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Fyn kinase contributes to tyrosine phosphorylation of the GABA_A receptor γ 2 subunit

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Abstract

Phosphorylation of GABA_A receptors is an important mechanism for dynamically modulating inhibitory synaptic function in the mammalian brain. In particular, phosphorylation of tyrosine residues 365 and 367 (Y365/7) within the GABA_A receptor γ 2 subunit negatively regulates the endocytosis of GABA_A receptors and enhances synaptic inhibition. Here we show that Fyn, a Src family kinase (SFK), interacts with the γ 2 subunit in a phosphorylation-dependent manner. Furthermore, we demonstrate that Fyn binds within a region of the γ 2 intracellular domain that is centered on residues Y365/7, with the phosphorylation of Y367 being particularly important for mediating this interaction. Tyrosine phosphorylation of the γ 2 subunit is significantly reduced in the hippocampus of Fyn knock-out mice, suggesting that Fyn is an important kinase that contributes to the phosphorylation of this subunit *in vivo*. Tyrosine phosphorylation of the γ 2 subunit is not completely abolished in Fyn kinase mice, suggesting that other SFKs, such as Src, also contribute to maintaining and regulating the endogenous phosphorylation level of γ 2-containing GABA(A) receptors. In summary, we demonstrate Fyn as one of the SFKs that binds to and phosphorylates the γ 2 subunit of the GABA_A receptor. This has important implications for the regulation of synaptic GABA_A receptors via signaling pathways that lead to the activation of Fyn kinase.

Introduction

GABA_A receptors are heteropentameric chloride-selective ligand-gated ion channels that mediate fast synaptic inhibition in the adult central nervous system (Jacob et al., 2008). They are also the therapeutic sites of action for many important classes of drugs, including benzodiazepines, barbiturates and general anesthetics (Rudolph and Mohler, 2004). Multiple subtypes of GABA_A receptors exist, however, the majority of synaptic GABA_A receptors in adult brain are composed of 2 α , 2 β and 1 γ subunit (Olsen and Sieghart, 2009).

Mechanisms that regulate GABA_A receptor function are of critical importance in modulating overall synaptic inhibition. Phosphorylation is one important way in which GABA_A receptor

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function can be dynamically regulated (Jacob et al., 2008). GABA_A receptor subunits have been shown to be phosphorylated by a number of serine/ threonine protein kinases, including protein kinase A (PKA), protein kinase C (PKC), Ca²⁺/ calmodulin-dependent protein kinase II (CaMKII) and protein kinase G (PKG) (Brandon et al., 2000; Houston et al., 2009; Vithlani and Moss, 2009), in addition to protein tyrosine kinases such as Src (Brandon et al., 2001; Moss et al., 1995; Valenzuela et al., 1995). Phosphorylation sites for these kinases have been mapped to the major intracellular domains (ICD) between transmembrane region (TM) 3 and TM4 of β 1-3 and γ 2 subunits (Brandon et al., 2000).

We have recently demonstrated an important role for tyrosine phosphorylation of the γ 2 subunit in regulating the efficacy of synaptic inhibition and in hippocampal-dependent learning (Tretter et al., 2009). This study was performing using a knock-in mouse in which the two major tyrosine phosphorylation sites within the γ 2 subunit (residues Y365 and Y367) were mutated to phenylalanines. Mice heterozygous for this mutation exhibited profound alterations in the membrane trafficking of GABA_A receptors (Tretter et al., 2009). This is consistent with previous *in vitro* studies demonstrating that residues Y365/7 are part of a classical tyrosine-based (Yxx Φ) binding motif for the clathrin adaptor protein 2 (AP2) complex (Kittler et al., 2008). Phosphorylation of these residues negatively regulates clathrin-dependent endocytosis of GABA_A receptors (Kittler et al., 2008).

In our current study, we aimed to identify novel γ 2 subunit-interacting proteins whose interaction is positively regulated by phosphorylation. Using a mass spectroscopy-based analysis we identified a protein tyrosine kinase, Fyn, that interacts directly with the ICD of the γ 2 subunit in a phosphorylation-dependent manner. Furthermore, we demonstrate that Fyn is an important mediator of tyrosine phosphorylation of the GABA_A receptor γ 2 subunit in the hippocampus.

Results

Fyn kinase interacts with the intracellular domain of the γ 2 subunit in a phosphorylation-dependent manner

Since tyrosine phosphorylation of the GABA_A receptor critically affects receptor trafficking and function (Kittler et al., 2008), we were interested in identifying proteins that interact with the receptor in a tyrosine phosphorylation-dependent manner. The γ 2 subunit is found in the majority of synaptic GABA_A receptors and it is known to be tyrosine phosphorylated at residues Y365 and Y367 (Moss et al., 1995). These residues are contained within the ICD of the γ 2 subunit. Thus, in order to screen for proteins binding to this subunit in a phosphorylation-dependent manner, we synthesized peptides encoding the intracellular region around tyrosine residues 365 and 367 and immobilized them to sepharose beads. One set of peptides was chemically di-phosphorylated on residues Y365 and Y367, while another set of peptides remained unphosphorylated. We then tested the binding of these tyrosine motif peptides in the di-phosphorylated and unphosphorylated forms to proteins found in brain lysates by performing an *in vitro* binding assay, followed by SDS-PAGE and Coomassie blue staining. Numerous proteins were observed to bind to both unphosphorylated and phosphorylated versions of the peptide encoding the region surrounding the tyrosine residues (Fig. 1A). Of greatest interest to us for this study were proteins that bound to the phosphorylated peptide but not the unphosphorylated peptide. In this regard, one major band interacting only with the phospho-peptide was observed at 55 kDa (Fig. 1A). This band was excised and subjected to Maldi-TOF mass spectrometry. The sequence of the protein led to its identification as Fyn kinase.

To confirm that Fyn kinase interacts with the intracellular region of the γ 2 subunit in a phosphorylation-dependent manner, we repeated the *in vitro* binding assay. After overnight

incubation of the immobilized unphosphorylated and phosphorylated peptides with hippocampal lysates, we performed SDS-PAGE on the bound proteins followed by Western blot analysis using an antibody specific to Fyn kinase (Fig. 1B). This confirmed that Fyn did indeed bind to the phosphorylated $\gamma 2$ peptide but not to the unphosphorylated peptide or to beads alone (Fig. 1B, lane 3 *versus* lanes 1 and 2).

To identify whether one or both of the tyrosine residues within the $\gamma 2$ subunit were important for mediating the binding of Fyn, we designed peptides that were chemically phosphorylated on only one of the two residues (ie. Y365 or Y367). When we repeated the *in vitro* binding assay using these singularly phosphorylated peptides, we saw that there was a significant reduction in the binding of Fyn to the peptide phosphorylated on residue Y365 compared to the peptide phosphorylated at both Y365 and Y367 (Fig. 1B, lane 4 *versus* lane 3). No reduction in binding was observed to the peptide singularly phosphorylated on Y367 (Fig. 1B, lane 5). Thus, these results demonstrate that Fyn kinase associates with the ICD of the $\gamma 2$ subunit in a tyrosine phosphorylation-dependent manner, and furthermore, that phosphorylation at tyrosine residue Y367 within the $\gamma 2$ subunit is the critical factor in mediating this interaction.

Tyrosine phosphorylation of the $\gamma 2$ subunit is mediated by Fyn kinase

Tyrosine phosphorylation of the $\gamma 2$ subunit has been observed in both cultured neurons and rodent brain (Brandon et al., 2001), however the identity of the tyrosine kinases responsible for this phosphorylation remain unknown. Having established that Fyn kinase binds to the intracellular region of the $\gamma 2$ subunit in a tyrosine phosphorylation-dependent manner, we next set-out to determine whether this kinase is responsible for phosphorylating the $\gamma 2$ subunit. To do this, we performed experiments using cultured hippocampal neurons that had been pre-treated for 30 minutes prior to lysis with the tyrosine phosphatase inhibitor, sodium pervanadate (NV). As had been previously established (Brandon et al., 2001), this treatment was necessary to observe robust levels of tyrosine phosphorylation of the $\gamma 2$ subunit by Western blot analysis (Fig. 2A, lane 2 *versus* lane 1). Fyn kinase is a member of the Src family kinase (SFK) family and we observed a dramatic reduction in the level of tyrosine phosphorylation of the $\gamma 2$ subunit upon pre-treatment of cultured neurons with NV and PP2, a pharmacological inhibitor of SFKs, compared to NV treatment alone (Fig. 2A, lane 3 *versus* 2, and Fig. 2B). Pre-treatment with NV and PP3 (an inactive analog of PP2) resulted in similar levels to NV treatment alone (Fig. 2A, lane 4 *versus* 2, and Fig. 2B). Thus, this result demonstrates that SFKs are responsible for tyrosine phosphorylation of the $\gamma 2$ subunit.

SFKs comprise a number of kinases. Five members of this family, namely Src, Fyn, Yes, Lck and Abl, are known to be expressed within the forebrain (Kalia and Salter, 2003; Salter and Kalia, 2004; Sugrue et al., 1990; Yagi et al., 1993). Pharmacological inhibition of the individual kinases within this family is currently not possible. Thus, to unequivocally demonstrate an involvement of Fyn kinase in the phosphorylation of the $\gamma 2$ subunit, we decided to assess the level of tyrosine phosphorylation within hippocampal lysates derived from Fyn knock-out mice. These mice have been widely characterized in previous studies (Grant et al., 1992; Stein et al., 1992). Hippocampal slices were prepared from homozygous Fyn mice and pre-treated with NV for 30 min prior to lysis and Western blot analysis. A significant reduction in the level of tyrosine phosphorylation of the $\gamma 2$ subunit was observed in Fyn knockout mice compared to wild-type littermates (Fig. 3). Phosphorylation was not completely abolished, suggesting that other SFKs are also involved in phosphorylating the $\gamma 2$ subunit.

To begin to analyze which other SFKs are associated with GABA_ARs via interactions with $\gamma 2$ subunit, unphosphorylated and phosphorylated peptides from the $\gamma 2$ subunit were exposed to solubilized hippocampal extracts and then immunoblotted with antibodies against Src, a SFK that has been previously shown to phosphorylate residues Y365/7 within the $\gamma 2$ subunit (Moss et al., 1995). Src was able to bind to peptides diphosphorylated on Y365/367 (Fig. 4A,

lane 3) but not to the unphosphorylated peptide (Fig. 4A, lane 2) or to a peptide that was singularly phosphorylated at residue Y365 (Fig. 4A, lane 4). Thus, these results suggest that both Fyn and Src are able to bind to the GABA_A γ 2 subunit, dependent on the phosphorylation status of residues Y365/7.

Given these *in vitro* binding experiments, an assessment was made of the ability of SFKs to associate with GABA_ARs from detergent solubilized cortical neurons. Lysates were immunoprecipitated with control IgG antibodies, or antibodies against the GABA_A β 3/ γ 2 subunits. Precipitated material was then subjected to *in vitro* kinase assays in the presence of ³²P γ -ATP to measure the autophosphorylation of associated tyrosine kinases. These experiments were performed in the presence of calphostin C to block PKC, as this family of enzymes are also intimately associated with GABA_ARs (Brandon et al., 1999). Under these conditions phosphorylation of a band of approximately 55 kDa was seen in material immunoprecipitating with γ 2/ β 3 antibodies that was not seen with IgG (Fig. 4B). Furthermore, phosphorylation of this band was blocked by genistein, a protein tyrosine kinase inhibitor, suggesting the presence of a tyrosine kinase (Fig. 4B). Consistent with this, phospho-amino acid analysis revealed that the respective band was phosphorylated solely on tyrosine residues (Fig. 4C). Unfortunately, the relatively low sensitivity of immunoblotting and the co-migration of IgG prevented the use of Src or Fyn antibodies in these experiments.

Collectively, these results illustrate that both Fyn and Src are associated with GABA_ARs via phospho-dependent binding to residues Y365/7 within the ICD of the γ 2 subunit. Given the important role that these residues play in regulating endocytosis of GABA_ARs and the efficacy of synaptic inhibition (Tretter et al., 2009) (Kittler et al., 2008), the ability of Fyn and Src to modulate their phosphorylation was assessed *in vitro*. For this experiment, purified glutathione-S-transferase (GST) fusion proteins encoding the intracellular domain of the γ 2 subunit or a mutant in which residues Y365/7 had been converted to phenylalanine residues were exposed to purified Fyn or Src kinase. Both kinases robustly phosphorylated the γ 2 subunit fusion protein, but not GST alone (Fig. 5, lane 2 versus 1). This phosphorylation was dependent on residues Y365/7, as phosphorylation was abolished or dramatically reduced in the mutant Y365/7F fusion protein (Fig. 5, lane 3).

Discussion

In this study, we have identified Fyn kinase as a protein that interacts with the GABA_A receptor γ 2 subunit in a tyrosine phosphorylation-dependent manner. Fyn binds to the ICD of the γ 2 subunit only when this region is phosphorylated at tyrosine residues 365 and 367, with phosphorylation of Y367 within the γ 2 subunit playing the major role in determining binding efficacy. To the best of our knowledge, this is the first identification of a protein whose binding to a GABA_A receptor subunit is positively modulated by phosphorylation. The previously identified association of the clathrin adaptor protein AP2 with the γ 2 subunit is negatively modulated by tyrosine phosphorylation (Kittler et al., 2008). It has also been shown that the binding affinity of PKC decreases with serine phosphorylation of GABA_A receptor β subunits (Brandon et al., 2002). The identification of proteins whose association with GABA_A receptor subunits are dynamically regulated by the phosphorylation state of the receptor is important for furthering our understanding of the mechanisms that neurons use to achieve an alteration in synaptic inhibition.

We also demonstrate in this study that Fyn phosphorylates endogenous γ 2 subunit-containing GABA_A receptors, since tyrosine phosphorylation of the γ 2 subunit is significantly reduced in the hippocampus of Fyn knockout mice. An involvement of Fyn kinase in the regulation of GABAergic function has been previously suggested by a number of studies utilizing Fyn mutant mice. In addition to impairments in NMDA receptor phosphorylation, LTP and spatial

memory (Grant et al., 1992; Miyakawa et al., 1997; Tezuka et al., 1999), Fyn knockout mice also have an altered sensitivity to GABAergic drugs, such as THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol), etomidate and muscimol (Boehm et al., 2004). Furthermore, Kitazawa et al. (1998) have demonstrated altered GABAergic function in olfactory bulb neurons from Fyn knockout mice. A reduction in GABAergic inhibition in the hippocampus of transgenic mice overexpressing constitutively active mutant Fyn has also been demonstrated (Lu et al., 1999). Together, these results have implicated Fyn kinase as an important modulator of GABAergic function, however, prior to our study there has been no evidence to indicate that Fyn directly interacts with GABA_A receptor subunits or any other protein important for GABAergic function.

Tyrosine phosphorylation of the $\gamma 2$ subunit was not completely abolished in the hippocampus of Fyn null mice, suggesting that other tyrosine kinases may also be involved. The majority of $\gamma 2$ tyrosine phosphorylation was inhibited by the pharmacological inhibition of Src family protein tyrosine kinases. Thus, other SFKs that are expressed in the forebrain, such as Src, Lyn, Lck, Abl or Yes, are likely to contribute to maintaining and regulating the endogenous phosphorylation level of $\gamma 2$ -containing GABA_A receptors (Salter and Kalia, 2004). Indeed, we demonstrate that Src is able to bind to the ICD of the $\gamma 2$ subunit in a phosphorylation-dependent manner. Furthermore, both Src and Fyn can phosphorylate residues Y365/7 in the $\gamma 2$ subunit. Due to the importance of these particular tyrosine residues in regulating GABA_A receptor endocytosis and synaptic inhibition (Tretter et al., 2009) (Kittler et al., 2008), both of these kinases are likely to have important roles in the dynamic regulation of GABA_A receptor function.

As previously mentioned, tyrosine phosphorylation at residues Y365/7 within the $\gamma 2$ subunit plays a critical role in the regulation of synaptic GABA_A receptor numbers. Enhancing tyrosine phosphorylation of the $\gamma 2$ subunit via activation of either Src or Fyn kinase, would be expected to lead to an increase in the number of synaptic GABA_A receptors at the membrane, due to a decreased association of the phosphorylated GABA_A receptors with the clathrin internalization machinery (Kittler et al., 2008). Indeed insulin and BDNF, both of which activate downstream tyrosine phosphorylation signaling pathways and SFKs (Iwasaki et al., 1998; Xu et al., 2006), result in the rapid recruitment of GABA_A receptors to postsynaptic sites in neurons (Jovanovic et al., 2004; Wan et al., 1997). Thus, recruitment of active SFKs to GABA_A receptors may play a critical role in rapidly modulating synaptic inhibitory levels.

In conclusion, we have identified Fyn as one of the tyrosine kinases responsible for phosphorylation of the $\gamma 2$ subunit in the hippocampus. Other SFKs, such as Src, which we have demonstrated to associate with and phosphorylate the $\gamma 2$ subunit in vitro, are also likely to play important roles in vivo. These findings have important implications for the regulation of synaptic GABA_A receptors via signaling pathways that lead to the activation of SFKs.

Experimental Methods

Antibodies

Anti-Fyn (mouse) and anti-Src (mouse) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and Millipore (Billerica, MA), respectively. Anti-GABA_A receptor $\gamma 2$ subunit (rabbit) and $\beta 3$ (rabbit) antibodies were a gift from Werner Sieghart (Medical University of Vienna, Austria). Anti- $\gamma 2$ (pY367) phospho-specific rabbit antibodies have been described in Tretter et al. (2009).

Drugs

Sodium pervanadate was prepared freshly on the day of treatment. Briefly, a 30 mM solution of sodium orthovanadate (Sigma) was activated by the addition of hydrogen peroxide (0.2% concentration) and incubated for at least 15min at room temperature before use at a final concentration of 50 μ M (cultured neurons) or 100 μ M (hippocampal slices). The Src family tyrosine kinase inhibitor PP2 was obtained from Calbiochem and 10 mM stock solutions were dissolved in DMSO and used at a concentration of 10 μ M. The inactive analog PP3 was used at an equivalent concentration. The protein tyrosine kinase inhibitor genistein was obtained from Calbiochem and 20 mM stock solutions were dissolved in DMSO and used at a concentration of 20 μ M. The PKC inhibitor Calphostin C was obtained from Calbiochem and 50 mM stock solutions were dissolved in DMSO and used at a concentration of 50 μ M.

Animals

Fyn knockout mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and these mice have been previously described in Stein et al. (1992). Heterozygous mating pairs were maintained to produce wild-type, heterozygous and homozygous littermates that were used in the present study. Timed-pregnant Sprague-Dawley rats were also purchased from Jackson (Bar Harbor, MN). All animal procedures were approved by Tufts University Institutional Animal Care and Use Committee.

Neuronal cultures

Rat embryos at embryonic day 18 were removed and decapitated into 1 $\times$ Hanks' balanced salt solution (HBSS, Invitrogen) on ice. Embryonic brain tissue was removed and transferred into fresh HBSS on ice and cortical and hippocampal regions were further dissected out. Dissected tissue was placed in 0.25% trypsin at 37°C for 15 min with gentle shaking. Tissues were washed with HBSS two times for 5 min and passed through medium-gauge pipette tips 10 times to dissociate. Non-dissociated debris was allowed to settle to the bottom for 10 min. Dissociated neurons were counted by hemocytometer and plated on 0.1 mg/ml poly(L)-lysine-treated 60-mm culture dishes at a density of 0.5 million/dish (hippocampal) and 1 million/dish (cortical) in attachment medium containing 10% fetal bovine serum, 1 mM sodium pyruvate and 25 mM glucose in minimum Eagle's medium (Invitrogen). 24 hr after plating, the attachment medium was replaced with warm maintenance Neurobasal medium (Invitrogen) supplemented with 2% B-27 neural supplement (Invitrogen), 33 mM D-glucose (Sigma) and 2 mM L-glutamine (Invitrogen). Neuronal cultures were kept in an incubator conditioned at 37°C and 5% CO₂. 1 ml of media was removed from the culture and 2 ml of fresh maintenance media was added to the culture dish every week to replenish nutrients and to prevent evaporative media loss. Neurons were used for experiments between 14-22 DIV.

Hippocampal acute slice preparation

The hippocampus was rapidly removed from mice (6-9 weeks of age) and rinsed with ice-cold artificial cerebral spinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 25 mM NaHCO₃, 1.1 mM NaH₂PO₄, 2 mM MgSO₄ and 10 mM D-glucose, equilibrated with 95% O₂ and 5% CO₂. The hippocampus was then rapidly cut into 400 μ m slices using a tissue chopper. Hippocampal slices were incubated in ACSF at 32°C for 1 h recovery prior to experimentation.

Preparation of protein extracts

After drug treatments, hippocampal cultured neurons or acute slices were lysed in SDS buffer (containing 1% SDS, 20 mM Tris pH 7.4, 50 mM NaF, 1 mM EDTA, 1 mM sodium orthovanadate, 250 μ g/ml AEBSF, 10 μ g/ml leupeptin, 10 μ g/ml antipain and 1 μ g/ml pepstatin). Insoluble material was removed by centrifugation at 13.2k rpm at 4°C for 10 min.

Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, IL).

In vitro binding assays

Hippocampi were dissected from adult mice (2-4 months of age) and lysed in lysis buffer (50 mM Tris pH 7.4, 10 mM EDTA, 320 mM sucrose, 1% Igepal CA-630, 0.5% deoxycholate, 150 mM NaCl, 1 mM sodium orthovanadate, 250 µg/ml AEBSF, 10 µg/ml leupeptin, 10 µg/ml antipain and 1 µg/ml pepstatin). Insoluble material was removed by centrifugation. Protein extracts were exposed to sepharose-coupled peptides at 4°C overnight. The following peptides of the GABA_A receptor γ2 subunit were used: ATHLQERDEEYGYECLDGKDC phosphorylated on Y365 and/or Y367, or unphosphorylated peptide. Beads were washed three times in lysis buffer, and proteins binding to the beads were separated by SDS-PAGE. For the identification of bound proteins, gels were Coomassie blue stained and bands were excised and processed for MALDI-TOF. Proteins were also detected by immunoblotting.

Immunoblotting

Protein samples were resolved on an 8% SDS-PAGE gel at constant voltage of 140 V and transferred to a nitrocellulose membrane (Biorad, Hercules, CA) and transferred to a nitrocellulose membrane. Membranes were blocked in a milk solution (5% milk in TBS-T: 20 mM Tris pH 7.4, 137 mM NaCl and 0.1% Tween 20) or a BSA solution (4% BSA in TBS-T) for the detection of non-phosphorylated and phosphorylated proteins, respectively. Membranes were then incubated overnight at 4°C with primary antibodies diluted in milk or BSA solution. Membranes were washed 3 times with TBS-T followed by incubation with anti-rabbit (GE Healthcare) or anti-mouse (Jackson ImmunoResearch) horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:5000 in milk or BSA solution for 1 h at room temperature and washes were repeated 3 times. Immunoreactivity was detected by using Visiglo Plus HRP chemiluminescence detection kit (Amresco, Solon, Ohio) and processed using a Fujifilm LAS 3000 detector. Immunoreactivity signal of proteins was quantified using the NIH ImageJ program.

Immunoprecipitation

18-24 Div cortical neurons were solubilized in a buffer containing 1% (CHAPS) and 150 mM NaCl, 10 mM triethanolamine (pH 7.6), 5mM EGTA, 5mM EDTA, 50 mM NaF, 10 mM Na pyrophosphate, 1 mM Na orthovanadate, 0.2 mMPMSF, and 10 µg/ml leupeptin, pepstatin, antipain, and aprotinin. GABA_A receptors were immunoprecipitated with anti-γ2 and β3 antibodies coupled to protein A-sepharose. Immunoprecipitated receptors were subjected to in vitro kinase assays as outlined below.

GST protein purification—GST-γ2 and mutant GST-γ2Y365/7F constructs have been described previously (Brandon et al., 2001). Fusion proteins were expressed in *Escherichia coli* and purified on immobilized glutathione by affinity chromatography. Bound material was subjected to in vitro kinase assays as outline below.

In vitro kinase assays—Immobilized proteins were washed extensively in kinase buffer (10 mM Tris, pH 7.5, 10 mM MgCl, 10 mM MnCl and 10 mM DTT) before being subjected to in vitro kinase assays in the presence of 5000 Ci/mmol ³²P-ATP (final ATP concentration 1.0 µM). Kinase assays were performed for 20 min at 30°C in the absence/ presence of the following drugs: calphostin C (50 µM) and genistein (20 µM) or recombinant purified Src or Fyn kinases purchased from Millipore (100ng). Phosphorylated proteins were then separated by SDS-PAGE and visualized by autoradiography.

Phospho-amino acid analysis—Gel slices excised from SDS-PAGE gels were washed and digested with 0.1 mg/ml trypsin and hydrolysed with 6 N HCl. The resulting phospho-amino acids were separated by thin-layer chromatography and visualized by autoradiography.

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Fig. 1. Phosphorylation-dependent binding of Fyn to the YECL peptide of the $\gamma 2$ subunit is critically dependent on tyrosine residue 367 within $\gamma 2$

A. Coomassie blue staining of hippocampal proteins copurifying with the unphosphorylated (lane 2) and di-phosphorylated (lane 3) YECL peptide of the $\gamma 2$ subunit. An arrow indicates the presence of a protein of 55 kDa that binds to the peptide only when it is phosphorylated (lane 3) but not in the absence of phosphorylation (lane 2) or to beads alone (lane 1). Mass spectroscopy analysis of this band identified the protein as Fyn kinase. B. Western blot analysis using anti-Fyn antibodies reveals a copurification of Fyn from hippocampal lysates with the YECL-peptide when it is phosphorylated on Y365/7 (lane 3) but not in the absence of phosphorylation (lane 2) or to beads alone (lane 1). A clear reduction in Fyn binding is observed for the peptide phosphorylated on Y365 (lane 4), whereas phosphorylation of Y367 (lane 5) results in similar binding levels as to the phosphorylated Y365/7 peptide (lane 3).

Fig. 2. Tyrosine phosphorylation of the GABA_A receptor $\gamma 2$ subunit is mediated through Src-family tyrosine kinases

A. Hippocampal neuronal cultures were treated with vehicle (control, lane 1), sodium pervanadate (NV, lane 2), NV plus PP2 (lane 3) or NV plus PP3 (lane 4). Homogenates were resolved by SDS-PAGE and analyzed by Western blotting using anti- $\gamma 2$ (pY367) (top panel) and anti- $\gamma 2$ (bottom panel) and antibodies. B. Bar histogram shows data normalized to control and plotted as a percentage of control $\pm$ S.D. $n = 3$ (** $P < 0.01$, *** $P < 0.001$, one sample t -test).

Fig. 3. Tyrosine phosphorylation of the $\gamma 2$ subunit is reduced in Fyn knockout mice

A. Hippocampal slices from wild-type (WT) mice (lanes 1 and 2) or Fyn knockout (KO) mice were treated with vehicle (lanes 1 and 3) or sodium pervanadate (NV, lanes 2 and 4). Homogenates were resolved by SDS-PAGE and analyzed by Western blotting using anti- $\gamma 2$ (pY367) (top panel), anti- $\gamma 2$ (middle panel) and anti-Fyn (bottom panel) antibodies. B. Bar histogram shows the ratio of $\gamma 2$ (pY367)/ $\gamma 2$ normalized to wild-type levels and plotted as a percentage of control $\pm$ S.D. $n = 3$ (* $P < 0.05$, one sample t -test).

Fig 4. Tyrosine kinases associate with the GABA_A receptor γ2 subunit

(A) Western blot analysis using anti-Src antibodies reveals a copurification of Src from hippocampal lysates with the YECL-peptide when it is phosphorylated on Y365/7 (lane 3) but not in the absence of phosphorylation (lane 2) or to beads alone (lane 1). A clear reduction in Src binding is observed for the peptide phosphorylated on Y365 (lane 4), whereas phosphorylation of Y367 (lane 5) results in similar binding levels as to the phosphorylated Y365/7 peptide (lane 3). (B) Tyrosine kinases immunoprecipitate with neuronal GABA_ARs. Detergent-soluble extracts of cortical neurons were immunoprecipitated with γ2/β3 subunit antibodies or control IgG antibodies. Precipitated material was subjected to *in vitro* kinase assays in the presence of Calphostin C (50 μM) to inhibit PKC and in the absence/ presence of genistein (Gen, 20 μM) to inhibit tyrosine kinases. After separation by SDS-PAGE, the major phosphorylated band at 55 kDa was visualized by autoradiography. (C) This 55 kDa band was subsequently subjected to phospho-amino acid analysis and the migration of phospho-serine, threonine and tyrosine residues are shown.

Fig. 5. Src and Fyn kinases phosphorylate residues Y365/7 in the GABA_A receptor γ2 subunit
 Glutathione S-transferase (GST) and GST-fusion proteins encoding the ICD of the γ2 subunit (GST-γ2) and a phospho-mutant of the γ2 subunit (GST-γ2Y365/7F) were phosphorylated using 100ng of purified Src or Fyn kinase in an in vitro kinase assay, followed by SDS-PAGE analysis and visualization of phosphorylated products by autoradiography.