H-Ras modulates NMDA receptor function via inhibition of Src tyrosine kinase activity

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Summary

Tyrosine phosphorylation of the NR2A and NR2B subunits of the NMDA receptor by Src protein tyrosine kinases (PTKs) modulates receptor channel activity and is necessary for the induction of long term potentiation (LTP). Deletion of H-Ras increases both NR2 tyrosine phosphorylation and NMDA receptor-mediated hippocampal LTP. Here we investigated whether H-Ras regulates phosphorylation and function of the NMDA receptor via Src family PTKs. We identified Src as a novel H-Ras binding partner. H-Ras bound to Src but not Fyn, both in vitro and in brain via the Src kinase domain. Cotransfection of H-Ras and Src inhibited Src activity and decreased NR2A tyrosine phosphorylation. Treatment of rat brain slices with Tat-H-Ras depleted NR2A from the synaptic membrane, decreased endogenous Src activity and NR2A phosphorylation, and decreased the magnitude of hippocampal LTP. No change was observed for NR2B. We suggest that H-Ras negatively regulates Src phosphorylation of NR2A and retention of NR2A into the synaptic membrane leading to inhibition of NMDA receptor function. This mechanism is specific for Src and NR2A and has implications for studies in which regulation of NMDA receptor-mediated LTP is important such as synaptic plasticity, learning and memory and addiction.

Keywords: H-Ras / NMDA / phosphorylation / Src / LTP / NR2A
Introduction

The N-methyl-D-Aspartate (NMDA) receptor is a ligand-gated calcium channel that plays an essential role in neuronal development, addiction, and learning and memory (1,2). Tyrosine phosphorylation modulates NMDA receptor function, for example, inhibition of tyrosine kinase activity decreases NMDA receptor-mediated currents whereas treatment with tyrosine phosphatase inhibitors increases these currents (3). Tyrosine phosphorylation of NR2A and NR2B occurs via Fyn and Src PTKs (4,5), resulting in potentiation of NMDA channel activity (6,7). NMDA receptor phosphorylation by Src and Fyn is modulated by many mechanisms including PKC (8) and the scaffolding protein RACK1 (9). Thus, the modulation of NMDA receptor function by Src PTKs likely involves the convergence of diverse signaling pathways generating a complex mechanism of receptor regulation.

Glutamate activation of the NMDA receptor and the subsequent increase in intracellular calcium are essential for the induction of long term potentiation (LTP), a candidate mechanism underlying synaptic plasticity (10), thought to mediate learning and memory (11,12). NMDA receptor-mediated LTP is associated with an increase in tyrosine phosphorylation of NR2 subunits via a mechanism that requires Src family PTKs (5). In the CA1 region of the hippocampus, activation of Src occurs within 5 minutes of LTP induction, and LTP can be prevented by application of Src-specific inhibitors (13). In addition, Fyn knockout mice show impaired LTP, which can be rescued by introduction of a Fyn transgene (14,15). Thus, both Src and Fyn are necessary for the induction of LTP (13-16).
Recently, increased tyrosine phosphorylation of NR2A and NR2B and subsequent enhanced LTP was observed in the hippocampus of H-Ras null mice (17). H-Ras functions as a molecular switch, existing in an active GTP-bound or inactive GDP-bound form (18). It is an upstream initiator of the MAP kinase pathway, which in neurons can be activated by the influx of calcium resulting from activation of the NMDA receptor (19-21). Many components of the H-Ras-MAPK pathway are associated with the postsynaptic density (PSD; 22) a region of the synapse packed with signal transduction complexes, including Src family PTKs, that colocalize with the NMDA receptor (23-25). A number of Ras family regulatory proteins have also been identified in the PSD suggesting that the H-Ras-MAPK pathway may have varied synapse-specific functions (26-30). Since NMDA receptor channel activity is positively regulated by Src tyrosine phosphorylation that is essential for LTP, we hypothesized that H-Ras negatively regulates NMDA phosphorylation and function via inhibition of a Src PTK. Here we present data showing that Src and H-Ras interact in vitro and in the brain. The interaction is specific for Src as Fyn, another member of the Src family expressed in the PSD, was unable to bind H-Ras. H-Ras binds via the Src kinase domain and inhibits Src kinase activity, decreasing phosphorylation and subsequently the membrane level of NR2A. Furthermore, overexpression of H-Ras in hippocampal slices results in a decrease in NMDA-mediated LTP. Taken together our results imply that H-Ras negatively regulates NMDA receptor channel activity by decreasing the number of NR2A-containing NMDA receptors in the synaptic membrane.
Experimental Procedures

Materials - Active recombinant Src and Fyn tyrosine kinases, H-RasGST-agarose, pUSE-SrcWT, pUSE-H-RasWT, Raf-1 H-Ras Binding Domain GST beads, anti-Src agarose, monoclonal anti-Src, anti-Fyn and anti-H-Ras antibodies were purchased from Upstate Biotechnologies (Lake Placid, NY). Anti-Src[pY418] antibodies were purchased from Biosource (Camarillo, CA). Phosphatase inhibitor cocktails, polyclonal anti-H-Ras and anti-c-Src antibodies were purchased from Sigma (St. Louis, MO). Anti-HA antibodies, all secondary antibodies, protease inhibitor tablets and Expand PCR system were purchased from Roche (Indianapolis, IN). Sequencing and generation of primers was carried out by the Gallo Center molecular biology core. Restriction enzymes and TNT in vitro translation kit were purchased from Promega (Madison, WI). pGBK-T7 was purchased from Clontech (Palo Alto, CA). \(^{35}\)S-methionine (15mCi/ml, 3000 Bq) and “Amplify” were from Amersham (Piscataway, NJ). Lipofectamine PLUS was purchased from Gibco (Carlsbad, CA). L(-tk) cells stably transfected with NR1+NR2A were a generous gift from Merck, Sharp and Dohme Research Laboratories.

Animals - Src+/- and Fyn-/- mice (129\*ImJ/C57BL6J hybrids) were purchased from Jackson Laboratories. Fyn-/- mice were mated in house with 129 wild type mice to generate Fyn+/- mice. Src+/- and Fyn+/- mice were mated to generate Src +/- and Fyn-/- mice. The genotyping of mice was determined by reverse transcription-PCR analysis of products derived from tail mRNA. The mean age of animals used in this study was 4 weeks. Male Sprague-Dawley rats, 3-4 weeks old, were purchased from Simonsen.
In Vitro Translations - [35S]methionine-labeled proteins were generated in rabbit reticulocyte lysates (TNT kit, Promega) using the appropriate cDNAs. The translation reactions were analyzed by SDS-PAGE and fluorography.

In vitro pull down assay - 30 units of Src kinase or 75 units of Fyn kinase were incubated with 5μg H-RasGST-agarose or GST-sepharose for 2 hr at 4°C with mixing. Agarose pellets were washed (1 x PBS; 1% Triton X-100), proteins resolved by SDS-PAGE and analyzed by western blotting using anti-Src (1:500), anti-Fyn (1:500) or anti-H-Ras (1:5000) monoclonal antibodies. The H-RasGST-agarose pull-down and competition assays using radiolabeled in vitro translated proteins were incubated and resolved as above. Gels were fixed in 40% methanol, 10% acetic acid, incubated in Amplify and dried down. Radiolabeled proteins were detected by overnight fluorography (-80°C).

Plasmid Construction - Src deletion mutant cDNAs, Src-SH3 (a.a. 84-145), Src-SH2 (a.a. 151-238) and Src-KD (a.a. 270-523), were amplified from pUSE-SrcWT using the Expand PCR system and cloned into pGBKT7. H-RasWT cDNA was amplified from mRNA generated from NT2 cells and cloned into pTAT-HA via EcoRI and XhoI sites.

Preparation of brain homogenates - Src -/- mice, Fyn -/- mice and 3-4 week old male Sprague-Dawley rats were euthanized with Halothane, whole brain dissected and homogenized in homogenization buffer (250 mM sucrose, 20 mM Tris-HCl pH 7.5, 2 mM EDTA, 10 mM EGTA and protease and phosphatase inhibitors as per manufacturer’s instructions). Membrane and cytosolic fractions were prepared as described previously (31). Briefly, homogenates were centrifuged at 1000g for 2 minutes yielding pellet (P1) and supernatant (S1) fractions. The S1 fraction was further centrifuged (10,000g, 30 minutes, 4°C) generating fractions P2 (crude synaptosomal
membranes) and S2 (cytosol and light membranes). Pellets were washed in cold PBS then solubilized in solubilization buffer (1% deoxycholate, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM EGTA, protease and phosphatase inhibitors as per manufacturer’s instructions).

**Immunoprecipitation** - Immunoprecipitation was performed with 5 µg of the appropriate antibodies and 500 µg protein homogenate as described previously (9).

**Transfection** - L(-tk) cells were cultured on 100mm plates and expression of NR1 and NR2A was induced as previously described (32). When 60% confluent, cells were transfected with a total of 10 µg of pUSE-SrcWT and pUSE-H-RasWT cDNA using Lipofectamine PLUS in accordance with the manufacturer’s instructions.

**Preparation of cell homogenates** - L(-tk) cells were washed once with cold PBS, harvested and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1% deoxycholate, and protease and phosphatase inhibitors as per manufacturer’s instructions). Samples were sonicated briefly and lysis was allowed to proceed for 30 min on ice. Determination of the protein concentration was made using BCA kit (Pierce). In addition, samples were also normalized with respect to SrcWT by western blot and densitometry (NIH Image v1.62).

**Generation of phospho-NR2A specific polyclonal antibody** - The NR2A-derived peptide RLLEGNFY(PO3)GSLFSV corresponding to amino acids 1318-1331 was generated by SynPep and used to immunize rabbits. Test bleeds were made over the course of three months after which terminal bleeds were taken. The antigenicity of the bleeds was analyzed by slot blot using the immunizing peptide and showed increasing recognition of the antigen over three months. The terminal bleed was tested in the same way and then
subsequently purified using the phospho-peptide coupled to an inert support. Specificity for the phosphorylated NR2A epitope was analyzed by slot blotting the immunizing phospho-peptide and a non-phospho form of the same epitope. No cross-reaction with the non-phospho-peptide was detected.

**Raf-1 binding assay** - Raf-1 RBD GST-agarose and rat total brain homogenate (250 μg) were diluted in PBS and incubated for 30 minutes at 4°C as per manufacturer’s instructions. Raf-1-H-Ras complexes were resolved by SDS-PAGE and analyzed by western blotting using anti-H-Ras antibodies.

**Preparation of Tat-H-Ras fusion protein** - pTAT-H-RasWT was expressed in and purified from *E. coli* as previously described (33). Tat fusion protein was detected using anti-HA antibodies.

**Immunohistochemistry** - Following incubation with 1μM Tat-H-Ras, L(-tk) cells were washed in cold wash buffer (PBS, 0.1% Triton X-100), fixed in ice-cold methanol for 3 minutes and blocked in wash buffer containing 0.3% normal goat serum for 4 hours at room temperature. Immunofluorescence was performed using rat monoclonal anti-HA antibodies (1:100) incubated overnight at 4°C. Staining was detected with secondary antibodies conjugated to Texas Red (1:500) incubated for 2 hours at room temperature in the dark. Slides were mounted using Vectashield and viewed with a Zeiss 510 meta laser-scanning confocal microscope. Images shown are individual middle sections of projected Z series and were processed using Adobe Photoshop (Adobe Systems Inc).

**Preparation of Tat-H-Ras treated brain slices** - Coronal whole brain slices were prepared from 3-4 week old male Sprague-Dawley rats (300 μm), Src-/− and Fyn-/− mice (250 μm). The slices were allowed to recover for at least 1 hour in an artificial cerebrospinal fluid
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(aCSF) perfusion medium saturated with 95%O₂/5%CO₂ containing (in mM): 126 NaCl, 1.2 KCl, 1.2 NaH₂PO₃, 1.2 MgCl₂, 2.4 CaCl₂, 18 NaHCO₃ and 11 glucose. Slices were incubated in either aCSF (Control) or 1µM Tat-H-Ras diluted in aCSF (treated) for 2 hours at room temperature after which homogenates were made and fractionated as described above.

Electrophysiology - Transverse hippocampal slices (350 µm) were prepared from 3-5 week old male Sprague-Dawley rats. Slices were maintained for at least 2 hours in artificial cerebrospinal fluid (aCSF) that contained 126mM NaCl, 1.2mM KCl, 1.2mM NaH₂PO₄, 0.01mM MgCl₂, 2.4mM CaCl₂, 18mM NaHCO₃ and 11mM glucose, saturated with 95%O₂/5%CO₂ at 25°C. Following recovery, slices were submerged and continuously superfused with aCSF at 25°C. Field excitatory post-synaptic potentials (fEPSPs) were recorded from stratum-radiatum of CA1 region with glass microelectrodes filled with 2M NaCl. Picrotoxin (100 µM) was added to the bath solution to block GABA₃ receptor-mediated IPSPs. To evoke fEPSPs, Schaffer collateral/commissural afferents were stimulated with 0.1-Hz pulses using steel bipolar microelectrodes at intensities adjusted to produce an evoked response that was 40-50% of the maximum-recorded fEPSP for each recording. LTP was induced by high frequency stimulation (100 Hz, 1-sec duration, 2 trains at 10 sec interval) at the same intensity as the test stimulus and synaptic responses were monitored for 60 min following LTP induction. Data were collected using an Axopatch-1D amplifier (Axon instruments), filtered at 2 kHz and digitized at 5-10 kHz. Compiled data were analyzed and expressed as the mean percent of fEPSP slope ± SEM over the baseline levels.
Results

Src and H-Ras interact in vitro and in brain

We set out to determine whether H-Ras negatively regulates the NMDA receptor through a Src-dependent mechanism. Previously Stancato and colleagues observed that H-Ras coimmunoprecipitates with Src in Sf9 insect cells (34). As both Src and H-Ras are present in the PSD (24), we hypothesized that H-Ras may regulate Src activity via a direct interaction. To confirm that Src and H-Ras interact directly, purified Src was incubated with H-RasGST-agarose or GST-sepharose alone. H-RasGST-agarose was capable of "pulling down" Src (Figure 1a, lane 3) whereas no interaction was observed between Src and GST-sepharose (Figure 1a, lane 4) suggesting a direct interaction between Src and H-Ras in vitro. To determine the specificity of this interaction, the pull-down experiment was repeated using another member of the Src family, Fyn, which shares 84% sequence homology with Src and is also expressed in the PSD (35). There was no significant interaction detected between Fyn and H-Ras (Figure 1b, lane 3) suggesting that the interaction between H-Ras and Src is specific and not a general property of the Src family of PTKs.

Next, to confirm that the Src-H-Ras interaction occurred in brain, we performed co-immunoprecipitation studies in rat brain homogenate (Figure 1c). We found that anti-Src antibodies were capable of co-immunoprecipitating H-Ras (lane 1) and conversely, that anti-H-Ras antibodies formed an immune complex with Src (lane 2). In summary, we show that Src and H-Ras can interact in vitro and in rat brain.
H-Ras interacts with Src via its kinase domain

Src is comprised of a unique N-terminal region, an SH3 domain, an SH2 domain and a C-terminal catalytic domain (36). To further characterize the Src–H-Ras interaction, we made constructs of various domains and expressed them as $^{35}$S-methionine-labeled proteins by in vitro translation (Figure 2a). Radiolabeled Src-SH3, Src-SH2 and Src-KD were incubated with H-RasGST-agarose and analyzed by fluorography. SrcKD, but not SrcSH2 or SrcSH3, bound to H-RasGST-agarose, indicating that the kinase domain of Src contains a binding site for H-Ras (Figure 2a, lane 6). To confirm the identification of the binding site, a competition assay was carried out in which H-RasGST-agarose was preincubated with either control (in vitro translated empty plasmid) or non-radiolabeled in vitro translated kinase domain (KD; Figure 2b). Radiolabeled kinase domain ($^{35}$S KD) was then added to the reaction and the incubation allowed to proceed. Assuming that the kinase domain is indeed the binding site for H-Ras, fewer binding sites would be available for $^{35}$S KD binding after preincubation with unlabeled KD. Indeed, there was a decrease in $^{35}$S KD detected after H-RasGST-agarose preincubation with unlabeled KD (lane 2) when compared with H-RasGST-agarose preincubation with control (lane 1). Therefore, the Src-H-Ras interaction was mediated at least in part, through binding between H-Ras and the kinase domain of Src.

H-Ras inhibits Src kinase activity and subsequent NR2A phosphorylation

The activation state of Src is affected by its structure. Phosphorylation of the C-terminal tyrosine of Src (Y529) by an upstream kinase, Csk, results in an intramolecular interaction between the phosphorylated residue and the Src-SH2 domain, inactivating Src
Dephosphorylation or binding by other molecules displaces this folding allowing Src autophosphorylation of Y418 and activation (reviewed in 39,40). Since H-Ras interacts via the kinase domain of Src (Figure 2a&b), we tested whether the binding of H-Ras to Src affected Src kinase activity. L(-tk) mouse fibroblast cells were transiently transfected with combinations of SrcWT and H-RasWT cDNA. Immunoprecipitation using anti-Src agarose confirmed that in these cells, Src co-immunoprecipitated with H-Ras (Figure 3a, lane 2). Therefore Src activity was measured in the presence of H-Ras using anti-Src[pY418], an antibody that recognizes active autophosphorylated Src. In cells transfected with SrcWT alone, anti-Src[pY418] detected a robust signal corresponding to Src at 60kD (Figure 3b, top panel, lane 1) suggesting that transfected Src was active. Cotransfection of H-RasWT with SrcWT resulted in a sharp decrease in active Src (Figure 3b, top panel, lane 2). Our results show that H-Ras significantly inhibits the ability of Src to autophosphorylate Y418, implying that Src kinase activity is abrogated by the binding of H-Ras to the Src kinase domain.

Since NR2A is a substrate for tyrosine phosphorylation by Src (6), we analyzed the effect of H-Ras overexpression and the consequent inhibition of Src activity on the tyrosine phosphorylation state of NR2A. To do so, we used L(-tk) cells stably transfected with NR1 and NR2A and transiently transfected them with combinations of SrcWT and H-RasWT cDNA. In addition, we generated a polyclonal rabbit antibody against tyrosine 1325-phosphorylated NR2A (41) and tested for the effects of H-Ras overexpression on basal level phosphorylation of NR2A. As predicted, there was a decrease in phosphorylated NR2A in SrcWT/H-RasWT transfected cells compared with those
transfected with Src alone (Figure 3c). Thus, co-transfection with H-RasWT decreased Src kinase activity, consequently inhibiting Src-mediated NR2A phosphorylation.

**Active Tat-H-Ras transduces into cultured cells and brain and inhibits endogenous Src kinase activity**

In order to determine whether H-Ras modulates endogenous Src kinase activity and NR2A-mediated channel function, we used the Tat fusion protein transduction system (42) to elevate H-Ras protein levels in brain slices. We generated a Tat-H-Ras construct and expressed and purified the fusion protein from *E. coli* as described previously (33). First, we used immunofluorescence and confocal microscopy to confirm that the fusion protein was capable of entering cells. Control and Tat-H-Ras (1μM) transduced L(-tk) cells were fixed and analyzed using an antibody against an HA tag engineered into the Tat fusion protein (Figure 4a). There was a clear antibody signal detected in cells treated with Tat-H-Ras (right panel) but not in control (left panel). Since the images shown represent the middle sections of a projected Z series, Tat-H-Ras has been successfully transduced through the cell membrane. Next, we tested Tat-H-Ras transduction in brain slices. Coronal rat whole brain slices were incubated in the absence or presence of Tat-H-Ras, homogenized and fractionated to P2 (crude synaptosomes) and S2 (cell cytosol, light membranes) fractions. The integrity of the fractions was confirmed using an antibody against PSD-95 (data not shown), a protein enriched in the PSD (43) and Tat-H-Ras was detected in the P2 pellet fraction (Figure 4b, lane 3) correlating with previous data showing that H-Ras is localized to the membrane (44,45). In order to determine whether transduced Tat-H-Ras was functional, we utilized the Raf-1 binding
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Initiation of the MAPK pathway requires H-Ras to be active (i.e. GTP-bound) before it can bind to and activate Raf-1, a kinase downstream of H-Ras (46). Thus, the activation state of Tat-H-Ras can be determined by its binding to the H-Ras binding domain (RBD) on Raf-1. We found that transduced Tat-H-Ras did indeed bind to Raf-1 RBD (Figure 4b, lane 3) suggesting that Tat-H-Ras was successfully transduced into brain, was activated and correctly compartmentalized. As transfection of H-Ras caused a decrease in Src kinase activity in L(-tk) cells (Figure 3a), we examined Src kinase activity in Tat-H-Ras treated brain slices using anti-Src[pY418] antibodies. The pY418 signal was reduced in slices treated with Tat-H-Ras (Figure 4c, top panel, lanes 1 and 3) implying that transduction of Tat-H-Ras decreases Src kinase activity through in vivo interaction with the Src kinase domain.

Tat-H-Ras decreases NR2A retention and NR2A phosphorylation in synaptic membranes

Interestingly, we found that the level of NR2A in the crude synaptosomal membrane fraction (P2) was decreased after transduction of Tat-H-Ras (Figure 5a, top panel, lanes 1 and 3). In addition, there was a concomitant decrease in phosphorylated NR2A on Tat-H-Ras treatment (Figure 5a, middle panel, lanes 1 and 3) consistent with the observations made for NR2A in L(-tk) cells (Figure 3c). Since Src is capable of phosphorylating NR2B as well as NR2A in vitro (47) and since inhibition of Src-mediated phosphorylation by H-Ras decreased NR2A in the synaptic membrane, we examined whether H-Ras affects retention of NR2B in the synaptic membrane. There was no change in NR2B levels in the synaptic membrane after Tat-H-Ras treatment (Figure
5b) suggesting that Src-mediated tyrosine phosphorylation plays a major role in specifically retaining NR2A-containing NMDA receptors in the membrane.

**Retention of NR2A in the membrane is mediated by a Src-specific mechanism**

In order to confirm that the observed membrane depletion of NR2A was mediated via a Src tyrosine kinase mechanism, we transduced Tat-H-Ras into brain slices from Src -/- and Fyn -/- mice. Tat-H-Ras transduced slices from Fyn -/- mice showed decreased NR2A in the crude synaptosomal P2 fraction (Figure 6a) as observed in Tat-H-Ras treated brain slices (Figure 5a, top panel, lanes 1 vs. 3). However, no change was seen in membrane-associated NR2A in Src -/- mice (Figure 6b) implying that the retention of NR2A in the membrane is mediated by Src and not Fyn. There was no change in levels of NR2B in the membrane on Tat-H-Ras treatment (Figure 6c) consistent with the results observed in rat brain slices (Figure 5b). Therefore, not only is H-Ras inhibition a Src-specific effect, the downstream consequences of this inhibition are specific for NR2A-containing NMDA receptors. Furthermore, even though the overall amount of NR2A in the Src -/- was significantly less than in the Fyn -/- mice (data not shown), NR2A was increased in the S2 fractions of Src -/- mice (Figure 6b, lanes 2 and 4) compared with the Fyn -/- mice (Figure 6a, lanes 2 and 4) supporting the hypothesis that Src is required for efficient membrane retention of NR2A.

**Tat-H-Ras inhibits the induction of LTP in hippocampal slices**

Manabe et. al. report an increase in NMDA receptor-mediated LTP in H-Ras -/- mice (17). Src is required for the induction of LTP (13) and mice lacking NR2A show
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reduced responses to LTP-inducing stimuli (48). As Tat-H-Ras inhibited Src kinase activity and reduced NR2A levels in the membrane, we predicted that elevation of intracellular Ras levels by treatment with Tat-H-Ras would result in inhibition of LTP. Field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 region of the hippocampus from control and Tat-H-Ras treated slices. A stable baseline was established (Figure 7b, traces 1 and 3) and LTP induced by tetanic stimulation of afferent fibers (Figure 7a). The magnitude of LTP in Tat-H-Ras slices was 45% of that measured in control untreated slices (Figure 7a&b, traces 2 vs. 4) or slices treated with a control Tat fusion protein (Tat-KIP27; data not shown). Thus, elevating levels of H-Ras in brain inhibits Src kinase activity, reduces NR2A membrane retention and phosphorylation and consequently inhibits NMDA receptor-mediated long term potentiation.
Discussion

Tyrosine phosphorylation of the NMDA receptor by Src PTKs plays an important role in the modulation of receptor function and is essential for the regulation of synaptic plasticity (1). We present here data to suggest a mechanism for the regulation of Src via the small GTP-binding protein H-Ras, which can act as a novel “switch off” mechanism for Src-mediated phosphorylation of the NMDA receptor (Figure 8). We found that H-Ras interacts with Src both in vitro and in brain. H-Ras bound to the Src kinase domain and decreased Src autophosphorylation. Transduction of Tat-H-Ras into brain slices reduced endogenous Src kinase activity, NR2A phosphorylation and NR2A retention in the synaptic membrane. Finally, treatment with Tat-H-Ras was capable of inhibiting NMDA receptor-mediated LTP in hippocampal slices. Our results suggest that the increase observed in NMDA receptor-mediated LTP in H-Ras null mice (17) may be due to increased Src phosphorylation of NR2A and the maintenance of NR2A-containing NMDA receptors in the membrane. The absence of H-Ras prevents the inhibition of Src kinase activity and consequently, the loss of NR2A from the membrane. Our results also correlate with LTP data from SynGAP +/- mouse hippocampal slices (49). SynGAP is a GTPase activating protein enriched in the PSD that negatively regulates the activity of synaptic Ras (26,27). In mice heterozygous for SynGAP, implying more Ras in its GTP-bound active form, the induction of LTP is inhibited (49). This supports our observations suggesting that increased Ras in the hippocampus negatively regulates LTP (Figure 7).

The mechanism by which inhibition of Src phosphorylation leads to a change in the level of NR2A in the synaptic membrane remains unknown. Since Src family-mediated NR2 phosphorylation is required for trafficking of NR2 subunits to the
postsynaptic membrane (31,50), it is possible that inhibition of tyrosine phosphorylated NR2A prevents its initial insertion into the membrane. However we do not see a corresponding increase of NR2A in the cytosolic S2 fraction after Tat-H-Ras treatment (Figure 5a, top panel, lane 4), which would occur if NR2A were unable to localize to the membrane.

Recently it has been proposed that protein turnover in the PSD regulates synaptic activity and structure (51,52). A known mediator of protein degradation in the PSD is calpain, a protease that is activated in response to increases in intracellular calcium (53). Calpain degradation promotes restructuring of the PSD (54) and some of the major constituents of the PSD e.g. PSD-95 and the intracellular C-termini of both NR2A and NR2B, are substrates for calpain-mediated proteolysis (55,56). Src and Fyn tyrosine phosphorylation exert a protective effect from calpain degradation on NR2A and NR2B respectively (57). Therefore inhibition of Src phosphorylation of NR2A by H-Ras may promote its degradation by calpain and result in the loss of NR2A from the synaptic membrane.

Interestingly, the inhibitory effect of H-Ras is specific for Src since Fyn was not capable of binding H-Ras in vitro (Figure 1b) and the reduction of NR2A in the membrane fraction after Tat-H-Ras treatment was still apparent in Fyn-/− mice (Figure 6a). In addition, since no loss of NR2B from the membrane was observed in either rat (Figure 5b) or Src −/− mouse brain slices (Figure 6c) after Tat-H-Ras treatment, our results suggest that the action of H-Ras is specific for NR2A. Although the sequences of Src and Fyn are highly homologous, conformational differences conferred by their N-terminal unique domains may allow selective binding to other proteins. In addition,
differences in compartmentalization of binding partners may account for specific binding
to subsets of highly related proteins. A number of scaffolding proteins have been isolated
in the PSD (23,24). Previously we showed that Fyn but not Src is capable of binding the
scaffolding protein RACK1 in a trimolecular complex with NR2B (9). The formation of
this complex prevents Fyn from phosphorylating NR2B and negatively regulates the
function of NR2B- but not NR2A-containing NMDA receptors (9). During the current
study we showed that Src but not Fyn is capable of binding to H-Ras, causing a decrease
in Src kinase activity and subsequent reduction in phosphorylation and retention of
NR2A but not NR2B in the membrane. In vitro, Src and Fyn are capable of
phosphorylating both NR2A or NR2B (6,35,47). We therefore suggest that it is
compartmentalization that regulates the specificity of Src and Fyn for in vivo
phosphorylation of the NR2 subunits.

In summary, we show here that Src tyrosine phosphorylation of NR2A is
negatively regulated by H-Ras. The H-Ras inhibition of Src and subsequent alteration in
NMDA receptor composition at the membrane contribute to negative regulation of
synaptic strength. LTP is understood to represent a cellular basis for learning and
memory (58) and there is a positive correlation between increased synaptic strength and
learning and memory paradigms. Ras has been implicated in both regulation of LTP (17)
and in learning and memory (59-61). Therefore the negative regulation of NMDA
receptors by H-Ras may have implications for modulation of synaptic plasticity and
learning and memory.
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References

Figure Legends

Figure 1 Src and H-Ras interact *in vitro* and in brain.

a) Src (30 Units) was incubated in the presence of H-RasGST-agarose (5 µg; lane 3) or GST-sepharose (5 µg; lane 4). Protein complexes were washed and analyzed by western blot using anti-Src (top panel) and anti-H-Ras (bottom panel) antibodies. Src (6 Units; lane 1) and H-Ras (1 µg) are shown as controls for input. N=3

b) The experiment was repeated for Fyn (75 Units; lane 3) and the membrane probed with anti-Fyn monoclonal antibodies. An input control of Fyn (15 Units) is shown (lane 2). N=3

c) Rat whole brain homogenates (500 µg) were incubated overnight with anti-Src (5 µg; lane 1), anti-H-Ras monoclonal antibodies (5 µg; lane 2) or normal mouse IgG (5 µg; lane 3) and immune complexes were resolved by SDS-PAGE. Antibodies alone (lanes 4 and 5) and total homogenate (50 µg; lane 6) were added as a control. Src (top panel) and H-Ras (bottom panel) were detected using the appropriate polyclonal antibodies. N=3.

Figure 2 H-Ras interacts via the Src kinase domain

a) ^35S-labeled deletion proteins were incubated with H-RasGST-agarose (5 µg; lanes 4-6). Complexes were washed, resolved by SDS-PAGE and analyzed by fluorography. Control *in vitro* translated products (1/10th volume of binding assay; lanes 1-3) are also shown. N=3

b) H-RasGST-agarose was preincubated with equivalent amounts of either control *in vitro* translation (empty plasmid; lane 1) or unlabeled Src kinase domain (KD; lane 2) for 1hr. An equivalent amount of radiolabeled KD was added to both reactions and the
incubation continued for a further 2 hours. Protein complexes were washed, resolved by SDS-PAGE and analyzed by fluorography as above. N=3

**Figure 3 Binding of Src and H-Ras causes inhibition of Src kinase activity and decreases NR2A phosphorylation in L(-tk) cells**

a) L(-tk) cells transfected with SrcWT ± H-RasWT were lysed and 500 μg total protein incubated overnight with anti-Src-agarose (5 μg). Immune complexes (lanes 1 and 2) were washed, resolved by SDS-PAGE and analyzed by western blotting with anti-Src or anti-H-Ras monoclonal antibodies. Total protein (50 μg) was also included as a control for expression of transfected protein (lanes 3 and 4). N=3

b) L(-tk) cells transfected with SrcWT and H-RasWT were lysed and normalized with respect to Src by western blotting. Lysates (approx. 50 μg) from SrcWT and SrcWT/H-RasWT transfections were resolved by SDS-PAGE then analyzed by western blot using anti-Src[pY418], an antibody that recognizes active Src (top panel). A loading control was run simultaneously (bottom panel). N=3

c) Cell lysates (50 μg) were resolved as before and analyzed using anti-phospho NR2A (top panel) and anti-NR2A antibodies (middle panel). The bar histogram represents percentage NR2A phosphorylation normalized to total NR2A ± SD. N=3.

**Figure 4 Active Tat-H-Ras is successfully transduced into brain slices and inhibits endogenous Src kinase activity**

a) L(-tk) cells were transduced with Tat-H-Ras (1 μM) and analyzed by confocal microscopy using an anti-HA antibody and secondary antibodies conjugated to Texas
Red. Cells were scanned using a Zeiss 510 meta laser-scanning confocal microscope and viewed at a magnification of 25x. Images shown are individual middle sections of projected Z series and are representative of 3 separate experiments.

b) Control (lanes 1 and 2) and Tat-H-Ras treated P2 and S2 fractions (lanes 3 and 4; 500 μg) were incubated with Raf-1 RBD GST-agarose. Protein complexes were resolved by SDS-PAGE and analyzed by western blotting using anti-H-Ras monoclonal antibodies. Tat-H-Ras migrates with an apparent molecular mass of approx. 32 kD (lane 3), slightly larger than endogenous H-Ras (21 kD; lanes 1 and 3). N=3

c) Control (lanes 1 and 2) and Tat-H-Ras (lanes 3 and 4; 1 μM)-treated rat brain homogenate fractions (P2 and S2; 50 μg) were resolved by SDS-PAGE and analyzed by western blotting using anti-Src[pY418] polyclonal antibodies (top panel). The membrane was stripped and reprobed with anti-Src monoclonal antibodies (bottom panel). The bar histogram shows data normalized to total Src and plotted as a percentage of control ± SD. N=3

Figure 5 Tat-H-Ras decreases NR2A phosphorylation and NR2A membrane retention in synaptic membranes

a) Control (lanes 1 and 2) and Tat-H-Ras (lanes 3 and 4; 1 μM)-treated P2 and S2 fractions (500 μg) were subjected to immunoprecipitation using anti-NR2A antibodies (5 μg). The resulting immune complexes were resolved by SDS-PAGE and analyzed by western blotting using anti-NR2A (top panel) or anti-pY20 (middle panel) antibodies. The bottom panel shows control input (50 μg) prior to immunoprecipitation, analyzed using anti-actin antibodies. N=3
b) Homogenates were prepared as in a) and protein analyzed using anti-NR2B (top panel) and anti-actin (bottom panel) antibodies. N=3

**Figure 6 NR2A membrane retention requires a Src-specific mechanism**

Control (lanes 1 and 2) and Tat-H-Ras (lanes 3 and 4; 1 μM)-treated slice homogenates from Fyn -/- (a) and Src -/- (b&c) mice (P2 and S2; 50 μg) were resolved by SDS-PAGE and analyzed by western blotting using anti-NR2A (a&b; top panels) and anti-NR2B (c; top panel) antibodies. Bar histograms show data normalized to actin (bottom panels) and plotted as a percentage of control ± SD. N=3

**Figure 7 LTP is inhibited in Tat-H-Ras treated rat hippocampal slices.**

a) The averaged time course of LTP induction in control (n = 3 slices, 3 rats) and Tat-H-Ras (n = 3 slices, 3 rats) treated slices. Initial fEPSP slopes were measured, and the values were normalized in each experiment using the averaged slope value measured during the control period (time, −15 to 0 min). Tetanic stimulation was applied at time 0. Data is plotted ± SEM.

b) Sample traces of field EPSPs (average of 12 consecutive responses) of control and Tat-H-Ras (1 μM) treated slices recorded at the times indicated in a. The stimulus artifacts are truncated.
**Figure 8 Model**

a) Src tyrosine kinase phosphorylates NR2A allowing its retention in the synaptic membrane.

b) Inhibition of Src kinase activity by interaction with H-Ras causes a decrease in the phosphorylation of membrane-bound NR2A. Subsequently there is a reduction in NR2A in the synaptic membrane and a concomitant inhibition in NMDA receptor-mediated LTP.
Figure 2

(a) SH3 SH2 KD SH3 SH2 KD

RasGST-Agarose+

(b) Con+35S KD KD+35S KD

1 2 3 4 5 6

1 2
Figure 5

a

Con | Tat-Ras
---|---
P2 | P2 S2 | P2 S2

250 - 
160 - 

IP: αNR2A
WB: αNR2A

Con | Tat-Ras
---|---
P2 | P2 S2 | P2 S2

250 - 
160 - 

IP: αNR2A
WB: αPY

Con | Tat-Ras
---|---
P2 | P2 S2 | P2 S2

50 - 
35 - 

Actin

b

Con | Tat-Ras
---|---
P2 | P2 S2 | P2 S2

250 - 
160 - 

NR2B

50 - 
35 - 

Actin

1 2 3 4
Figure 7

(a) Normalized EPSP slope over time for Control and Tat-H-Ras.

(b) Voltage traces showing 0.5 mV and 10 msec.

Legend:
- Control
- Tat-H-Ras

Note: Graphs depict changes in normalized EPSP slopes and voltage traces over time.
Figure 8

(a) 

Src

H-Ras

P

Activity

(b) 

Src

H-Ras

P

Activity

Removed from membrane
H-Ras modulates NMDA receptor function via inhibition of Src tyrosine kinase activity
Claire Thornton, Rami Yaka, Son Dinh and Dorit Ron

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