. * Indicates $p<0.05$, Student's t-test.

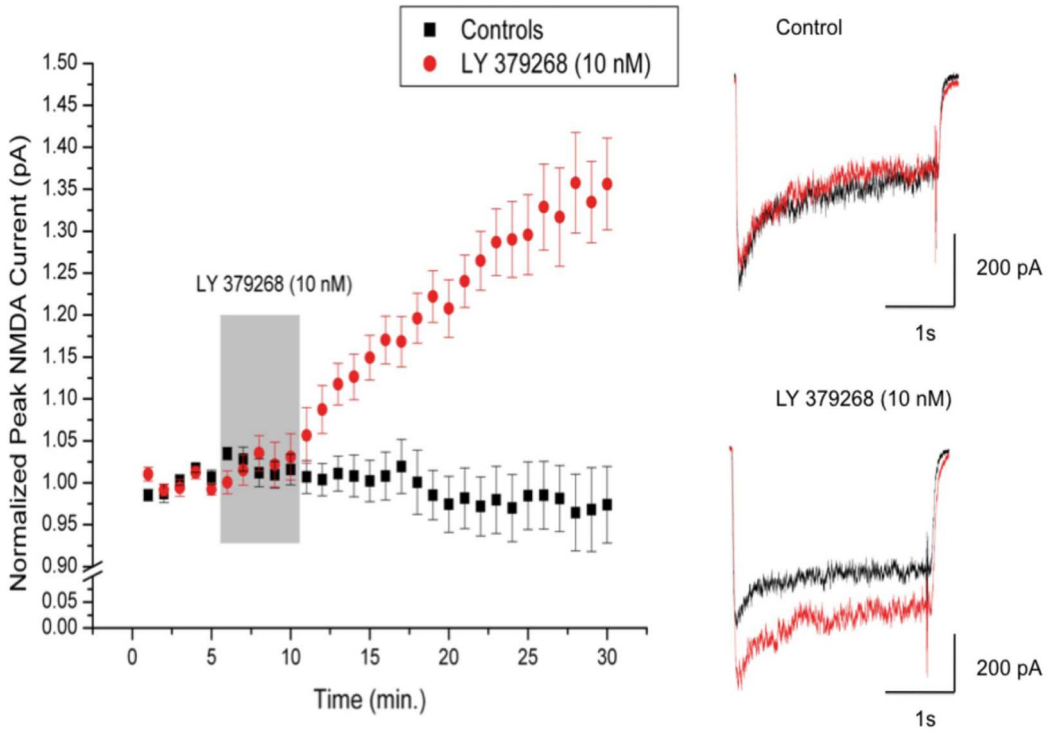

Figure 2 | Application of LY 379268 to acutely isolated CA1 neurons potentiates NMDAR currents. Application of LY 379268 (10 nM) to acutely isolated CA1 neurons (N=18) resulted in an increase of NMDA-evoked peak currents that began after washout of the agonist and persisted for the rest of recording. NMDA-evoked currents in control cells (N=10) remained stable throughout the time period of the recording. Cells treated with 10 nM LY 379268 had significantly larger NMDA-evoked peak currents (control, 97.4 ± 10%, N=10; 10 nM LY 379268, 120.8 ± 14.6%, N=18; $p<0.001$, unpaired t-test; data obtained at 20 min. of recording). The shaded region indicates the period of LY 379268 application. Right, sample traces of NMDA-evoked currents for control and LY 379268-treated cells. Traces represent points immediately before LY379268 application (t=5 min.) and 10 min. after the end of LY 379268 application (t=2 min.).

electrophysiological findings, we determined the relative activation of Src versus Fyn in hippocampal slices treated with or without LY 379268 (30 nM). The mGluR2/3 agonist enhanced phosphorylation of Src at Y416 (Fig. 4b), a site whose phosphorylation is required for activation this kinase[27]. The LY 379268-mediated increase in Src Y416 phosphorylation was prevented by co-application of the mGluR2/3 antagonist LY 341 495 (Fig. 4b). In contrast, LY 379268 failed to enhance the tyrosine phosphorylation of the analogous activation site of Fyn kinase, Y420 (Fig. 4b)[28]. These findings illustrate that Src, and not Fyn, regulates the mGluR2/3-mediated modulation of NMDAR currents in dissociated CA1 neurons.

We previously reported that several GPCRs acting via Gαq activate Src kinase to phosphorylate the GluN2A subunit of this receptor[22,23]. In this regard, we employed applications of zinc (300 μM) to selectively inhibit responses to heteromeric NMDARs containing two GluN2A subunits[23,29]. The enhancement of NMDAR currents by LY 379268 was absent when GluN2A subunits were blocked with Zn^{2+} (Fig. 5a). In contrast, the mGluR2/3-mediated modulation of NMDARs was still observed in the presence of the selective GluN2B antagonist, Ro 25–6981 (500 nM) (Fig. 5a). We also examined the potential phosphorylation of GluN2A and GluN2B subunits and found that applications of LY 379268 enhanced tyrosine phosphorylation of GluN2A but not GluN2B subunits (Fig. 5b). Thus, activation of mGluR2/3 selectively potentiates GluN2A-containing NMDAR currents.

Gαs-dependent receptors enhance PKA and target Fyn kinase activation to enhance phosphorylation of NMDARs[23]. Given that Fyn kinase was not activated by LY 379268 and that mGluR2/3 classically signal via inhibition of PKA, we initially confirmed that LY 379268 does indeed inhibit cAMP in treated hippocampal slices. Applications of LY 379268 reduced basal levels of cAMP in a concentration-dependent manner with an IC_{50} of 10.932 nM (Fig. 6a).

Src is strongly regulated in CA1 hippocampal neurons by C-terminal Src kinase (Csk), which phosphorylates Src on the C-terminal tyrosine Tyr527 and maintains it in an inactive conformation[30]. Furthermore, the tyrosine kinase activity of Csk is increased by PKA-mediated phosphorylation[31], suggesting that a decrease in PKA activity might inhibit Csk and lead to a disinhibition of Src activity. To test this possibility, we first determined whether activation of mGluR2/3 inhibits Csk activity. Treatment of hippocampal membranes with LY379268 led to a significant reduction of Csk activity, as assessed by phosphorylation of Ser364 (Fig. 6b). Furthermore, LY379268 treatment significantly reduced Csk-mediated phosphorylation of Src at its C-terminal regulatory tyrosine Y527 (Fig. 7a). Decreased phosphorylation of Y527 on Src prevents the intramolecular interaction with the SH2 domain and leads to an open conformation of Src[32,33]. Thus, activation of mGluR2/3 may activate Src through inhibition of its regulatory partner Csk. To determine whether inhibition of PKA may activate Src and occlude the effects of LY379268 on NMDAR currents, we applied a highly selective PKA inhibitor in the patch pipette and examined the modulation of NMDAR currents by LY 379268. Under this condition LY 379268 failed to enhance NMDAR currents (Fig. 7b) suggesting that Src is activated, at least in part, by a reduction in PKA activity.

Discussion

We have shown that activation of group II mGluRs enhances postsynaptic NMDAR function in spite of reducing excitatory transmission. Application of the selective mGluR2/3 agonist LY 379268 increases the ratio of NMDA-to-AMPA EPSCs at Schaffer

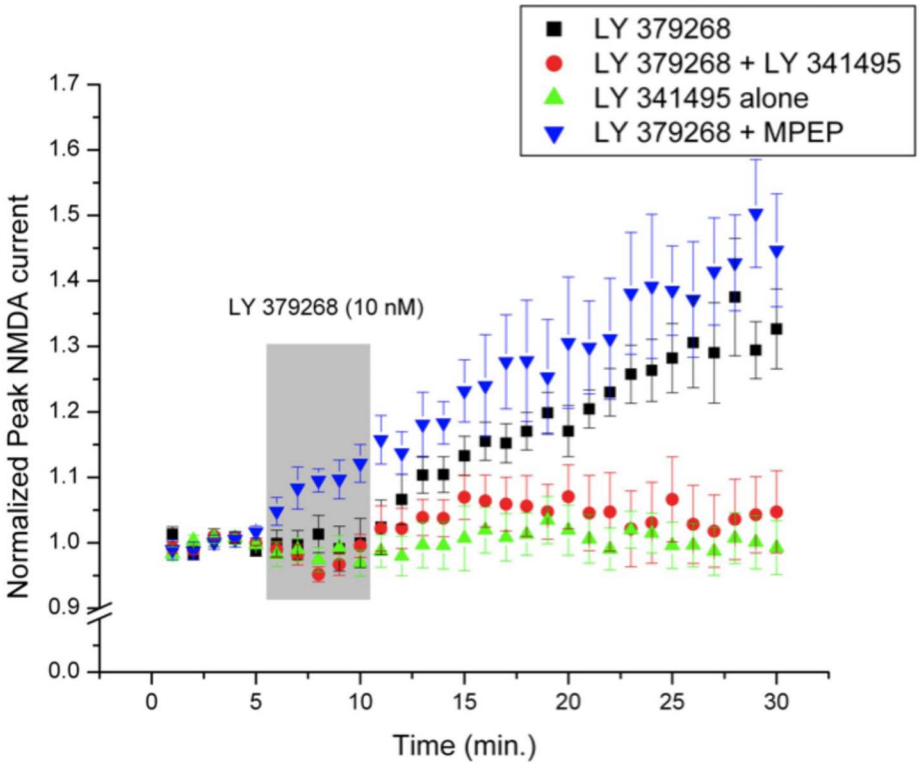

Figure 3 | The LY 379268-mediated increase of NMDA-evoked currents is mediated by group II metabotropic glutamate receptors. Upregulation of I_{NMDA} by LY 379268 (N=11) was blocked by co-application of the selective mGluR2/3 antagonist LY 341495 (N=9) (LY plus LY 341495, 99 ± 1%, n=7; LY alone, 131 ± 2%, n=10). The mGluR2/3 antagonist on its own did not change the amplitude of NMDAR-evoked currents (N=7). Application of the group I mGluR antagonist MPEP hydrochloride failed to prevent the LY 379268-mediated potentiation of NMDAR currents (N=9) (LY plus MPEP, 143 ± 2%, n=8; LY alone, 131 ± 2%, n=10). Antagonists (LY 341495, 10 nM; MPEP hydrochloride, 10 μM) were applied in all extracellular solutions for the duration of the experiment. The shaded region indicates the period of LY 379268 application.

Collateral-CA1 synapses and enhances NMDA-induced currents in acutely isolated CA1 pyramidal neurons. The enhancement of NMDA-evoked currents was mediated by mGluR2/3 as it was inhibited by the selective group II mGluR antagonist LY 341 495. The Src family kinase Src, and not Fyn, was required for this potentiation as it was blocked by the selective Src-interfering peptide, Src(40–58), but not by the comparable Fyn interfering peptide, Fyn(39–57). Furthermore, LY 379268 increased the activity of Src kinase (increased phosphorylation of Tyr-416) but not that of Fyn (phosphorylation of Tyr-420 unchanged), leading to a selective tyrosine phosphorylation of GluN2A- versus GluN2B-containing NMDARs. Unlike Gαq-dependent signaling, the potentiation by LY 379268 was insensitive to a blocker of PKC and was occluded by an inhibitor of PKA. This suggests that the mechanism of Src activation by LY 379268 differs substantially from Gαq-coupled receptors which signal via sequential activation of PKC, Pyk2 and Src[20,22,34].

We provide evidence that group II mGluRs activate Src by inhibition of PKA and Csk activities. Activation of the cAMP-PKA pathway has previously been shown to down regulate Src kinase activity in a Csk-dependent manner[31,35]. Activation of mGluR2/3 couples to the inhibition of PKA and decreases phosphorylation of Ser364 on Csk, thereby inhibiting Csk activity. This in turn reduces the ability of Csk to phosphorylate the regulatory Y527 on Src and promotes an active conformation of Src. However, we cannot rule out the potential role of activation of an unidentified tyrosine phosphatase, which would also reduce phosphorylation of Src at Tyr527. Src kinase activity can also be directly stimulated by Gαi/o[36]. Activated Gαi has been shown to interact directly with the catalytic domain of Src, thus changing the conformation of c-Src and allowing increased accessibility of the active site to substrates. Activation of Src by Gαi-protein-coupled receptors has also shown to mediate Ras-dependent activation of mitogen-activated protein kinases (MAPKs) in various cell types[37,38]. Stimulation of the Gαi-coupled α-2 adrenergic receptor leads to liberation of Gßγ subunits and phospholipase C activation[37]. The resulting increase in intracellular Ca^{2+} activates calmodulin and Pyk2, which then activates c-src. Whether or not mGluR2/3 activates such a signaling pathway in parallel with an inhibition of PKA and Csk activities to up regulate Src kinase activity is unknown.

Agonists of group II mGluRs have opposing actions on pre- and post-synaptic sites: decreasing glutamate release presynaptically[14] and potentiating NMDAR function postsynaptically (our present findings). Thus, the relative contributions of pre- to post-synaptic effects of mGluR2/3 agonist *in vivo* could be complicated. One possibility is that reduced glutamate release will prevent the activation of extrasynaptic NMDARs and preferentially activate subsets of synaptic NMDARs. The NMDAR appears to be an important postsynaptic target for mGluR2/3 in the hippocampus. Activation of mGluR2/3 is also reported to decrease the AMPA-component of EPSPs, consistent with a reduction in presynaptic glutamate release[39,40]. We have shown that mGluR2/3 activation decreases the ratio of AMPA-to-NMDA EPSCs, suggesting that LY 379268 increases the relative contribution of NMDARs to AMPARs in the synapse. The paradoxical effects of mGluR2/3 agonists on presynaptic glutamate release and postsynaptic NMDAR function can also be explained by the activation of extrasynaptic tonic NMDAR conductances. Ambient glutamate release from non-synaptic sources, such as glial cells, induces a tonic NMDA current primarily mediated by extrasynaptic NMDARs[41]. Given that acutely isolated neurons from the CA1 are heavily enriched in extrasynaptic NMDARs, we propose

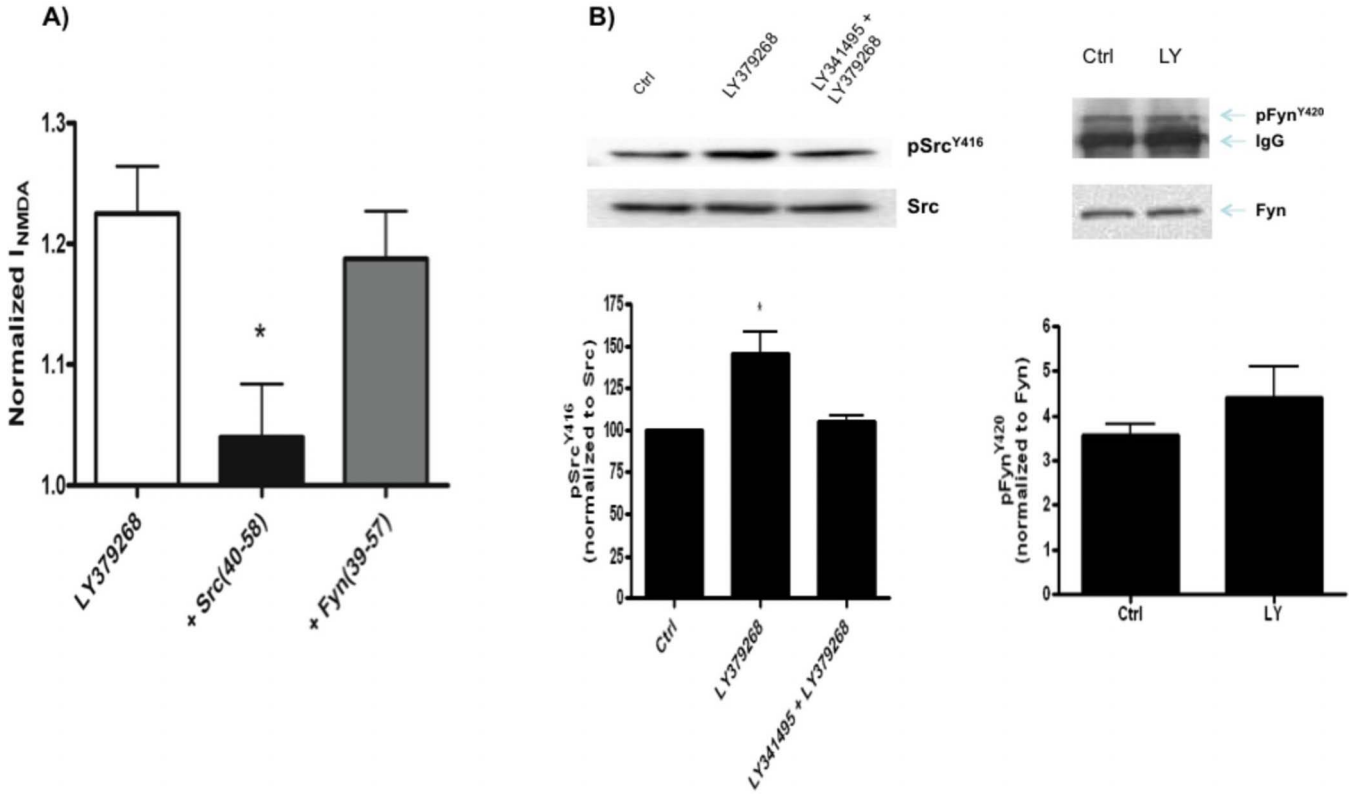

Figure 4 | mGluR2/3 activation recruits Src kinase to potentiate NMDAR currents in acutely isolated CA1 cells. A) Quantification of normalized I_{NMDA} recorded from hippocampal CA1 neurons treated with LY 379268. mGluR2/3-mediated potentiation of I_{NMDA} (N=11) is prevented by intracellular application of Src(40–58) (N=10) but not Fyn(39–57) (N=11) (LY plus Src(40–58), 103 ± 2%, n=9; LY plus Fyn(39–57), 125 ± 2%, n=10; LY alone, 135 ± 2%). LY 379268 (10 nM) was co-applied with NMDA/glycine solutions using the multi-barreled perfusion system. Fyn(39–57), 25 ng/ml, and Src(40–58), 25 ng/ml, were included inside the patch pipette. Peak currents were averaged for 5 min prior to application of LY 379268 and were compared with currents averaged for values between 20 and 25 minutes. *Indicates $P<0.01$, one-way ANOVA. B) LY 379268 (30 nM) treatment increases the phosphorylation of Src(pSrcY416) but not Fyn (pFynY420). The enhanced Src phosphorylation was prevented by pre-treatment with the mGluR2/3 antagonist LY 341495 (30 nM). Below, summary of immunoblot analysis shows the averaged relative density of pSrcY416 and pFynY420 for each condition (n=4). *Indicates $P<0.05$, Student's t-test.

that LY 379268 may target these extrasynaptic NMDAR currents independently of vesicular glutamate release.

Several lines of evidence suggest that hypofunction of NMDARs underlies the pathophysiology of schizophrenia. Administration of dissociative anesthetics such as phencyclidine and ketamine to healthy volunteers produces behaviours similar to the positive, negative and cognitive symptoms of schizophrenia[1,4]. Analysis of postmortem hippocampal tissue from schizophrenic patients reveals a decrease in GluN1 mRNA[42]. Candidate schizophrenia genes such as neuregulin 1 have been shown to promote rapid internalization of NMDARs from the cell surface and reduce whole-cell NMDAR currents[43]. In postmortem brains of schizophrenia subjects, enhanced ErbB4 signaling by neuregulin 1 mediates a suppression of GluN2A tyrosine phosphorylation, which promotes NMDAR internalization and decreases NMDAR signaling[3]. Thus, enhancing GluN2A-containing NMDAR function with group II mGluRs may counteract the effects of neuregulin 1 in these individuals and help stabilize NMDARs to the cell surface.

A recent study suggested that dysregulated Src activity mediates NMDAR hypofunction in schizophrenia induced by neuregulin-1-ErbB4 signaling, a candidate schizophrenia pathway[44]. Activation of neuregulin 1-ErbB4 signaling prevented the Src-induced potentiation of NMDAR-mediated synaptic currents in mouse prefrontal cortex and hippocampus. Thus, normalizing Src-mediated enhancement of NMDARs via activation of group II metabotropic glutamate receptors could represent a therapeutic avenue in the treatment of schizophrenia. The antipsychotic agent clozapine has also been shown to signal through Src kinase to potentiate NMDAR currents in the nucleus accumbens[45]. Thus, upregulation of Src activity may represent an important mechanism underlying the ability of these agents to relieve both positive and negative symptoms of schizophrenia.

Stimulation of group II mGluRs increases the function of postsynaptic NMDARs in hippocampal CA1 neurons via inhibition of PKA and activation of Src kinase. We propose that inhibition of the cAMP-PKA pathway decreases the activity of Csk, thereby decreasing the phosphorylation of its substrate Src at Tyr527. Reduced phosphorylation of Src at Tyr527 prevents the intramolecular interaction with the SH2 domain and leads to an active conformation of Src. However, we cannot rule out the potential role of activation of an unidentified tyrosine phosphatase, which might also reduce phosphorylation of Src at Tyr527. Up until now, the antipsychotic effects of group II mGluR agonists have been attributed to a decrease in presynaptic glutamate release. Given that schizophrenia is characterized by a hypoglutamatergic state, we propose that enhancing postsynaptic NMDAR function may be more relevant in restoring a balance of glutamatergic signaling in this disease. One candidate schizophrenia pathway leading to hypofunction of NMDARs implicates aberrant Src activity. Thus, we speculate that enhancing Src activity through group II mGluRs may represent one of the mechanisms for the antipsychotic effects of mGluR2/3 agonists.

Methods

Hippocampal slice preparation. Transverse hippocampal slices were prepared from 2- to 3-week old Wistar rats. Following anaesthetization of the animal, the brain was

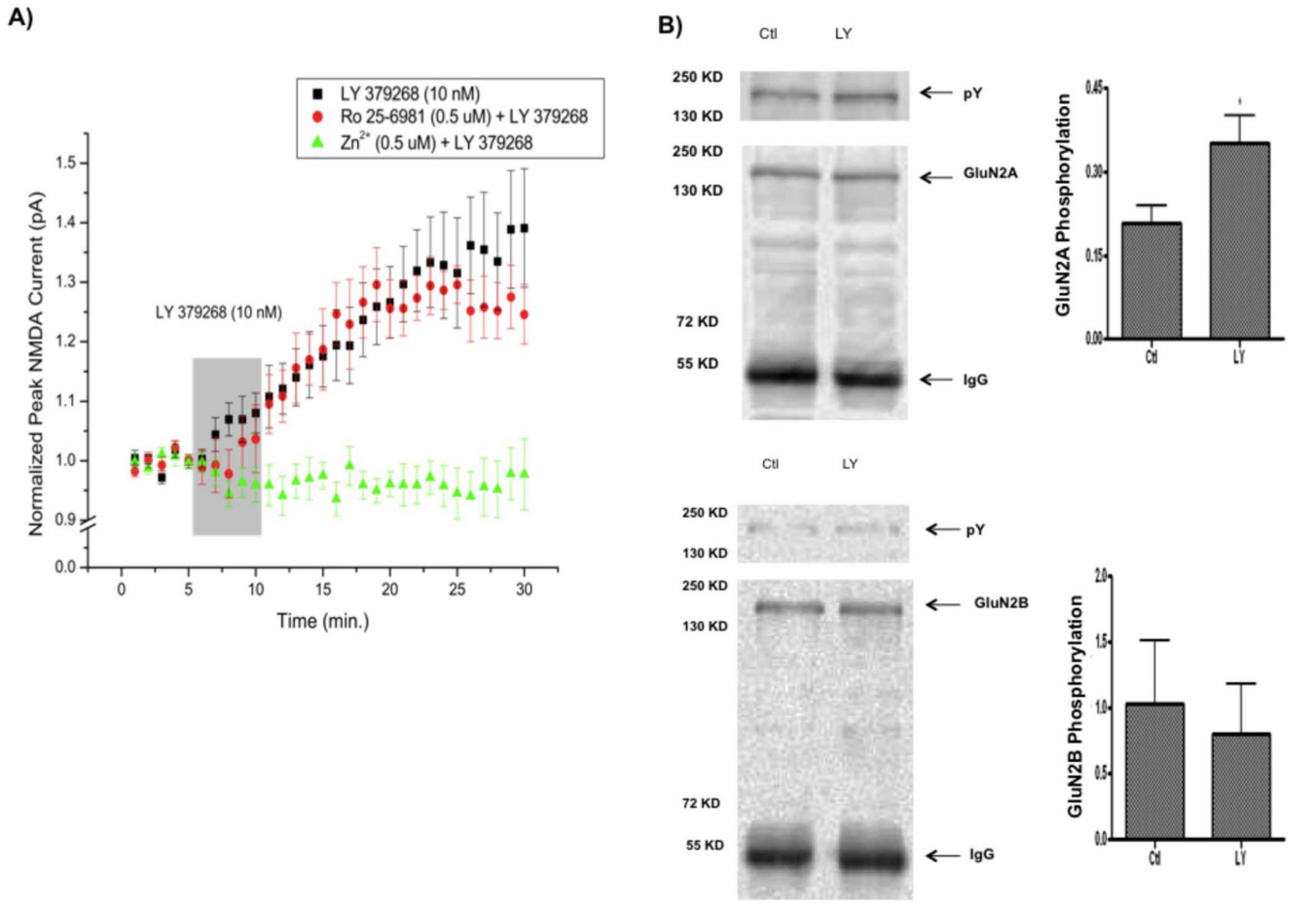

Figure 5 | Stimulation of group II metabotropic glutamate receptors potentiates GluN2A-containing NMDAR currents. A) Upregulation of I_{NMDA} by LY 379268 (N=7) was blocked by the GluN2A-antagonist Zn^{2+} (N=7) but not by the GluN2B-antagonist Ro 25–6981 (N=7) (LY plus Zn^{2+}, 97 ± 1%, n=6; LY plus Ro 25–6981, 125 ± 1%, n=6; LY alone, 135 ± 2%, n=16). The application of LY 379268 (10 nM) is indicated by the shaded region. Zn^{2+} (300 nM) and Ro 25–6981 (0.5 μM) were applied to the bath and to the perfusion solutions containing NMDA/glycine. B) LY 379268 (30 nM) treatment increases the tyrosine phosphorylation of immunoprecipitated GluN2A- but not GluN2B-containing NMDARs. The averaged relative density of pTyr for GluN2A (N=4) and GluN2B (N=4) obtained under each of the conditions is shown. * Indicates P<0.05, Student's t-test.

decapitated and quickly removed and placed in ice-cold oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.3 $MgCl_2$-$6H_2O$, 2.6 $CaCl_2$, 1.25 NaH_2PO_4-H_2O, 26 $NaHCO_3$, 10 glucose with an osmolarity between 300–310 mOsm. Coronal hippocampal slices 300 μm thick were prepared using a vibratome (VT100E, Leica). Slices were allowed to recover for at least 1 hour in oxygenated ACSF until needed.

Field excitatory postsynaptic potentials (fEPSP) recording from Schaffer collateral-CA1 synapses. $fEPSP_{NMDA}$ were evoked every 30 s (0.033 Hz) by electrical stimulation (100 μs duration) delivered to the Schaffer-collateral pathway using a concentric bipolar stimulating electrode (25 μm exposed tip) and recorded using glass microelectrodes (3–5 MΩ, filled with ACSF) positioned in the stratum radiatum of the CA1 area, when bicuculline methiodide (10 μM) and CNQX (20 μM) were present. The input-output relationship and paired-pulse ratio was determined in each slice by varying the stimulus intensity (50–200 μA) and recording the corresponding fEPSP. The stimuli intensity was made as that to evoke 30–50% of the maximal $fEPSP_{NMDA}$. After a 20min stable recording as baseline, LY379268 (30 nM) was bath applied. Signals were amplified (Axoclamp 700B, Molecular Devices, Sunnyvale CA, USA), recorded digitally (Digidata 1440A) and analyzed offline using Clampfit 10.

Whole-cell patch clamp recordings from hippocampal slices. A single slice was transferred to a recording chamber continually superfused with oxygenated ACSF (2 ml/min) composed of the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH_2PO4, 1.3 $MgCl_2$, 2.6 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, and 0.01 bicuculline methiodide (saturated with 95% O_2–5%CO_2 at 31–33°C). Recording electrodes (4–6 MΩ) were filled with internal solution containing (in mM): Cs-gluconate 132.5, CsCl 17.5, HEPES 10, EGTA 0.2, Mg-ATP 2 and GTP 0.3 (pH 7.25, 290 mOsm). Visual patch recordings of CA1 pyramidal neurons were performed using whole-cell configuration with holding potential at −60 mV. Synaptic responses were evoked with a concentric bipolar tungsten electrode located about 50 μm from the cell body layer in CA1 when the neuron was held at −70 mV and +40 mV, respectively. The AMPAR and NMDAR-mediated EPSC was measured at the peak and 20 ms after the start of stimulus artifact at −70 mV and +40 mV respectively, as previously described[46]. Signals were amplified using Multiclamp 700B, sampled at 5 kHz, and analyzed with Clampfit 10 software (Axon Instruments, Foster City, CA).

Cell isolation and whole-cell recordings. CA1 neurons were isolated from the hippocampus of postnatal rats (Wistar, 14–22 days) using previously described procedures[47]. To control for variation in response, recordings from control and drug-treated cells were conducted on the same day. The extracellular solution was composed of the following (in mM): 140 NaCl, 1.3 $CaCl_2$, 5 KCl, 25 HEPES, 20 glucose and 0.0005 tetrodotoxin, pH 7.4 (osmolality between 305 and 310 mOsm). Recording electrodes with resistances of 3–5 MΩ were constructed from borosilicate glass (1.5 μM diameter; World Precision Instruments, Sarasota, FL) using a two-stage puller (PP83; Narashige, Tokyo, Japan). Electrodes were filled with intracellular solution composed of the following (in mM): 140 CsF, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 2 tetraethylammonium, and 2K2ATP, pH 7.2–7.3 (osmolality between 290 and 300 mOsm). Where indicated, some drugs were included inside the patch pipette. Recordings were performed at room temperature (20–22°C). After formation of the whole-cell configuration, the neurons were voltage clamped at −60 mV and lifted into a stream of solution supplied by a computer-controlled, multi-barreled perfusion system (SF-77 B, Warner Instrument Corporation). The exchange time for solutions was ~30–50 ms. To monitor access resistance, a voltage step of −10 mV was made before each application of NMDA. If series resistance was increased to >20 MΩ, the cell was discarded. Currents were recorded using AxoPatch 1D amplifier. Data were filtered at 2 kHz and digitized at 10 kHz using Clampex software.

Zn^{2+}-buffered solutions. The tricine-buffered zinc solutions were prepared according to the empirically established binding constant by adding into 10 mM

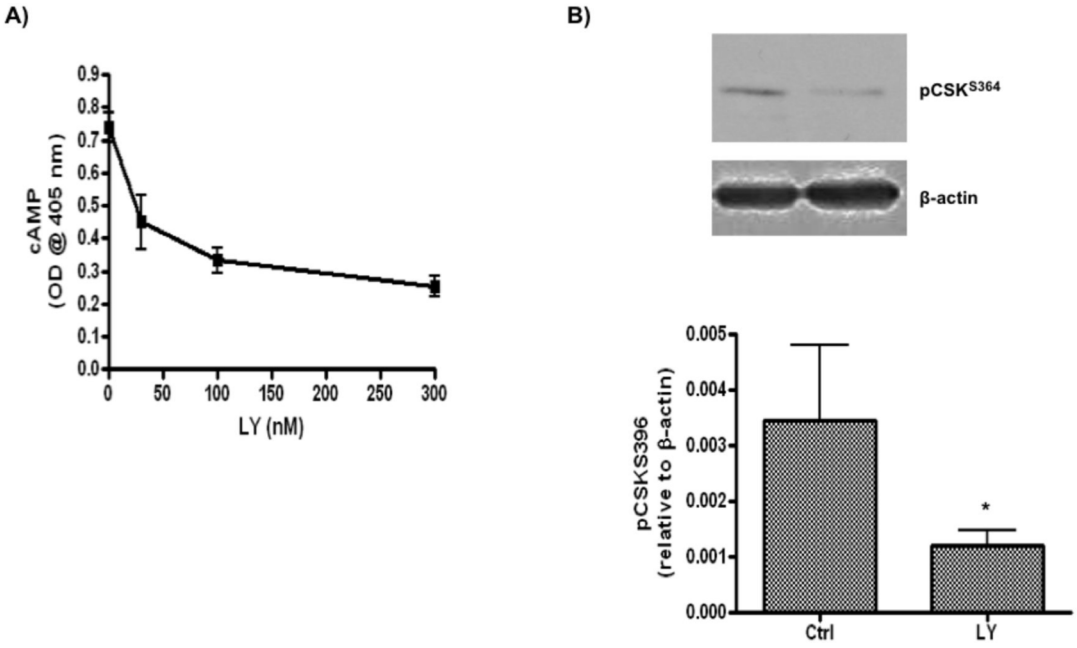

Figure 6 | Group II metabotropic glutamate receptors inhibit cAMP formation and prevent PKA-mediated phosphorylation of Csk. A) Dose-response curve showing a dose-dependent inhibition of cAMP formation with increasing concentrations of LY 379268. The IC_{50} for LY-mediated inhibition of cAMP was: 10.932 nM. B) LY 379268 (30 nM) treatment decreases the phosphorylation of Csk on its C-terminal Ser364. Below, summary of immunoblot analysis shows the averaged relative density of pCsk^{S396} for control (n=4) and LY 379268 (n=4) treatments. * Indicates $p<0.05$, Student's t-test.

tricine and as previously described[48]. The amount of $ZnCl_2$ required can be calculated based on the following formula: $[Zn]_{free} = [ZnCl_2]total/300$. (At pH 7.3; with 10 mM tricine for $[Zn]_{free}$ = 300 nM).

Immunoprecipitation and Western blotting. Hippocampal slices were prepared from Wistar rats (PN 15–20) and incubated with ACSF saturated with 95% O_2 and 5% CO_2 for at least 1 hour at room temperature. This was followed by treatment with LY 379268 (30 nM for 20 min) or vehicle control. For experiments testing Src(40–58), Fyn(39–57) or mGluR2/3 inhibition, the slices were pretreated with TAT-Src_{40-58} (10 μM), or TAT-Fyn_{39-57} (10 μM), or LY 341495 (30 nM) 30 minutes before drug exposures. After three washes with cold PBS, slices were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% Triton X-100, and 1% Sodium Deoxycholate) supplemented with 1 mM sodium orthovanadate and 1% protease inhibitor cocktail, 1% protein phosphatases inhibitor

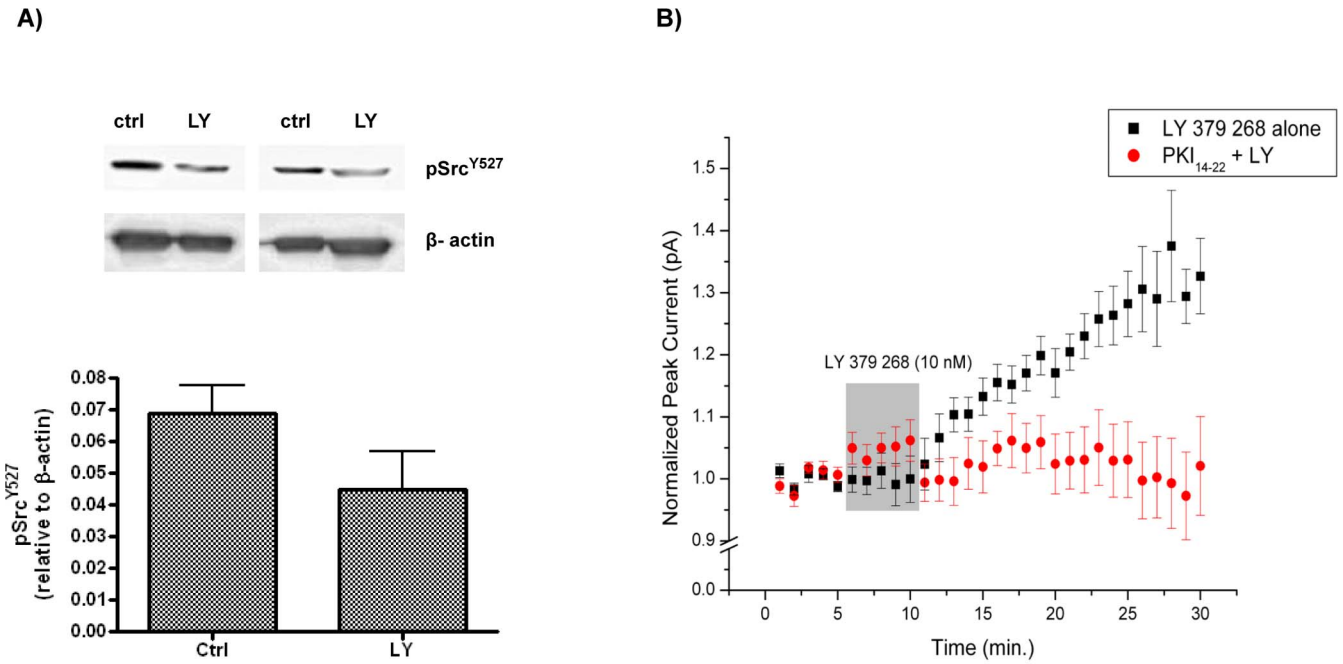

Figure 7 | Inhibition of PKA via group II metabotropic glutamate receptors activates Src kinase. A) LY 379268 (30 nM) treatment to hippocampal slices significantly reduces Csk-mediated phosphorylation of Src at its C-terminal regulatory tyrosine Y527. Decreased phosphorylation of Y527 prevents the intramolecular interaction with the SH2 domain on Src, promoting an open conformation of Src. Below, summary of immunoblot analysis shows the averaged relative density of pSrc^{Y527} for control (n=4) and LY 379268 (n=4) treatments. * Indicates $p<0.05$, Student's t-test. B) The upregulation of NMDAR-currents by LY 379268 (N=11) was occluded by the PKA inhibitory peptide PKI_{14-22} (N=9) (LY plus PKI_{14-22}, 99 ± 2%, n=9; LY alone, 135 ± 2%, n=16). PKI_{14-22} was included inside the patch pipette. The shaded region indicates the period of LY 379268 (10 nM) application.

cocktails, and subsequently spun at 16 000 r.c.f. for 30 minutes at 4°C (Eppendorf Centrifuge 5414R). The supernatant was collected and kept at −70°C. For immunoprecipitation, the sample containing 500 µg protein was incubated with antibodies (see below) at 4°C and gently shaken overnight. Antibodies used for immunoprecipitation were anti-GluN2A and anti-GluN2B (3 µg, rabbit IgG; Enzo Life Sciences, PA), anti-Src (1 : 500, mouse IgG, Cell Signaling Technology; Danvers, MA), and anti-Fyn (1 : 200, mouse IgG; Santa Cruz Biotechnology, Santa Cruz, CA). The immune complexes were collected with 20 µl of protein A/G Sepharose beads for 2 h at 4°C. Immunoprecipitates were then washed three times with ice-cold PBS, resuspended in 2-x Laemmli sample buffer and boiled for 5 minutes. These samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The blotting analysis was performed by repeated stripping and successive probing with antibodies: anti-pY(4G10) (1 : 2000, mouse IgG; Millipore Corp., Billerica, MA), GluN2A or GluN2B (1 : 1000, rabbit IgG; Cell Signaling Technology, Danvers, MA), $pSrc^{Y416}$ (1 : 1500, rabbit IgG; Cell Signaling Technology, Danvers, MA), $pSrc^{Y527}$ (1 : 1000, rabbit IgG; Cell Signaling Technology, Danvers, MA), $pCsk^{S364}$ (1 : 500, rabbit IgG; Antibodies-Online, Atlanta, GA), and $pGSK^{Ser9}$ (1 : 1000; rabbit IgG; Cell Signaling Technology, Danvers, MA).

cAMP assay. Hippocampal slices were prepared from Wistar rats (2–3 week old) and incubated in ACSF saturated with 95% O_2 and 5% CO_2 for at least 1 h at room temperature. Hippocampal tissue was then treated with 30 nM LY 379268 or vehicle control for 20 minutes. The treated tissue was then washed with cold ACSF 3 times and frozen immediately in liquid nitrogen. Equal amounts of frozen tissue were homogenized in 10 volumes of 0.1 M HCl and the debris was centrifuged at 10, 000 rpm for 10 min at 4°C (Eppendorf Centrifuge 5415R). Cyclic AMP levels were determined using the cAMP Immunoassay kit (Assay Designs, Ann Arbor, Michigan, USA). In order to increase the sensitivity of cAMP measurement, the supernatant was diluted 10-fold with distilled water. For the assay, 100 µl of the diluted sample, 50 µl of blue conjugate and 50 µl of yellow antibody were each added to the bottom of appropriate wells sequentially, and the reaction was incubated for 2 hours on a plate shaker at room temperature. The standards were prepared following the protocol supplied by the company. After incubation, the wells were emptied and washed 3 times with 400 µl of wash buffer. 200 µl of substrate solution was then added into each well. The reaction was stopped by adding 50 µl of stop solution and incubated at room temperature for 1 hour without shaking. The optical density of each well was then determined with a microplate reader set to 405 nm.

Drugs and peptides. The sources of drugs for this study are as follows: LY 379268, LY 341495, Ro 25-6981, MPEP hydrochloride, PKI_{14-22} amide (Tocris, Minneapolis, MN), TDZD-8, tricine, $ZnCl_2$, NMDA, glycine (Sigma, St. Louis, MO). Src(40–58) was provided by Dr. MW Salter (Hospital for Sick Children, Toronto, Ontario). The Fyn(39–57) peptide was synthesized by the Advanced Protein Technology Centre (Toronto, Ontario, Canada) with the following sequence: YPSFGVTSIPNYNNFHAAG, Fyn amino acids 39–57. We attached both Src(40–58) and Fyn(39–57) to Tat transduction domains (YGRLLRQRRR), which allowed us to apply these membrane permeant forms of Src versus Fyn interfering peptides to hippocampal slices for biochemical experiments.

Animal care. All animal experimentation was conducted in accordance with the Policies on the Use of Animals at the University of Western Ontario and approved by the Animal Care Committee of the University of Western Ontario.

Statistics. All population data are expressed as mean ± SD. Student's t-test was used to compare between two groups and one-way ANOVA with Tukey's post-hoc comparison was used to compare multiple groups.

1. Tsai, G. & Coyle, J. T. Glutamatergic mechanisms in schizophrenia. *Annu Rev Pharmacol Toxicol* **42**, 165–179 (2002).
2. Pilowsky, L. S. *et al.* First in vivo evidence of an NMDA receptor deficit in medication-free schizophrenic patients. *Mol Psychiatry* **11**, 118–119 (2006).
3. Hahn, C. G. *et al.* Altered neuregulin 1-erbB4 signaling contributes to NMDA receptor hypofunction in schizophrenia. *Nat Med* **12**, 824–828 (2006).
4. Krystal, J. H. *et al.* Subanesthetic effects of the noncompetitive NMDA antagonist, ketamine, in humans. Psychotomimetic, perceptual, cognitive, and neuroendocrine responses. *Arch Gen Psychiatry* **51**, 199–214 (1994).
5. Javitt, D. C. & Zukin, S. R. Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* **148**, 1301–1308 (1991).
6. Harrison, P. J. & Weinberger, D. R. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol Psychiatry* **10**, 40–68; image 45 (2005).
7. Imre, G. The preclinical properties of a novel group II metabotropic glutamate receptor agonist LY379268. *CNS Drug Rev* **13**, 444–464 (2007).
8. Patil, S. T. *et al.* Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: a randomized Phase 2 clinical trial. *Nat Med* **13**, 1102–1107 (2007).
9. Moghaddam, B. & Adams, B. W. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. *Science* **281**, 1349–1352 (1998).
10. Galici, R., Echemendia, N. G., Rodriguez, A. L. & Conn, P. J. A selective allosteric potentiator of metabotropic glutamate (mGlu) 2 receptors has effects similar to an orthosteric mGlu2/3 receptor agonist in mouse models predictive of antipsychotic activity. *J Pharmacol Exp Ther* **315**, 1181–1187 (2005).
11. Olszewski, R. T. *et al.* Phencyclidine and dizocilpine induced behaviors reduced by N-acetylaspartylglutamate peptidase inhibition via metabotropic glutamate receptors. *Biol Psychiatry* **63**, 86–91 (2008).
12. Crook, J. M., Akil, M., Law, B. C., Hyde, T. M. & Kleinman, J. E. Comparative analysis of group II metabotropic glutamate receptor immunoreactivity in Brodmann's area 46 of the dorsolateral prefrontal cortex from patients with schizophrenia and normal subjects. *Mol Psychiatry* **7**, 157–164 (2002).
13. Wright, R. A. *et al.* CNS distribution of metabotropic glutamate 2 and 3 receptors: Transgenic mice and [(3)H]LY459477 autoradiography. *Neuropharmacology* (2012).
14. Niswender, C. M. & Conn, P. J. Metabotropic glutamate receptors: physiology, pharmacology, and disease. *Annu Rev Pharmacol Toxicol* **50**, 295–322 (2010).
15. Anwyl, R. Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res Brain Res Rev* **29**, 83–120 (1999).
16. Manahan-Vaughan, D. Group 1 and 2 metabotropic glutamate receptors play differential roles in hippocampal long-term depression and long-term potentiation in freely moving rats. *J Neurosci* **17**, 3303–3311 (1997).
17. Weinberger, D. R., Berman, K. F., Suddath, R. & Torrey, E. F. Evidence of dysfunction of a prefrontal-limbic network in schizophrenia: a magnetic resonance imaging and regional cerebral blood flow study of discordant monozygotic twins. *Am J Psychiatry* **149**, 890–897 (1992).
18. Marsh, L., Suddath, R. L., Higgins, N. & Weinberger, D. R. Medial temporal lobe structures in schizophrenia: relationship of size to duration of illness. *Schizophr Res* **11**, 225–238 (1994).
19. Lu, W. Y. *et al.* G-protein-coupled receptors act via protein kinase C and Src to regulate NMDA receptors. *Nat Neurosci* **2**, 331–338 (1999).
20. Kotecha, S. A. *et al.* Co-stimulation of mGluR5 and N-methyl-D-aspartate receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons. *J Biol Chem* **278**, 27742–27749 (2003).
21. MacDonald, J. F., Kotecha, S. A., Lu, W. Y. & Jackson, M. F. Convergence of PKC-dependent kinase signal cascades on NMDA receptors. *Curr Drug Targets* **2**, 299–312 (2001).
22. Macdonald, D. S. *et al.* Modulation of NMDA receptors by pituitary adenylate cyclase activating peptide in CA1 neurons requires G alpha q, protein kinase C, and activation of Src. *J Neurosci* **25**, 11374–11384 (2005).
23. Yang, K. *et al.* Metaplasticity gated through differential regulation of GluN2A versus GluN2B receptors by Src family kinases. *EMBO J* (2011).
24. Monn, J. A. *et al.* Synthesis, pharmacological characterization, and molecular modeling of heterobicyclic amino acids related to (+)−2-aminobicyclo[3.1.0] hexane-2,6-dicarboxylic acid (LY354740): identification of two new potent, selective, and systemically active agonists for group II metabotropic glutamate receptors. *J Med Chem* **42**, 1027–1040 (1999).
25. Skeberdis, V. A. *et al.* Protein kinase A regulates calcium permeability of NMDA receptors. *Nat Neurosci* **9**, 501–510 (2006).
26. Gingrich, J. R. *et al.* Unique domain anchoring of Src to synaptic NMDA receptors via the mitochondrial protein NADH dehydrogenase subunit 2. *Proc Natl Acad Sci U S A* **101**, 6237–6242 (2004).
27. Smart, J. E. *et al.* Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60v-src) and its normal cellular homologue (pp60c-src). *Proc Natl Acad Sci U S A* **78**, 6013–6017 (1981).
28. Cheng, S. H. *et al.* Structural elements that regulate pp59c-fyn catalytic activity, transforming potential, and ability to associate with polyomavirus middle-T antigen. *J Virol* **65**, 170–179 (1991).
29. Paoletti, P., Ascher, P. & Neyton, J. High-affinity zinc inhibition of NMDA NR1-NR2A receptors. *J Neurosci* **17**, 5711–5725 (1997).
30. Nada, S., Okada, M., MacAuley, A., Cooper, J. A. & Nakagawa, H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. *Nature* **351**, 69–72 (1991).
31. Vang, T. *et al.* Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. *J Exp Med* **193**, 497–507 (2001).
32. Liu, X. *et al.* Regulation of c-Src tyrosine kinase activity by the Src SH2 domain. *Oncogene* **8**, 1119–1126 (1993).
33. Roussel, R. R., Brodeur, S. R., Shalloway, D. & Laudano, A. P. Selective binding of activated pp60c-src by an immobilized synthetic phosphopeptide modeled on the carboxyl terminus of pp60c-src. *Proc Natl Acad Sci U S A* **88**, 10696–10700 (1991).
34. Heidinger, V. *et al.* Metabotropic glutamate receptor 1-induced upregulation of NMDA receptor current: mediation through the Pyk2/Src-family kinase pathway in cortical neurons. *J Neurosci* **22**, 5452–5461 (2002).
35. Abrahamsen, H., Vang, T. & Taskén, K. Protein kinase A intersects SRC signaling in membrane microdomains. *J Biol Chem* **278**, 17170–17177 (2003).
36. Ma, Y. C., Huang, J., Ali, S., Lowry, W. & Huang, X. Y. Src tyrosine kinase is a novel direct effector of G proteins. *Cell* **102**, 635–646 (2000).
37. Della Rocca, G. J. *et al.* Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. *J Biol Chem* **272**, 19125–19132 (1997).
38. Luttrell, L. M. *et al.* Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. *J Biol Chem* **271**, 19443–19450 (1996).

39. Farazifard, R. & Wu, S. H. Metabotropic glutamate receptors modulate glutamatergic and GABAergic synaptic transmission in the central nucleus of the inferior colliculus. *Brain Res* **1325**, 28–40 (2010).
40. Poisik, O. *et al.* Metabotropic glutamate receptor 2 modulates excitatory synaptic transmission in the rat globus pallidus. *Neuropharmacology* **49 Suppl 1**, 57–69 (2005).
41. Le Meur, K., Galante, M., Angulo, M. C. & Audinat, E. Tonic activation of NMDA receptors by ambient glutamate of non-synaptic origin in the rat hippocampus. *J Physiol* **580**, 373–383 (2007).
42. Gao, X. M. *et al.* Ionotropic glutamate receptors and expression of N-methyl-D-aspartate receptor subunits in subregions of human hippocampus: effects of schizophrenia. *Am J Psychiatry* **157**, 1141–1149 (2000).
43. Gu, Z., Jiang, Q., Fu, A. K., Ip, N. Y. & Yan, Z. Regulation of NMDA receptors by neuregulin signaling in prefrontal cortex. *J Neurosci* **25**, 4974–4984 (2005).
44. Pitcher, G. M. *et al.* Schizophrenia susceptibility pathway neuregulin 1-ErbB4 suppresses Src upregulation of NMDA receptors. *Nat Med* **17**, 470–478 (2011).
45. Wittmann, M., Marino, M. J., Henze, D. A., Seabrook, G. R. & Conn, P. J. Clozapine potentiation of N-methyl-D-aspartate receptor currents in the nucleus accumbens: role of NR2B and protein kinase A/Src kinases. *J Pharmacol Exp Ther* **313**, 594–603 (2005).
46. von Engelhardt, J. *et al.* CKAMP44: a brain-specific protein attenuating short-term synaptic plasticity in the dentate gyrus. *Science* **327**, 1518–1522 (2010).
47. Wang, L. Y. & MacDonald, J. F. Modulation by magnesium of the affinity of NMDA receptors for glycine in murine hippocampal neurones. *J Physiol* **486 (Pt 1)**, 83–95 (1995).
48. Nozaki, C. *et al.* Zinc alleviates pain through high-affinity binding to the NMDA receptor NR2A subunit. *Nat Neurosci* **14**, 1017–1022 (2011).

Acknowledgements

This study was supported by a grant to JFM by CIHR. We thank Dr. MW Salter for the Src interfering peptide Src(40–58).

Author contributions

CT designed and performed whole-cell voltage clamp recordings from acutely isolated hippocampal neurons. YFX designed and performed whole-cell voltage clamp recordings from CA1 pyramidal neurons in slice. GL designed and performed all biochemical experiments. JFM conceptualized and supervised the project and contributed to the design of the experiments. CHT and JFM wrote the manuscript. All authors read and approved the final manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Trepanier, C., Lei, G., Xie, Y. & MacDonald, J.F. Group II metabotropic glutamate receptors modify N-methyl-D-aspartate receptors via Src kinase. *Sci. Rep.* **3**, 926; DOI:10.1038/srep00926 (2013).