The induction of long-term potentiation at CA3-CA1 synapses is caused by an NMDA receptor-dependent accumulation of intracellular Ca$^{2+}$, followed by Src family kinase activation and a positive feedback enhancement of NMDA receptors. Nevertheless, the amplitude of baseline transmission remains remarkably constant even though low frequency stimulation is also associated with an NMDA receptor-dependent influx of Ca$^{2+}$ into dendritic spines. We show here that an interaction between C-terminal Src kinase and NMDARs controls the Src-dependent regulation of NMDAR activity. C-terminal Src kinase associates with the NMDAR signalling complex in the adult brain, inhibiting the Src-dependent potentiation of NMDA receptors in CA1 neurons and attenuating the Src-dependent induction of long-term potentiation. In vitro C-terminal Src kinase associates directly with Src-phosphorylated NR2 subunits. An inhibitory antibody for C-terminal Src kinase disrupts this physical association, potentiates NMDA receptor-mediated excitatory postsynaptic currents and induces long-term potentiation at CA3-CA1 synapses. Thus, C-terminal Src kinase serves to maintain the constancy of baseline excitatory synaptic transmission by inhibiting Src kinase-dependent synaptic plasticity in the hippocampus.

Pyramidal neurons of the CA1 hippocampus receive excitatory synaptic input from the axons of CA3 pyramidal neurons, which form the Schaffer Collateral pathway. The induction of synaptic plasticity at these CA3-CA1 synapses requires stimulation of NMDARs (N-methyl-D-aspartate receptors) located at CA1 pyramidal cell dendritic spine synapses. It is widely accepted that spine depolarization relieves the Mg$^{2+}$ block of NMDARs, causing a concomitant increase in the influx of Ca$^{2+}$. Both long-term potentiation (LTP) and long-term depression (LTD) are triggered by the subsequent accumulation of intracellular Ca$^{2+}$ in the spines. For example, induction of LTP increases Ca$^{2+}$ up to 10 μM whilst LTD is associated with lower but more sustained increases (1). Appreciable NMDAR-dependent Ca$^{2+}$ influx occurs at spines even at resting membrane potential due to the relative incompleteness of the Mg$^{2+}$ block (2). Therefore an NMDAR-dependent influx of Ca$^{2+}$ occurs even at low, baseline frequencies of stimulation that induce neither LTP nor LTD. This raises a fundamental question: Why does this baseline influx of Ca$^{2+}$ fail to induce synaptic plasticity?

Part of the answer to this question may lie in the observations that the induction of LTP at these synapses also requires activation of the Src family tyrosine kinases, Fyn and Src (3). Src family kinases control the induction of LTP by potentiating the NMDAR-dependent influx of Ca$^{2+}$ (3) and activation of Src is “necessary and sufficient” to induce LTP at CA3-CA1 neurons (4). Src facilitates NMDAR function likely by increasing tyrosine phosphorylation of the NR2A subunit while Fyn does so by phosphorylating the NR2B subunit (3). The precise mechanism whereby Src and/or Fyn are activated during the induction of LTP is poorly understood. The influx of Ca$^{2+}$ likely enhances the tyrosine kinase Pyk2, which associates with and activates Src (3;5). Therefore, activation of NMDARs and an influx of Ca$^{2+}$ are coupled to stimulation of Src, which is in turn responsible for increasing both NMDAR activity and the subsequent influx of Ca$^{2+}$. During high frequency stimulation, relief of the Mg$^{2+}$ block of NMDARs is insufficient in itself for induction of long-term potentiation at CA3-CA1 synapses. This early Src-
dependent positive feedback amplifies the NMDAR-dependent Ca\(^{2+}\) signal (6), allowing the (e.g. CamKII) responsible for enhancing the sur-

Surprisingly, during baseline stimulation intracellular applications of selective inhibitors of Pyk2 or Src are without effect on the amplitude of NMDAR-mediated excitatory postsynaptic currents (EPSCs) and thus fail to modify AMPAR-mediated EPSCs. However, they are highly effective in preventing the induction of LTP (4;9). These results are not consistent with a NMDAR-mediated Ca\(^{2+}\) entry into postsynaptic spines during the baseline stimulation (2). Indeed, such elemental Ca\(^{2+}\) signals might be expected to be amplified by Src-mediated facilitation of NMDARs (positive feedback), which would subsequently and progressively facilitate AMPAR-mediated EPSPs. Intuitively, such a scheme would be undesirable for the maintenance of a stable neuronal network. A regulatory mechanism may therefore exist that holds Src in balance while leaving NMDARs unaffected until they are required for the induction of synaptic plasticity (e.g. LTP).

Csk family kinases (Csk, COOH-terminal Src kinase and Chk, Csk-homologous kinase) are cytosolic kinases which inhibit Src family kinases and as such are possible candidates for such a regulatory mechanism. Reports showing the down-regulation of Csk in the adult brain and a parallel up-regulation of Chk suggest that Chk would be the most likely candidate (10;11). However, we present evidence that Csk is present at CA3-CA1 synapses and that it interacts directly with the NMDAR complex to regulate Src’s ability to enhance NMDARs. Csk prevents an inappropriate activation of Src by the NMDAR-dependent influx of Ca\(^{2+}\) that occurs during baseline stimulation frequencies and, in turn, inhibits the induction of LTP. Ours is the first demonstration that Csk plays a critical role in regulating the balance between baseline synaptic excitatory transmission and plasticity in the adult CNS.

Experimental Procedures

All animal experiments were conducted following the guidelines of the Canadian Council on Animal Care and the NIH, and were approved by the Animal Care Committee of the University of Toronto and the Animal Care and Use Committee of Florida State University.

Acutely Isolated CA1 pyramidal neurons and electrophysiological recordings: Methods for acute activation of the appropriate downstream pathways face expression (7) and/or function of AMPARs neuron isolation from young adult rats have been previously described (12). In brief, the hippocam-

Acutely isolated CA1 pyramidal neurons present both advantages and disadvantages as a preparation for examining the function of NMDAR-mediated currents. The isolation procedure shears off much of the dendritic tree leaving only a few major primary and secondary dendritic branches. Applications of NMDA during whole cell recordings will therefore induce currents mediated by the activation of channels largely found within the extrasynaptic compartment. Nevertheless, some synaptic NMDARs are likely stimulated as many synapses are located on the large dendritic processes that come along with the cell soma. The predominant subtype of NMDAR in the extrasynaptic compartment of CA1 pyramidal neurons is NR1/NR2B. However, it is estimated that approximately 25% of the receptor compliment in this compartment is composed of NR1/NR2A receptors. This contrasts with the ~75% compliment of this receptor subtype at the synapse (13). Furthermore, the peak open probability of NR1/2A receptors is about 2 to 5 times greater than for NR1/NRB receptors (14). It is therefore anticipated that populations of both NR1/NR2A and NR1/NR2B receptors will contribute substantially to the peak whole cell currents recorded from isolated CA1 pyramidal neurons. Of course, we cannot rule out the possible presence of triheteromeric receptors.

Whole-cell clamped pyramidal neurons are lifted into the stream of a rapid perfusion sys-

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Hippocampal slice preparation and electrophysiological recording.

In brief, hippocampal slices (400 μm) were prepared from 3–4 week-old Wistar rats. Slices recovered for 1 h in oxygenated artificial cerebral spinal fluid (ACSF) containing (mM): NaCl (124), KCl (3), CaCl₂ (2.6), MgCl₂ (1.3), NaHCO₃ (26), NaH₂PO₄ (1.25) and glucose (10), osmolarity 310–320 mOsm, pH 7.4. Recording electrodes were filled with an intracellular solution containing (mM): CsCl or CsF (140), HEPES (10), MgCl₂ (2), BAPTA (1) and K₂ATP (4), pH 7.2 and osmolarity 290–300 mOsm. DC resistances of recording electrodes were 4–6 MΩ. Input resistance was monitored by applying a voltage step of −10 mV and any recordings with more than a 20% change during the recording were rejected. Only recordings with a pipette-membrane seal > 2 GΩ were included.

For evoking NMDA receptor-mediated whole-cell responses, the standard and NMDA receptor agonist-containing extracellular solutions were rapidly applied through double-barrel glass to neurons using the SF-77B fast-step perfusion system (Warner Instruments). Whole-cell NMDA-mediated currents were recorded using an Axopatch 1D or Axopatch 200B amplifier (Molecular Devices) under voltage-clamp and a holding potential of -60 mV except where otherwise indicated. For recordings from acutely isolated CA1 pyramidal neurons NMDA was applied at 50 μM in the continuing presence of 0.5 μM glycine. On-line data acquisition and off-line analysis were performed using pClamp8 software (Molecular Devices).

**Hippocampal slice preparation and electrophysiological recording** - Techniques used for the hippocampal slice preparation and electrophysiological recording have been previously described (9;17). In brief, hippocampal slices (400 μm) were prepared from 3–4 week-old Wistar rats. Slices recovered for 1 h in oxygenated artificial cerebral spinal fluid (ACSF) containing (mM): NaCl (124), KCl (3), CaCl₂ (2.6), MgCl₂ (1.3), NaHCO₃ (26), NaH₂PO₄ (1.25) and glucose (10), osmolarity 310–320 mOsm, pH 7.4. Recording electrodes were filled with an intracellular solution containing (mM): K-gluconate (135.5), KCl (17.5), HEPES (10), EGTA (0.2), Mg-ATP (2), GTP (0.3) and QX 314 (5) with osmolarity 290–300 mOsm and pH 7.2. DC resistances were 3–5 MΩ. Series resistance (10–30 MOhm), monitored throughout the recording period, was estimated from the series resistance compensation of currents generated in response to 5 mV voltage steps. Cells were rejected if the resistance values changed by more than 20%. During recording, slices were continuously perfused at 3–5 ml/min with oxygenated and heated (30°C - 32°C) ACSF.

Synaptic responses were evoked with electrical stimulation at the border of the stratum radiatum–stratum lacunosum region. Stimuli were delivered at 0.05 or 0.1 Hz with the intensity adjusted to 25–30% of the maximal synaptic responses. Whole-cell EPSCs or EPSPs were recorded from the CA1 cell body layer using Multiclamp 700A amplifier and Clampex 9 software (Molecular Devices). During whole-cell recordings, field EPSPs (fEPSPs) were simultaneously recorded with electrodes filled with ACSF and placed within the stratum radiatum. Whole-cell EPSC recordings were conducted at -90 mV. After recording baseline synaptic activity for 10–30 mins, tetanic stimulations (two 100 Hz stimulus trains; 500 ms with 10s intertrain interval) were applied. The peak amplitudes of EPSCs and the slopes between 10–70% of the peak responses of EPSPs and fEPSPs were measured. Pharmacologically isolated NMDAR-mediated EPSPs were also recorded in some experiments with cells held at -60 mV in the presence of CNQX (10 μM) to block AMPARs and bicuculline to block GABAAr responses.

**Biochemical experiments** - Subcellular fractionation and PSD isolation: Subcellular fractionation of adult male rat (40 to 60 days) forebrains were performed as previously described (18;19). The approaches used for PSD isolation are described elsewhere (20-22). Briefly, the synaptosome fraction was isolated by discontinuous sucrose gradient centrifugation and was solubilized in 0.5% Triton X-100 then centrifuged to obtain the PSD-I pellet. This pellet was re-suspended, solubilized in 0.5% Triton X-100 and centrifuged to obtain the PSD-II pellet. Extracts from individual fractions were finally solubilized in 1% sodium dodecyl sulphate (SDS) and centrifuged at 15,000g for 5 min for western blot analysis.

**Immunoprecipitation and Western blot analysis** - Experiments were performed as described previously (9;17). In brief, proteins from the P2 (or P1) fraction of adult rat forebrain were dissolved in a lysis buffer containing (mM): Tris-HCl (50, pH 9.0), NaCl (150), 0.5% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, EDTA (2) and sodium orthovanadate (1). For attached cell homogenates, cells were mechanically dissociated and cell pellets were re-suspended in HNTG buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA).
TA, 10mM sodium pyrophosphate, 100 mM NaF and 1mM sodium orthovanadate). Solubilized proteins (600-800 μg) were incubated overnight at 4°C with antibodies, as indicated. The immune complexes were collected with protein G-sepharose beads (Amersham Biosciences) for 2 hs at 4°C. The blotting analysis was performed by repeated stripping and successive probing with antibodies as indicated. Antibodies used in these experiments include anti-NR1 (Pharmingen), NR2A C-terminal (Upstate), NR2A N-terminal (Santa Cruz), PTPα (17), Src (Oncogene), Src-pTyr416 (Cell Signaling), Src-pTyr527 (Cell Signaling), Chk (Santa Cruz), phosphotyrosine (Upstate), kv3.1b (BD Bioscience) and GluR2/3 (Upstate). Monoclonal (“anti-Csk”, mouse, BD Bioscience) and polyclonal (rabbit, Santa Cruz) antibodies against Csk were used to avoid signal overlapping of Csk with IgG heavy chain in Western blotting. To avoid confusion, only the monoclonal Csk antibody raised against the SH2 and SH3 domain of Csk was labeled as “anti-Csk” in this paper. Non-selective mouse or rabbit IgG was utilized for immunoprecipitation as negative control.

cDNA transfection- HEK293 or COS7 cells were transfected using the calcium phosphate method (Invitrogen) as per the manufacturer’s instruction with expression vectors (pcDNA3 or pRcCMV) containing cDNAs encoding NR1-1a, full length or C-terminal truncated NR2A, v-Src (a gift of Dr. T. Pawson, University of Toronto), Csk (a gift of Dr. K. Harder, Royal Melbourne Hospital), wild-type or mutant neuronal Src (n-Src, Y535F or K303R/Y535F, provided by Dr. S. Hanks, Vanderbilt University), and Src(40-58) (a gift from Dr. M.W. Salter, Hospital For Sick Children, Toronto). To avoid confusion, the n-Src mutants of chicken c-Src used in our experiments are referred to as Y527F and K295R/Y527F. Transfected cells were maintained in DMEM with 10% FBS for 24 hours before lysis.

GST pull-down assay- pGEX2th-Csk-ST plasmid was kindly provided by Dr. G. Sun at the University of Rhode Island. Csk S109C mutation was made using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) as described by the manufacturer. Point mutation was confirmed by DNA sequencing. The GST and GST fusion proteins conjugated with Csk or Csk S109C were produced in E. Coli (BL-21 strain, Invitrogen) with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1mM) induction. The bead-bound GST fusion proteins or GST alone (15-20 μg) were each incubated with solubilized membrane proteins (600-800 μg) extracted from the adult rat brain tissue or 5 μl 35S-methionine-labelled NR1 CT peptide and NR2A CT peptide in binding buffer (2% BSA in PBS) overnight at 4°C. Bound proteins were separated by electrophoresis and detected by Western blotting or autoradiography. 35S-methionine-labelled NR1 CT peptide and NR2A CT peptide were synthesized in TNT T7 Quick Coupled Transcription/Translation System (Promega) following the protocol recommended by the manufacturer. For dephosphorylation treatment, 5 μl 35S-methionine-labelled NR2A CT peptide was incubated with 100 units lambda protein phosphatase (λ-PPase, NEB) in the reaction buffer prior to the pull-down assays.

RESULTS

Due to the lack of highly selective inhibitors of Csk kinases we employed a Csk antibody (“anti-Csk”; mouse monoclonal antibody, 2.5 μg/ml, BD Biosciences) that prevents Csk’s ability to interact with NR2 subunits (see Fig. 7B) thereby inhibiting Csk’s ability to regulate the phosphorylation of NMDARs by Src. Such an approach is analogous to the use of anti-Src to selectively inhibit Src kinase activity in CA1 neurons (4). Intracellular applications of anti-Csk caused a time-dependent increase in the magnitude of the EPSP slope to 189 ± 11% of that recorded immediately after breakthrough (Fig. 1A,B). The slope of the CA3-CA1 EPSP largely reflects the underlying AMPAR conductance when extracellular Mg2+ is present (e.g. 1.3 mM). The enhancement of EPSPs induced by the anti-Csk antibody application subsequently occluded the induction of LTP (Fig. 1B). Furthermore, the potentiation of EPSPs by anti-Csk was blocked by pre-treating slices with the NMDAR antagonist AP5 (50 μM) or with the Src family kinase inhibitor PP2 (10 μM, 1 h) (Fig. 1A), indicating that endogenous Csk regulates synaptic plasticity through a Src family kinase and NMDAR dependent mechanism.

We also examined the effects of recombinant Csk supplied through our patch pipette on EPSCs recorded in the whole-cell voltage-clamp configuration. Intracellular applications of Csk had no time-dependent effect on the amplitude of baseline EPSCs (Fig. 1C), suggesting that endogenous Csk is sufficient to limit Src activity and prevent plasticity of baseline EPSCs. In contrast, recombinant Csk substantially attenuated the induction of LTP. In control recordings lacking
Csk, the amplitude of EPSCs were significantly enhanced to 285 ± 32% (mean ± SEM, n=9) after tetanic stimulation (Fig. 1C). However, application of recombinant Csk (0.1 μg/ml) into neurons through the recording pipettes substantially attenuated LTP of EPSCs (Fig. 1C). Recordings of fEPSPs performed simultaneously with whole-cell recordings of EPSCs were used to confirm that the magnitude of LTP did not differ in slices from each treatment group (Fig. 1D).

We next considered if intracellular applications of Csk or its inhibitor (anti-Csk) would alter pharmacologically isolated NMDAR EPSCs. Csk caused no change in the amplitude of these NMDAR-mediated synaptic currents (Fig. 2A). In contrast, intracellular applications of anti-Csk caused a substantial and time-dependent increase in the amplitude of these currents that was not mimicked by a non-selective IgG (Fig. 2B). This anti-Csk induced enhancement was prevented by including the selective Src kinase inhibitory peptide (Src40-58) in the patch pipette together with the antibody (Fig. 2B). These results imply that endogenous Csk kinases in CA1 neurons constitutively inhibit the Src-dependent potentiation of NMDAR EPSCs. Furthermore, this inhibition is likely complete, as supplementing the neurons with exogenous Csk kinase were without effect on these baseline responses.

To investigate the inhibitory effect of endogenous Csk on excitatory synaptic transmission, we first determined the subcellular distribution of Csk family kinases in the brain. Sequential fractionation of forebrain homogenates was performed from adult rats (40 to 60 days old). The NR1 subunit, PSD-95 (9;18;23) and Src (9;18) were abundant in both the crude synaptosomal membrane (P2) and the synaptosomal membrane (LP1) fractions but little was detected in either the synaptic vesicle-enriched (LP2), the low-speed supernatant (S1, S2) or in the cytosolic (S3) fractions (Fig. 3A). In contrast, Csk and Chk were found in all the fractions but less Chk was apparent in the P2 and LP1 fractions where a strong NR1 signal was detected (Fig. 3A). Highly selective antibodies distinguishing between Csk and Chk were employed (Supplementary Fig. 1).

Western blot analysis of the PSD fraction, which is highly enriched with the NR1 subunit and PSD-95, revealed that Src, Csk and to a lesser degree Chk are also present in this fraction (Fig. 3A). The vesicle-associated membrane protein-2 (VAMP-2) was absent from this PSD fraction. Thus, we hypothesized that Csk family kinases and NMDARs may be closely associated in neurons.

To examine this hypothesis, we performed co-immunoprecipitation experiments using synaptosomal membrane preparations (P2 and LP1 fractions) solubilized under non-denaturing conditions. Both Csk kinases and NMDARs were located in these fractions (Fig. 3A). An antibody against the NR1 subunit of the NMDAR precipitated neither Kv3.1b(24) nor GluR2/3(23;25) but did bring down NR2 subunits, PSD-95(17;25), Src(24;25), and protein tyrosine phosphatase α (PTPα)(17) (Fig. 3B). None of these proteins were detected in the immunoprecipitates obtained using a non-selective IgG (Fig. 3B). Importantly Csk, but not Chk, was found in NR1 immunoprecipitates (Fig. 3C). Conversely, we also found that antibodies raised against Csk co-immunoprecipitated NMDAR subunits, PTPα and Src but not Kv3.1b nor GluR2/3 (Fig. 3D).

In addition, NMDAR subunits, including NR1, NR2A and NR2B, were pulled down from the brain using GST-fusion proteins conjugated with Csk (GST-Csk), but not by GST alone (Fig. 3E). Neither Kv3.1b nor GluR2/3 was pulled down by GST-Csk and much less NMDAR protein was pulled down by Csk containing a defective SH2 domain (serine 109 mutated to a cysteine, (GST-Csk S109C))(26;27) when compared to that using GST-Csk (Fig. 3E). These data indicate that Csk in the adult brain, but not Chk, complexes with NMDARs via its SH2 domain and forms a signalling complex together with PTPα and Src.

To further test the hypothesis that Csk regulates Src’s abilities to control NMDAR activity we turned to CA1 pyramidal neurons acutely isolated from hippocampal slices prepared from young adult rats. Recordings from these neurons are performed within one hour of isolation and are therefore not to be confused with primary cultures of neurons. Concentrations of extracellular agonists and antagonists are tightly controlled using a rapid perfusion system.

Unlike the in situ slice condition, previous studies have shown that isolated CA1 neurons demonstrate enhanced Src kinase activity as NMDA-evoked currents are partially reduced by inhibitors of Src family kinases (4,9). Extracellular application of the Src kinase inhibitor SU6656 (2 μM) inhibited NMDAR currents by about 40% (Fig. 4A,B). Furthermore, including Csk (0.1 μg/ml) in the patch pipette led to a time-dependent depression of NMDAR currents to about 66% as...
compared to controls (Fig. 4C,D). Csk did not alter the reversal potential or the decay time constant of NMDA currents (data not shown). In contrast, applications of boiled Csk had no effect on the amplitude of NMDA currents (data not shown). Following this blockade of Src kinase Csk failed to inhibit NMDA currents (Fig. 4D). However, inhibition was spared when neurons were pre-treated with the inactive analogue of PP2, PP3 (10 μM) (Fig. 4D). In order to determine whether Csk was specifically targeting Src as opposed to another Src family kinases such as Fyn, we also included the Src-selective inhibitor peptide Src(40-58) in the patch pipette (3). The inhibition of NMDAR-mediated currents by Csk was also occluded by this inhibitor (Fig. 4D).

Given our results with isolated CA1 neurons we determined if there was any direct relationship between Csk and the Src-dependent phosphorylation of NR2A subunits expressed in vitro in HEK293 or COS7 cells. When both these proteins were over expressed in these cells, the NR2A subunit co-immunoprecipitated Csk (Fig. 5A). To examine the effects of increased tyrosine phosphorylation of NR2A on its association with Csk, we co-expressed Csk and NR2A along with v-Src. v-Src lacks the Csk targeting sequence in the C-tail (28) and was detected using an antibody that recognizes the autophosphorylated form of Src (Tyr416) (Fig 5B). Over-expression of v-Src did not alter the expression of NR2A or Csk but substantially enhanced the tyrosine phosphorylation of NR2A (Fig. 5A,B). It also increased the amount of Csk that co-precipitated with NR2A (Fig. 5A,B). The NR2A antibody failed to co-precipitate Csk in the absence of co-expressed NR2A (Fig. 5A) thus serving as a negative control. The greater the level of v-Src expression, the greater was both the phosphorylation of NR2A subunits and the co-precipitation of Csk (Fig. 5B right panel and 5D). Over expression of the constitutively active form of Src (v-Src) also enhanced co-precipitation of NR2A together with endogenous Csk (Supplementary Fig. 2A).

To determine if this increase in Csk-NR2A co-precipitation was the result of an enhancement of Src kinase activity or, alternatively, if it was simply due to an increased expression of Src protein, we co-transfected cDNA encoding either constitutively active Src (C-tail Tyr527 mutated to phenylalanine (Y527F)) or inactive Src (lysine 295 in kinase domain was mutated to arginine, in addition to the Tyr527 mutation (Y527F/K295R)(29). The level of expression of Src was similar in these two groups of cells (Fig. 5C left panel) while the amount of Csk that co-precipitated with the NR2A subunit in cells expressing inactive Src was only 12 ± 3% (n=4) of that in cells expressing active Src (Fig. 5C right panel and 5E). Similarly, the co-expression of active Src also significantly enhanced the association of Csk with the NR2B subunit when compared to the co-expression of inactive Src (Supplementary Fig. 2B). Csk was not precipitated with non-specific IgG from the lysates of cells co-transfected with active Src (Fig. 5C and Supplementary Fig. 2B). These findings demonstrate that association of Csk with NR2A (and NR2B) is dependent upon Src kinase activity.

Given the potential interactions between Csk and Src-induced phosphorylation of the NR2A subunit we explored the regions of NR2A that might directly interact with Csk. Wild-type or C-tail truncated mutants of NR2A subunits were co-expressed with NR1-1a, active Src (Y527F) and Csk (Fig. 6A). Csk was co-immunoprecipitated using antibodies directed against the N-terminal of NR2A subunits (Fig. 6B) or against NR1 subunits (Fig. 6C). Truncation of NR2A (aa1-837) did not alter the assembly of NMDAR subunits (Fig. 6B,C). However, it substantially reduced co-immunoprecipitation of Csk (Fig. 6B-E), confirming that Csk associates with the NMDAR complex through interactions involving the NR2A subunit C-terminal region.

We further characterized the interaction between Csk and NR2A by performing in vitro GST pull-down assays. Since increased tyrosine phosphorylation of NR2A increased its association with Csk we explored whether the SH2 domain of Csk is specifically responsible for binding to the NR2A subunit. SH2 domains serve as a binding site for phosphotyrosine residues (28;30). The 35S-labelled peptide corresponding to the C-tail (aa838-1464) of the NR2A subunit was precipitated by GST-Csk but not by GST alone (Fig. 7A). When the experiment was repeated using GST fusion proteins conjugated with Csk containing a defective SH2 domain (aa1-109C) (GST-Csk S109C), much less NR2A C-tail peptide was pulled-down.
Furthermore, when the NR2A C-tail peptide was pre-treated with lambda protein phosphatase (λPPase, 100 units, New England Biolabs), which reduced tyrosine phosphorylation of the NR2A C-tail peptide (Fig. 7C), the amount of NR2A C-tail peptide precipitated with GST-Csk was significantly reduced (Fig. 7A,B).

Next, we examined the effects of the Csk antibody, anti-Csk (2.5 μg/ml), which was raised using a peptide sequence corresponding to the SH3-SH2 domains of Csk, on these interactions. Anti-Csk dose-dependently reduced the amount of NR2A C-tail peptide pulled down with GST-Csk (Fig. 7A,B). However, it did not alter the in vitro kinase activity of Csk (data not shown). Applications of non-selective IgG produced no change in the pull-down of the NR2A C-tail peptide (Fig. 7A,B). Therefore, Csk targets the tyrosine phosphorylated NR2A subunit via its SH2 domain and application of anti-Csk prevents Csk’s interactions with the NR2A C-tail. By this means the antibody disrupts Csk’s interactions with NMDAR and thereby serves as a functional inhibitor of Csk action on NMDAR activity.

The activity of endogenous Src kinases, as well as the basal level of protein tyrosine phosphorylation of the NR2A subunit, is very low in HEK293 cells (Fig. 7D-G) (32). Therefore, we examined the potential effect of Csk on tyrosine phosphorylation of the NR2A subunit in this cell line. Increasing Csk expression did not alter the tyrosine phosphorylation of the NR2A subunit (Fig. 7F,G), demonstrating that Csk does not directly phosphorylate NMDARs. Transfection of wild-type Src into HEK293 cells substantially elevated both Src kinase activity and overall protein tyrosine phosphorylation (Fig. 7D,E). Co-expression of Csk in these cells inhibited Src kinase activity (decreased Tyr416 phosphorylation and increased Tyr527 phosphorylation of Src) and reduced overall protein tyrosine phosphorylation (Fig. 7D,E). Expression of constitutively active Src (Y527F) further augmented Src kinase activity and protein tyrosine phosphorylation, but in this situation Csk did not alter either Src kinase activity or overall tyrosine phosphorylation (Fig. 7D,E). This result is consistent with a lack of inhibition of constitutively active Src by Csk. Tyrosine phosphorylation of the NR2A subunit was also depressed when Csk was co-transfected in cells expressing wild-type Src but not in cells over-expressing constitutively active Src (Fig. 7F,G). These results demonstrate that Csk modulates Src-induced NMDAR tyrosine phosphorylation of the NR2A subunit.

**DISCUSSION**

The importance of Src family kinases in the development of the CNS is well known but their specific role in synaptic transmission in the adult is less well recognized. Disruption of the csk gene in mice (Csk -/-) leads to enhanced Src family kinase activity, resulting in severe defects in neural tube development and early (E9-10) embryonic lethality (27;33) thereby illustrating that Csk is essential for CNS development. In contrast, Chk null mice are viable and demonstrate no histological CNS abnormalities (34). Nevertheless, a role for Csk in the adult central nervous system had all but been ruled out based on the dramatic down-regulation of Csk expression and parallel up-regulation of Chk that occurs with CNS maturation (10;11;35-37). Unexpectedly, our results show that it is Csk that is a critical signalling molecule in controlling excitatory synaptic transmission and plasticity in the hippocampus.

In central neurons, Src increases the open probability of NMDA channels (24) and, in the absence of added extracellular Mg²⁺, enhances NMDAR-mediated currents in cultured hippocampal, slice hippocampal and acutely isolated CA1 neurons (4;9;17). This is likely achieved through phosphorylation of tyrosine residues on NR2A and NR2B subunits (3). For example, Src directly phosphorylates NR2A subunits and Fyn phosphorylates NR2B subunits (3). We have shown that application of recombinant Csk depresses NMDAR responses in acutely isolated CA1 pyramidal neurons taken from young rats. This depression was dependent upon Src family kinase activity as it was entirely occluded by pharmacological blockade using Src family kinase inhibitors (PP2, SU6656). It was also dependent upon the specific activation of Src kinase as it was blocked by the Src-selective inhibitory peptide, Src(40-58) (3). Our results are consistent with Csk inhibiting Src and Fyn regulation of NR2A- and NR2B-containing receptors, respectively. Furthermore, we demonstrated that endogenous Csk depressed NMDAR-mediated EPSCs evoked by stimulation of Schaffer Collaterals.
We also provided evidence that Csk co-precipitates with the NMDAR complex of the adult brain along with Src and PTPα. To elucidate other potential physical associations, we examined the interaction of Csk with NMDAR subunits expressed in non-neuronal cells. We demonstrated co-immunoprecipitation of these proteins following over-expression of Csk and NR2A or NR2B subunits. Furthermore as Src activity was increased and phosphorylation of NR2A and NR2B was enhanced, the association of Csk with the NR2A and NR2B subunits was also augmented. We conclude that the SH2 domain of Csk binds to NR2A and NR2B subunits in a tyrosine-phosphorylation-dependent manner. This association likely brings Csk into proximity with Src and Fyn.

Increased Csk activity substantially attenuated NR2A subunit tyrosine phosphorylation in cells co-transfected with Src. In contrast, in cells expressing a mutant Src in which the C-tail tyrosine was mutated to phenylalanine, failed to inhibit NR2A tyrosine phosphorylation. The endogenous level of Src family kinase activity and of basal tyrosine phosphorylation of co-expressed NR2A subunits (32) is very low in HEK293 cells. This allowed us to examine the effect of increasing Csk activity under conditions of very low levels of basal Src activity. Over expression of Csk failed to enhance tyrosine phosphorylation of NR2A subunits under these conditions indicating that Csk does not directly phosphorylate this subunit. Therefore, we conclude that Csk diminishes NMDAR tyrosine phosphorylation via the selective depression of Src kinase activity.

At CA3-CA1 synapses the induction of LTP requires the activation of Src family kinases Src and Fyn. The induction protocol itself activates these kinases, facilitates NMDAR activity and in turn leads to the up-regulation of the synaptic AMPARs responsible for the enhanced EPSPs (38). However, inhibiting Pyk2, Src or PTPα fails to alter the baseline AMPAR EPSPs recorded in CA1 pyramidal neurons even though they effectively prevent induction of LTP (4;9;17). This implies that Src activity, NMDARs and the subsequent potentiation of EPSP amplitude is either subthreshold or that Src activity is inhibited by endogenous Csk during baseline stimulation. Consistent with the later explanation, intracellular application of Csk did not alter baseline EPSPs in CA1 neurons but substantially reduced the Src-dependent induction of LTP at CA3-CA1 synapses. Basal Csk activity is apparently sufficient to keep Src in check since “anti-Csk” enhanced baseline currents and occluded subsequent induction of LTP. These results are also consistent with previous findings showing that intracellular applications of a Src-activator peptide lead to enhanced NMDAR and AMPAR currents and the occlusion of subsequent LTP (4) while Src inhibitors did not alter the strength of excitatory transmission themselves but did inhibit the induction of LTP (4).

Our results show that the association of Csk with the NMDAR complex is responsible for preventing the Src-dependent up-regulation of NMDARs, which would have occurred during baseline excitatory transmission at CA3-CA1 synapses. Csk also serves as an intrinsic mechanism to provide a “brake” on the induction of LTP by its phosphorylation of Src at its C-tail regulatory site. An additional “brake” to this induction is provided by striatal tyrosine enriched phosphatase (STEP), which in contrast dephosphorylates tyrosine residues required for the activation of Src (39). On the other hand, protein tyrosine phosphatase α (PTPα) dephosphorylates the regulatory C-tail tyrosine of Src family kinases and directly counteracts the inhibition of Src by Csk (17). Both Csk and PTPα therefore target phosphorylation of the C-tail tyrosine of Src kinases and provide a counter balancing mechanism for the dynamic regulation of the induction of LTP at CA3-CA1 synapses.

Recent evidence indicates that Src family kinases serve as a convergent point of multiple transmitter pathways that in turn regulate NMDAR function in the hippocampus (3;40-42). Postsynaptic receptors on CA1 neurons, which are capable of enhancing the activity of Csk, are anticipated to depress NMDAR activity contingent upon their up-regulation by Src family kinases. Csk is itself activated via a PKA-dependent phosphorylation of serine 364 (43;44). In this regard, the catalytic subunit of PKA depresses the up-regulation of single NMDA channel activity induced by the Src activator phosphopeptide EPQ(pY)-EEPIA but fails to alter activity in the absence of this activator when applied to outside-out patches taken from cultured hippocampal neurons (45). This PKA-dependent and Src-contingent inhibition of NMDAR currents is mimicked by stimulation of platelet-derived growth factor receptors (45), indicating a potential physiological mechanism whereby Csk regulates the level of baseline excitatory synaptic transmission in central neurons (42).
Csk interacts with NMDARs to inhibit their phosphorylation by Src. This Csk interaction is also dependent upon the degree of tyrosine phosphorylation of the NMDARs themselves. The greater the increase in phosphorylation of the receptor by Src, the greater will be the interaction with Csk. Therefore, Csk serves as a “governor” of the Src-dependent positive feedback of NMDARs that occurs during high frequency stimulation. By this means Csk not only regulates baseline CA1 transmission but also serves to limit the enhancement of NMDARs associated with LTP.

**FOOTNOTES**

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REFERENCES

FIGURE LEGENDS

Fig 1. Csk limits Src-dependent LTP at CA1 Synapses. A. The slopes of EPSPs were recorded from neurons during intracellular applications of anti-Csk (2.5μg/ml, closed squares, n=6) or during applications of the antibody in slices pre-treated with NMDAR antagonist AP5 (50μM, open squares, n= 7) or SFK inhibitor PP2 (10μM, open circles, n=5). Slopes of EPSPs were normalized with respect to the values recorded at breakthrough. Anti-Csk substantially enhanced EPSP slopes during baseline stimulation (P<0.1, Mann Whitney test). B. In two groups of cells anti-Csk (2.5μg/ml, closed squares, n=6) or non-selective mouse IgG (mlgG, 2.5μg/ml, open squares, n=6) was applied and subsequently LTP was induced. C. Summary data (mean ± SEM) showing the peak amplitudes of EPSCs (normalized with the mean values of EPSC peak amplitudes during the first 2mins: I/I0) recorded from neurons during intracellular application of Csk (0.1μg/ml, closed squares, n=6) or no Csk (open squares, n=9) and the attenuation of LTP (P<0.01, Mann-Whitney test). Insets show examples of EPSCs recorded before (1) and after (2) the tetanus from neurons as indicated. D. Slopes of field (fEPSPs) normalized to the mean values of fEPSP slopes during the first 2mins. The fEPSPs were recorded simultaneously with the EPSC recordings shown in c with application of Csk (closed squares, n=6) or no Csk (open squares, n=9). Inserts show: examples of fEPSPs recorded before (1) and after (2,3) the tetanus. Large open arrows in c and d indicated tetanic stimulations. Time zero corresponds to the first evoked synaptic response (EPSP or EPSC as the case may be) recorded within 2 minutes after breakthrough, this brief delay being necessary to stabilize the recording.

Fig. 2. Endogenous but not exogenous Csk down-regulates baseline NMDAR-mediated EPSCs in CA1 pyramidal neurons. A. Intracellular application of Csk (closed squares, n = 7) or boiled Csk (open square, n = 5) did not influence EPSCsNMDA. B. Intracellular application of anti-CSK (closed squares, n = 6) causes a progressive increase in the amplitude of pharmacologically isolated NMDAR-mediated EPSCs (EPSCsNMDA) recorded in CA1 pyramidal neurons from hippocampal slices (P<0.01, Mann Whitney test). Inclusion of a mouse non-selective IgG (mlgG, open square, n = 6) in the intracellular pipette solution was without effect. Co-application of the Src inhibitory peptide (Src(40-58)) blocked the effects of anti-Csk on EPSCsNMDA (anti-Csk/Src(40-58), open circle, n = 6). In both A and B the amplitude of EPSCsNMDA was normalized to their averaged amplitudes for the first minute after breakthrough: I/I0. Representative traces in A and B were obtained at the times indicated by (1) and (2).

Fig. 3. Csk, PTPα, Src and the NMDAR form a complex in the CNS. A, Subcellular fractionation profiles of the NMDAR NR1 subunit, PSD-95, Src, Csk family kinases, and VAMP-2 are illustrated. In each case 30μg protein was loaded into the lanes except the PSD lanes, where 5μg was loaded. B-D, The gels shown were loaded with solubilized membrane protein (20µg, SM) and immunoprecipitates of antibodies as indicated on the top of blots. E, left panel shows the Coomassie blue staining of purified GST and GST fusion proteins after SDS-PAGE. The gel in right panel was loaded with solubilized membrane proteins (120µg, SM) and proteins precipitated by GST fusion proteins as indicated on the top of blots. The filters were sequentially stripped and immuno-blotted with antibodies against proteins as indicated next to the arrows. All experiments were repeated more than three times. Numbers to the left of blots indicate the position and size (kDa) of molecular mass markers. H: Homogenate; S1: low-speed supernatant 1; P2: crude synaptosomal membrane; S2: low-speed supernatant 2; LP1: synaptosomal membrane; LP2: synaptic vesicle-enriched fraction; S3: cytosolic fraction; PSD-I: one Triton PSD pellet; PSD-II: two Triton PSD pellet. IP: immunoprecipitation;

Fig. 4. Csk inhibits Src-dependent NMDAR-mediated whole-cell currents in isolated hippocampal neurons. A. Acute inhibition of NMDA evoked currents during and following acute application of SU6656 (2μM) at the time indicated by the bar. B. Quantification of this inhibition for a series of cells (n=5). C. Example of NMDAR-mediated whole-cell currents recorded from an acutely isolated CA1 neuron during intracellular application of Csk (0.1 μg/ml). Csk generated a time-dependent inhibition of these currents. D. Summary data showing normalized amplitudes of NMDAR-mediated currents recorded in acutely isolated CA1 neurons from young rats. Eight groups of cells were compared: 1) those with no Csk (open
squares, n= 6) 2) those with Csk (closed circles, n=7) 3) those with Csk combined with the SFK inhibitor PP2 (10 μM)(open circles; n=5) 4) those recorded with boiled Csk (open hexagons, n=5) 5) those recorded with Csk and the SFK inhibitor SU6656 (2 μM) (open diamonds, n=8) 6) those recorded with Csk and the inactive control PP3 (10 μM)(closed diamonds, n=5) 7) those recorded with Csk and the Src-selective inhibitory peptide, Src(40-58) (closed squares, n=6). PP2, PP3 and SU6656 were added to the bath 10 mins prior to recording NMDAR currents. Only Csk or Csk in the presence of PP3 demonstrated a time-dependent reduction in these currents. (P<0.01, Mann-Whitney test)

**Fig. 5.** Csk associates with the NR2A subunit by a Src-dependent mechanism. A. Gels were loaded (from left to right) with lysates (C) of HEK 293 cells and immunoprecipitates of NR2A antibody from these cells transfected with and without cDNAs of proteins listed underneath the blot as indicated with “+” and “−”, respectively. B. The gel (left) was loaded with lysates of cells co-transfected with various amounts of cDNAs encoding v-Src, and the gel to the right was loaded with NR2A immunoprecipitates from the lysates. C. The gel (left) was loaded with the lysates of cells co-transfected with cDNAs of active Src (Y527F) and inactive Src (Y527F/K295R), and the gels (right) were loaded with immunoprecipitates of non-selective IgG and NR2A antibody from the lysates. The filters were sequentially stripped and probed with antibodies against proteins as indicated to the right of blots. D,E. Summary data (mean ± SEM) showing the ratios of band intensities of Csk versus NR2A subunit protein detected in the NR2A immunoprecipitates. These were normalized with that found from cells co-transfected with 1 μg v-Src (in D) or active Src (in E) cDNA (dashed line). The number of times the experiments were repeated is indicated in brackets. **: P<0.01, Student’s t-test in comparison between cells expressing the active and inactive Src. IB: immuno-blotting; pTy: phosphotyrosine; pY416: tyrosine 416 phosphorylated Src.

**Fig. 6.** NR2A subunit C-terminal is involved in Csk association with NMDARs. A. Gels were loaded with lysates of cells co-transfected with cDNAs of full-length NR1-1a and NR2A or C-tail truncated NR2A subunits as indicated. B,C. The gels were loaded with NR2A (IP:NR2A(N)) and NR1 (IP:NR1) immunoprecipitates from the lysates shown in (A). The filters were sequentially stripped and probed with antibodies against proteins as indicated to the right of blots. D,E. Summary data showing the ratios between the band intensities of Csk versus NR2A detected in the NR2A immunoprecipitates (D), and of Csk versus NR1 in the NR1 immunoprecipitates (E). These were normalized to those detected in cells expressing full-length NR1 and NR2A. The number of times the experiments were repeated is indicated in brackets. NR2AΔCT, C-tail truncated NR2A; NR2A (N): antibody against N-terminal of NR2A subunit.

**Fig. 7.** Csk binds directly to the C-tail of the NR2A subunit in vitro and reduces NR2A subunit tyrosine phosphorylation in cells. A. The left panel are autoradiographs showing 35S-labelled peptide (Input, 10% of the total peptide used in the pull-down assays) corresponding to the C-tail of NR1 (35S-NR1 CT peptide, upper panel) or NR2A subunit (35S-NR2A CT peptide, middle and lower panels) pull-down by GST and GST fusion proteins conjugated with Csk (GST-Csk) or SH2 domain mutated Csk (GST-Csk S109C, middle panel). Peptides were synthesized with TNT T7 Quick Coupled Transcription/Translation System (Promega). B. Bar graphs showing the summarized data. GST-Csk/λPPase: GST-Csk precipitated 35S-NR2A CT peptide pre-treated with the phosphatase, λPPase (100 units); GST-Csk/10μg non-selective IgG: GST-Csk precipitated 35S-NR2A CT peptide in the presence of 10μg non-selective mouse IgG; GST-Csk/5μg anti-Csk: GST-Csk precipitated 35S-NR2A CT peptide in the presence of 5μg anti-Csk; GST-Csk/1μg anti-Csk: GST-Csk precipitated 35S-NR2A CT peptide in the presence of 1μg anti-Csk. C. The gel (left to right) was loaded with NR2A C-tail peptide synthesized without isotope (input, 3% of total protein used in IP experiments), non-selective-IgG and NR2A immunoprecipitates from these TNT® cell lysates with and without λPPase pre-treatment (1 hour at 30°C). D,E. The gels were loaded with lysates of cells transfected with cDNAs as indicated. F. The gel was loaded with NR2A immunoprecipitates from the cell lysates shown in (D) and (E). G. Summary data showing the ratios of band intensities of tyrosine phosphorylated NR2A versus total NR2A detected in the NR2A immunoprecipitates. These were normalized to that detected in cells transfected with cDNAs encoding wild-type Src but without Csk over-expression (dashed line). Numbers in brackets indicate the number of times the experiments repeated. * and **: P<0.05 and P<0.01, Student’s t-test in comparison to the amount of NR2A CT peptide precipitated by GST-Csk without any treatment in (B), and in comparison between wild-type
Src expressing cells with and without Csk DNA co-transfection in (G). pY527: tyrosine 527 phosphorylated Src; WT: wild-type; Y527F: active Src; pTy: phosphotyrosine.
Figure 1
Figure 2
Figure 3

A

B

C

D

E
Figure 4

A

B

C

D

Normalized currents (µA)

Time (min)

Normalized currents (µA)

Time (min)
Figure 7
Supplementary Information

Primary neuron culture, acute neuron isolation and electrophysiological recording

Acute isolation of neurons The hippocampi of Wistar rats were dissected and cut into approximately 1 mm thick slices in ice-cold oxygenated extracellular solution containing (mM): NaCl (140), KCl (5.4), 1.3 CaCl_2 (1.3), HEPES (25), glucose (33) and tetrodotoxin (0.001), pH 7.4, osmolarity was adjusted to 310–320mOsm. CA1 neurons were isolated from hippocampal slices by tapping the region with a pair of fine forceps.

Electrophysiological recordings Neurons were bathed in the extracellular solution containing (mM): NaCl (140), KCl (5.4), 1.3 CaCl_2 (1.3), HEPES (25), glucose (33), tetrodotoxin (0.001) and glycine (0.003), pH 7.4, osmolarity 310–320 mOsm. Recording pipettes were made from thin-walled borosilicate glass capillaries pulled to a diameter of 1-2μm at the tip, and filled with intracellular solution containing (mM): CsCl or CsF (140), HEPES (10), MgCl_2 (2), BAPTA (1) and K₂ATP (4), pH 7.2 and osmolarity was adjusted to 290–300 mOsm.

Whole-cell currents were recorded using an Axopatch 1D or Axopatch 200B amplifier (Molecular Devices) under the voltage-clamp conditions at a holding potential of -60mV except where indicated. Current-voltage (I/V) relationships of NMDA receptors were calculated by subtracting the currents recorded during voltage ramps from -60 to +60 mV in the absence of L-aspartate or NMDA application from that recorded in the presence of L-aspartate or NMDA application. On-line data acquisition and off-line analysis were performed using pClamp8 software (Molecular Devices).

Hippocampal slice preparation and electrophysiological recording

The ACSF contains (mM) NaCl (124), KCl (3), CaCl_2 (2.6), MgCl_2 (1.3), NaHCO_3 (26), NaH_2PO_4 (1.25) and glucose (10), osmolarity was adjusted to 310–320mOsmol, pH 7.4, at room temperature. Recording electrodes were filled with the intracellular solution containing (mM) K-glucuronate (135.5), KCl (17.5), HEPES (10), EGTA (0.2), Mg-ATP (2), GTP (0.3) and QX 314 (5) with osmolarity adjusted to 290–300mOsm and pH 7.2. DC resistance: 3–5mΩ.

Biochemical experiments

Subcellular fractionation Adult male rat forebrains were dissected and homogenized in homogenization buffer containing (mM): sucrose (320), Tris-HCl (10, pH 7.4), NaHCO_3 (1, pH 7.4), EDTA (2), sodium orthovanadate (1) and 1% (v/v) protease inhibitor cocktail. Homogenate (H) was subsequently centrifuged at 1,000g for 15min to remove nuclei and other large debris (P1). The supernatant (S1) was centrifuged at 10,000g for 30min to obtain a crude synaptosomal fraction (P2), which was then lysed in a hypo-osmotic buffer containing 35.6mM sucrose on ice for 30min before centrifuged at 25,000g for 30min to pellet a synaptosomal membrane fraction (LP1). The resulting supernatant was centrifuged at 165,000g for 60min to pellet a synaptic vesicle-enriched fraction (LP2). The supernatant (S2) above P2 was centrifuged at 165,000g for 60min to obtain a cytosolic fraction (S3) and a light membrane/microsome-enriched fraction (P3).

For preparing the PSD fraction, rat forebrain was homogenized in 1:10 (w/v) sucrose buffer (0.32M sucrose, 1mM NaHCO_3, 1mM MgCl_2, 0.5mM CaCl_2, 1% protease inhibitor cocktail) and centrifuged at 1,400g for 10min. The resulting supernatant was centrifuged at 13,800g for 20min to obtain the pellet which was then re-suspended in the 0.32M sucrose buffer and overlaid with discontinuous sucrose density gradient (0.85M, 1.0M, 1.2M sucrose). After centrifugation at 82,500g for 120min, the band between
1.0M and 1.2M sucrose was removed and incubated with equal volume of 1% Triton-X-100 for 15min. The PSD-I pellet was then obtained by centrifugation at 32,000g for 20min. The pellet was re-suspended in the 0.32M sucrose buffer and incubated a second time with equal volume of 1% Triton-X-100 for 15min, and then centrifuged at 201,800g for 60min to obtain the PSD-II pellet, which represents the concentrated and final PSD isolate. Extracts from individual fractions were solubilized in 1% sodium dodecyl sulphate (SDS) and centrifuged at 15,000g for 5min for Western blot analysis.

**Immunoprecipitation and Western blot analysis**  
Proteins from the P2 fraction of adult rat forebrain or cell pellets were dissolved in a lysis buffer containing (mM): Tris-HCl (50, pH 9.0), NaCl (150), 0.5% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, EDTA (2), sodium orthovanadate (1), and 1% (v/v) protease inhibitor cocktail.

Solubilized proteins (800μg) were incubated with antibodies as indicated overnight at 4°C. The immune complexes were collected with protein G-sepharose beads (Amersham Biosciences) for 2hs at 4°C. The blotting analysis was performed by repeated stripping and successive probing with antibodies as indicated.

For protein quantification, signal detected with ECLplus (Amersham Biosciences) in Western blot were scanned with Amersham Storm 860 fluorescent scanning system (Molecular Dynamics). Protein band intensity was calculated with the software, ImageQuant 5.0 (Molecular Dynamics).

**cDNA transfection** cDNAs transfected were full length NR1-1a (8μg), full length or C-terminal truncated NR2A (32μg), v-Src (as where indicated, a gift of Dr. T. Pawson, University of Toronto), Csk (1μg, a gift of Dr. K. Harder, Royal Melbourne Hospital), wild-type (2μg) or mutant neuronal Src (n-Src, 2μg, Y535F or K303R/Y535F, provided by Dr. S. Hanks, Vanderbilt University)1. cDNA sequence encoding the C-tail truncated forms of NR2A (NR2AΔCT) was amplified by PCR, and was constructed into EcoRI site of vector pcDNA3 (Invitrogen). Primers for constructing NR2AΔCT were 5′-CGGAATTCACCATGATGGGCAGATTGG-3′ and 5′-CGGAATTCCTACCAGATGAAGGTGATG-3′. 

**In-vitro GST pull-down assay** DNA sequences encoding the C-tails of NR1 (aa 834-938) and NR2A (aa 838-1464) were amplified by PCR. The PCR products were constructed into BamHI-EcoRI sites of vector pcDNA3 (Invitrogen) for in-vitro transcription and translation. Primers for constructing C-tails of NR1 and NR2A are 5′-TTTGGATCCATGGAGATCGCCTACAAGCG-3′ and 5′-TTTGAATTCTCAGCTCTCCCTATGACGGG-3′, 5′-CGGGATCCACCATGATGGAGCACCTCTTC-3′ and 5′-CGGAATTCACCATGATGGAGGACACCTCTTC-3′.

pGEX2th-Csk-ST plasmid was kindly provided by Dr. G. Sun at the University of Rhode Island 2. Csk S109C mutation was made using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) as described by the manufacturer with pGEX2th-Csk-ST as template. Point mutation was confirmed by DNA sequencing. The GST and GST fusion proteins conjugated with Csk or Csk S109C were produced in E. Coli (BL-21 strain, Invitrogen) with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1mM) induction.

**Characterization of Csk and Chk antibodies** Corresponding antibodies of high specificity, and showing no cross reactivity, are required in order to distinguish between
expression of Csk and Chk. We therefore examined the selectivity and cross reactivity of these antibodies initially by using GST-fusion proteins coupled to Csk and Chk. These antibodies demonstrated a high selectivity between Csk and Chk with no cross reactivity (Supplementary Fig. 2a). Furthermore, this selectivity was confirmed using cell lysates from the adult brain and the liver. The liver contains no Chk3,4,5 and served as a negative control (Supplementary Fig. 2d). We also examined the ability of anti-Csk to distinguish levels of expression of Csk in additional ways. Firstly, we examined its signal with differing levels of expression of recombinant Csk expressed in fibroblasts grown from Csk-/- transgenic mouse embryos (Supplementary Fig. 2b). Secondly, we tested the ability of the antibody to detect differential levels of expression Csk in adult brain lysates taken from wildtype and Csk+/- mice which are deficient in Csk (Supplementary Fig. 2c). These results validate our subsequent use of these antibodies.

Reference List


Supplementary Figure 1
Supplementary Figure 2