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The NMDA Receptor NR1 Subunit is Critically Involved in the Regulation of NMDA Receptor Activity by C-terminal Src kinase (Csk)

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Abstract Previous studies have shown that Csk plays critical roles in the regulation of neural development, differentiation and glutamate-mediated synaptic plasticity. It has been found that Csk associates with the NR2A and 2B subunits of N-methyl-D-aspartate receptors (NMDARs) in a Src activity-dependent manner and serves as an intrinsic mechanism to provide a "brake" on the induction of long-term synaptic potentiation (LTP) mediated by NMDARs. In contrast to the NR2A and 2B subunits, no apparent tyrosine phosphorylation is found in the NR1 subunit of NMDARs. Here, we report that Csk can also associate with the NR1 subunit in a Src activity-dependent manner. The truncation of the NR1 subunit C-tail which contains only one tyrosine (Y837) significantly reduced the Csk association with the NR1-1a/NR2A receptor complex. Furthermore, we found that either the truncation of NR2A C-tail at aa 857 or the mutation of Y837 in the NR1-1a subunit to phenylalanine blocked the inhibition of NR1-1a/NR2A receptors induced by intracellular application of Csk. Thus, both the NR1 and NR2 subunits are required for the regulation of NMDAR activity by Csk.

Keywords NMDA receptors · Src family kinases · C-terminal Src kinase · The NMDA receptor subunit

Introduction

Csk family kinases (Csk, C-terminal Src kinase and Chk, Csk-homologous kinase) are cytosolic kinases. They specifically phosphorylate a tyrosine residue (Tyr527 in chicken c-Src) in the C-tails of Src family kinases (SFKs) and thereby act as endogenous inhibitors of SFKs [1–4]. SFKs are involved in the regulation of many physiological and pathophysiological processes mediated through growth factors, G-protein-coupled receptors, or ligand-gated ion channels. As such, SFKs have become important targets of therapeutic drugs [5–7].

Large amounts of data have shown that through controlling the activity of SFKs Csk regulates neural development and differentiation [8–11], glutamate-mediated synaptic plasticity [12, 13] and toxicity [14], nociceptor function [15] and cancer cell progression [16]. Detailed studies have shown that the binding of Csk to the phosphotyrosine of SFKs' substrates is a key mechanism, which allows Csk to move close to and phosphorylate SFKs and thereby inhibit SFK activity [13, 17, 18]. The blockade of phosphorylation-dependent Csk binding has been found to play important roles in the regulation of SFK-mediated cellular functions [13, 17, 18]. Our previous study demonstrated that Csk associates with NMDARs and serves as an intrinsic mechanism to provide a "brake" on the induction of long-term synaptic potentiation (LTP) mediated by SFKs [13].

Xiao-Qian Fang and Jindong Xu contributed to this work equally.

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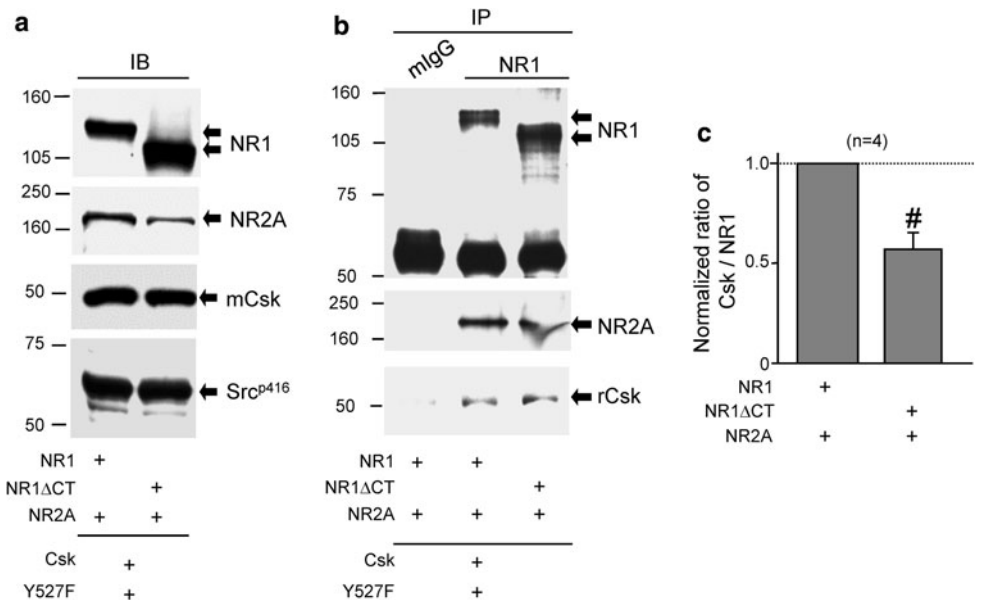

Fig. 1 The NR1 C-tail truncation reduces Csk co-precipitated with the NR1 subunit. **a** The gel was loaded with lysates of cells co-transfected with cDNAs as indicated with "plus". *NR1* full-length NR1-1a, *NR1ΔCT* NR1-1a (1-834), *NR2A* full-length NR2A, *Y527F* Src/Y527F. *IB* immunoblotting. Src^{p416}: probed with an antibody recognizing Src with phosphorylated Y416. The gel shown in **b**, was loaded with proteins immunoprecipitated from the lysates shown in **a** by using non-selective mouse IgG (*mIgG*) or a mouse anti-NR1 antibody as indicated. IP: immunoprecipitation. The filters shown in **a** and **b** were sequentially stripped and probed with antibodies against proteins as indicated to the *right of blots*. *mCsk* probed with a mouse anti-Csk antibody, *rCsk* probed with a rabbit anti-Csk antibody. **c** Summary data showing the ratios of the band intensities of Csk versus NR1, which were normalized with those detected in cells expressing full-length NR1 and NR2A. n in *brackets* indicates the times of the experiments repeated. $^{\#}P < 0.05$, *t*-test in comparison with that found in cells expressing full-length NR1-1a and NR2A subunits

A functional NMDAR in the central nervous system (CNS) contains at least two NR1 and two NR2 subunits and the NR1-1a and NR2A subunits of NMDARs are dominantly expressed in adult neurons [19]. The C-tails of NR2A and NR2B subunits are found to be primary targets for phosphorylation by SFKs, such as Src and Fyn [20–22]. Csk is found to associate with phosphorylated NR2A and NR2B subunits [13]. In contrast to NR2A and 2B subunits, there are only three tyrosine residues in the intracellular domain of the NR1 subunit. They are Y587, Y647 and Y837 in rat NMDARs, which are located, respectively in the transmembrane regions I and III, and the C-tail [23, 24] (ACCESSION NP_015566). No apparent tyrosine phosphorylation in the NR1 subunit is found [22, 25]. Csk is found to bind to the C-tail protein of the NR2A, but not the NR1, subunit in vitro [13]. Recently, however, it has been reported that Y837 in the NR1 subunit together with Y842 in the NR2A subunit may form a tyrosine "ring" and is critically involved in the Src-mediated regulation of NMDARs [26]. Mutating these tyrosines to phenylalanine blocks clathrin-mediated endocytosis of NMDARs [26]. In this work we investigated the interaction of Csk with the NR1 subunit and found that through the targeting of Y837 in the NR1 subunit Csk can associate with NMDARs and regulate the receptor activity.

Experimental Procedure

Cell Culture and cDNA Transfection

HEK-293 or COS-7 cells were grown in Dulbecco's Modified Eagles Media (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) in culture dishes (diameter: 60 or 100 mm). The cells were transfected by using either calcium phosphate (Invitrogen) or LipoD293^{TM} (SignaGen) as per the manufacturer's instruction with expression vectors (pcDNA3 or pRcCMV). With the calcium phosphate method, cDNAs transfected are full length NR1-1a, C-terminal truncated NR1-1a (aa 1-834) or NR1-1a with the mutation of Y837 to phenylalanine (Y837F) (8 μg), full length or C-terminal truncated NR2A (aa 1-837, 32 μg), v-Src (as where indicated, a gift of Dr. T. Pawson, University of Toronto), Csk (1 μg, a gift of Dr. K. Harder, Royal Melbourne Hospital), wild-type (2 μg) or mutant neuronal Src (n-Src, 2 μg, Y535F or K303R/Y535F, provided by Dr. S. Hanks, Vanderbilt University) [27]. Since we found that Csk may associate with NMDARs expressed in either HEK-293 or COS-7 cells (see Fig. 1), all experiments shown in the remaining figures were conducted using HEK-293 cells.

By using LipoD293™ transfection method, HEK-293 cells were incubated with LipoD293™ and cDNAs of NR1-1a or NR1-1a Y837F (4 μg), Csk (0.8 μg), and Src Y527F (1.6 μg). After 5 h incubation with the cDNAs and transfection agents, cells were transferred to DMEM supplemented with 10% fetal bovine serum and maintained in DMEM containing 10% FBS and NMDAR antagonist AP5 (0.5 mM) for 48 h before experiments. Since no difference in Csk association with NMDARs was found in cells transfected by using the calcium phosphate or LipoD293™, data were pooled. For electrophysiological recordings, cDNAs of green fluorescence protein (GFP, 0.15 μg), NR1-1a or NR1-1a Y837F (0.3 μg), NR2A (1.2 μg) or NR2A (aa 1-857, a gift from Drs. T. Yamamoto and T. Tezuka, Tokyo University, 0.9 μg), n-Src (0.15 μg) and PSD-95 (0.15 μg) were co-transfected with LipoD293™ into HEK-293 cells in culture dishes (diameter: 35 mm).

cDNAs encoding the C-tail truncated forms of NR1-1a (aa 1-834) and NR2A (aa 1-837) were amplified by PCR, and then constructed into *BamHI-EcoRI* sites or *EcoRI* site of vector pcDNA3 (Invitrogen). Primers for constructing NR1-1a (1-834) and NR2A (aa 1-837) are 5′-TTTGGATC CATGAGCACCATGCACC-3′ and 5′-TTTGAATTCT-CATCACTCAATGAAAATGAGGAAAATCCC-3′, 5′-C GGAATTCACCATGATGGGCAGATTGG-3′ and 5′-C GGAATTCCTACCAGATGAAGGTGATG-3′. Mutated NR1-1a (Y837F) was generated using the QuikChange XL site-directed mutagenesis kit (Strategene) according to the manufacturer's instructions. Primers for NR1 mutagenesis are 5′-CATTGAGATCGCCTTCAAGCGACACAAGGA TGC-3′ and 5′-GCATCCTTGTGTCGCTT GAAGGCGAT CTCAATG-3′. To avoid confusion, the n-Src mutants used in these experiments are referred to as Y527F and K295R/Y527F of chicken c-Src.

Immunoprecipitation and Western Blot Analysis

Immunoprecipitation and Western blotting experiments were performed as described previously [13, 28]. Cells were mechanically dissociated and 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 for 60 min. Solubilized proteins (800 μg) were incubated with antibodies as indicated overnight at 4°C. The immune complexes were collected with protein G-Sepharose beads (Amersham Biosciences) for 2 h 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).

Whole-cell Patch Clamp Recordings

For whole-cell patch clamp recordings HEK-293 cells were bathed in a standard extracellular solution containing (in mM): Na_2SO_4 (100), Cs_2SO_4 (10), $CaCl_2$ (4.8), HEPES (25), glucose (32), glycine (0.01), pH: 7.35 and osmolarity: 310–320 mOsm. Free Ca^{2+} concentration in this solution was at 1.2 mM as confirmed by measurement with a Ca^{2+} selective electrode (Thermo Electron Corporation, Beverly, MA). Recording pipettes were pulled to a diameter of 1–2 μm at the tip and filled with intracellular solution composed of (in mM): CsCl (145), BAPTA (0.5), HEPES (10), $MgCl_2$ (2), K-ATP (4), osmolarity 290–300 mOsm (DC resistance: 4–7 MΩ). Whole-cell currents were evoked by application of NMDA (300 μM) dissolved in the extracellular solution for 3 s via a multi-barrel fast-step perfusion system (SF-77B perfusion fast-step system, Warner Instrument). Recordings were conducted under the voltage-clamp condition at a holding potential of −60 mV except where indicated. Whole-cell currents were recorded using Axopatch 200B amplifiers (Molecular Devices). On-line data acquisition and off-line analysis were performed using pClamp9 software (Molecular Devices). The peak amplitude and the steady-state amplitude (the mean amplitude of currents during the last 300 ms of NMDA application) were measured. Current–voltage (I/V) relationships were calculated by the subtraction of currents during the voltage ramp from −60 to +60 mV without NMDA application from currents during the analogous ramp over the steady-state of NMDA-evoked responses.

Results

Csk Associates with the NR1 Subunit Within the Heteromeric NMDAR Complex

To investigate how Csk may interact with NMDARs in cells, we expressed NMDAR subunit proteins (NR1-1a and NR2A) or their mutants in a non-neuronal expression system (such as HEK-293 or Cos-7 cells). These cells provide an excellent model for expressing various proteins and their mutants, and therefore, have been widely used in studies of protein structure and function. Since our previous study has shown that the Csk interaction with the NR2A subunit depends upon the phosphorylation of the NR2A subunit [13], we conducted immunoprecipitation experiments from HEK-293 cells in which cDNAs of Csk and constitutively active Src (Src/Y527F) were

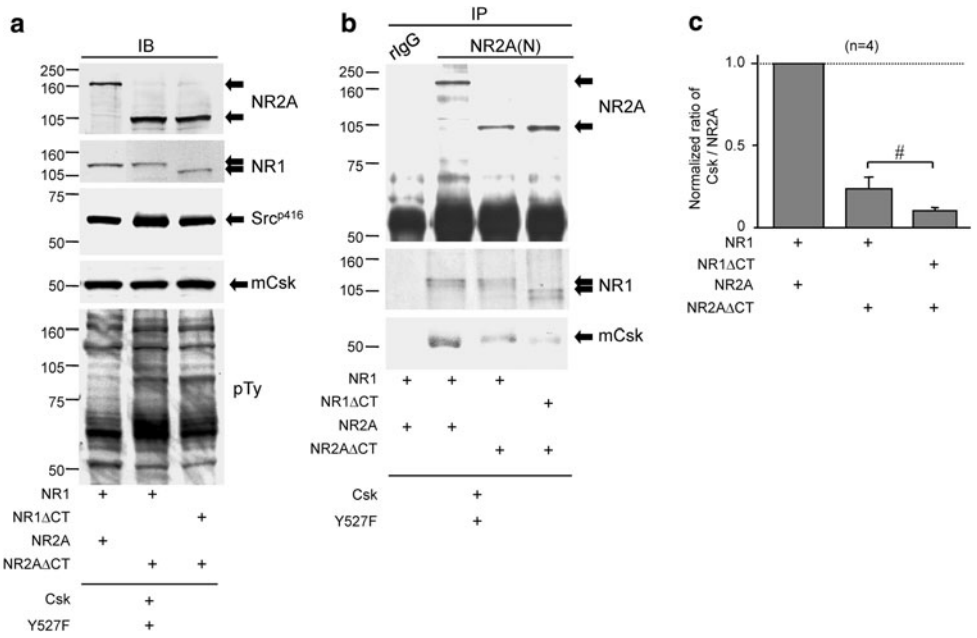

Fig. 2 The NR1 C-tail truncation reduces Csk co-precipitated with the NR2A subunit. **a** The gel was loaded with lysates of cells co-transfected with cDNAs as indicated. NR2AΔCT: NR2A (1-837). The gel shown in **b**, was loaded with proteins immunoprecipitated from the lysates shown in **a** by using non-selective rabbit IgG (*rIgG*) or a rabbit anti-NR2A antibody. The filters shown in **a** and **b** were sequentially stripped and probed with antibodies against proteins as indicated to the *right of blots*. **c** Summary data (mean ± SEM) showing the ratios of the band intensities of Csk versus NR2A detected in the NR2A immunoprecipitates, which were normalized with those detected in cells expressing full-length NR1 and NR2A. $^{\#}P < 0.05$, independent *t*-test

co-transfected. Figure 1a shows protein expression detected in cells transfected with cDNAs as indicated. Consistent with our previous finding, an antibody against the NR1 subunit co-precipitated both NR2A and Csk proteins (Fig. 1b). We measured the ratio of Csk versus NR1 proteins precipitated. We found that the truncation of the NR1 subunit C-tail significantly reduced the ratio of Csk/NR1 when compared with that in cells expressing wild-type NR1-1a and NR2A subunits (Fig. 1b, c). This finding suggests that Csk may associate with the NR1 subunit through targeting its C-tail. To confirm that Csk may associate with the NR1 subunit in the heteromeric NMDAR complex, we examined effects of the truncation of the NR1 C-tail on Csk association detected in NR2A immunoprecipitates.

Consistent with our previous findings, Csk proteins were co-immunoprecipitated by an NR2A antibody and this co-precipitation was significantly reduced by the truncation of the NR2A C-tail (Fig. 2b, c), confirming the Csk association with the NR2A subunit. Moreover, we found that the truncation of the NR2A C-tail produced greater reduction of Csk association when compared with that induced by the truncation of the NR1 C-tail (Figs. 1c, 2c). Our data showed that in cells expressing recombinant NMDARs in which C-tails of both the NR1-1a and NR2A subunits were truncated, co-immunoprecipitated Csk protein by an NR2A antibody was significantly reduced when compared with that detected in cells expressing the receptors in which only the C-tail of the NR2A subunit was removed (Fig. 2b, c). Thus, we can conclude that Csk can associate with NMDAR complex through the C-tail of either the NR1 or NR2A subunit.

Csk-NR1 Association is Src Activity-Dependent

To understand how Csk interacts with the NR1 subunit, we examined Csk-NR1 association in cells transfected with cDNAs encoding NR1-1a and Csk. We found that an NR1 antibody could not precipitate Csk if the NR1 subunit was not co-expressed (Fig. 3a). Increasing Src activity by co-transfection of v-Src did not change the expression of either Csk or the NR1-1a subunit. However, the tyrosine phophsorylation of the tissues and the Csk-NR1-1a association were increased. No Src association with the NR1-1a subunit could be found even when v-Src was over expressed (Fig. 3a). To determine the role of Src in the Csk association with the NR1-1a subunit, we examined the relationship between Src expression and Csk-NR1 association. Figure 3b shows an example of the experiments. We found that with a higher level of Src kinase expression, more Csk was found to be associated with the NR1-1a subunit (Fig. 3b, c). Moreover, application of the SFK inhibitor, PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine, 10 μM) [29, 30], to cells 12 h after co-transfection with NR1-1a, Csk and v-Src cDNAs

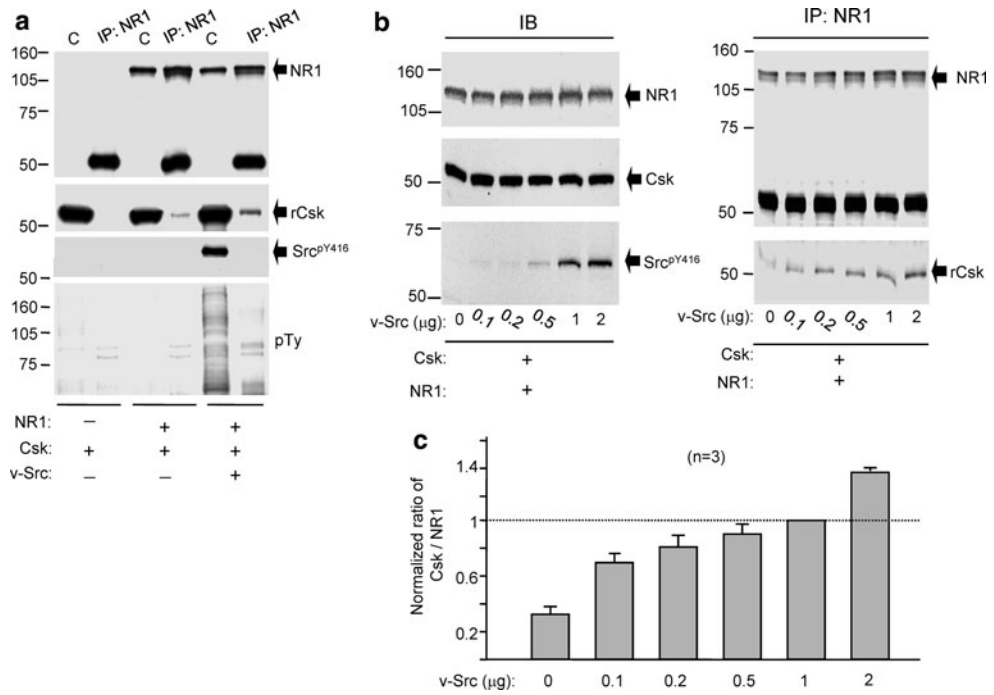

Fig. 3 Increases in Src expression enhance Csk association with the NR1 subunit. **a** The gel was loaded with cell lysates (30 μg, C) and NR1 immunoprecipitates (IP:NR1) from cells with and without virus Src (v-Src) co-expression. pTy: probed with the anti-phosphotyrosine antibody, 4G10. **b** The gel (*left*) was loaded with lysates of cells co-transfected with various amount of cDNAs of v-Src, and the gel (*right*) was loaded with NR1 immunoprecipitates from the lysates of cells co-transfected with various amount of v-Src. **c** Summary data showing the ratios of the band intensities of Csk versus NR1 detected in the NR1 immunoprecipitates, which was normalized with that detected in cells co-transfected with 1 μg v-Src cDNA (*dashed line*)

significantly reduced the amount of Csk co-precipitated with the NR1 subunit to 48 ± 9% ($n = 5$) of that without PP2 treatment (Fig. 4a). Consistent with this effect of PP2, co-expression of inactive Src (Y527F/K295R) also reduced Csk-NR1 association to 34 ± 5% ($n = 4$) of that detected in cells expressing constitutively active Src (Y527F) (Fig. 4b). The Csk-NR1 association was also significantly reduced by the mutation of Y837F in the NR1-1a subunit (Fig. 4c). Thus, we conclude that the association of Csk with the NR1 subunit in cells is Src kinase activity-dependent and that Y837 plays an important role in the Csk-NR1 association.

Y837 of the NR1 Subunit is Critical in the Regulation of NMDAR Activity by Csk

To clarify the role of the Csk-NR1 association in the regulation of NMDAR functions, NR1-1a/NR2A receptor-mediated whole-cell responses were recorded in cells co-transfected with cDNAs of wild-type Src and PSD-95. To deliver Csk into cells, Csk (2.5 μg/ml) was included in the intracellular solution filling recording electrodes. Figure 5a shows an example of recordings of NMDA-evoked responses in HEK-293 cells expressing the NR1-1a and NR2A subunit ($n = 7$). We found that NR1-1a/NR2A receptor-mediated whole-cell responses were gradually reduced when compared with that recorded immediately after breakthrough. Peak responses were reduced by more than 20% in 6 of 7 cells tested. At 10 min after breakthrough the peak and steady state amplitudes were reduced by 25 ± 5 and 26 ± 4% when compared with those recorded immediately after breakthrough (Fig. 5a, c). No change in the reversal potential of recorded currents could be found (Fig. 5b), confirming that NMDAR-mediated whole-cell conductance was reduced. We then examined the effects of the deletion of the NR2A C-tail expressed in HEK-293 cells ($n = 8$). We found that NMDA-evoked peak responses in cells expressing the NR1-1a and NR2A (1-857) subunits were reduced by more than 20% in only 2 of 8 cells tested. At 10 min after breakthrough the peak and steady state amplitudes were 95 ± 6 and 102 ± 7% of those recorded immediately after breakthrough (Fig. 5c). This data confirms that the Csk-NR2A interaction is important in the regulation of NMDARs by Csk.

Interestingly, we also noted that in cells ($n = 11$) expressing the NR1-1a (Y837F) and NR2A subunits NMDAR-mediated responses were reduced by more than 20% in 4 cells following the intracellular delivery of Csk. At 10 min after breakthrough the peak and steady state amplitudes were 97 ± 18 and 104 ± 13% of those recorded immediately after breakthrough (Fig. 5c). Taken together,

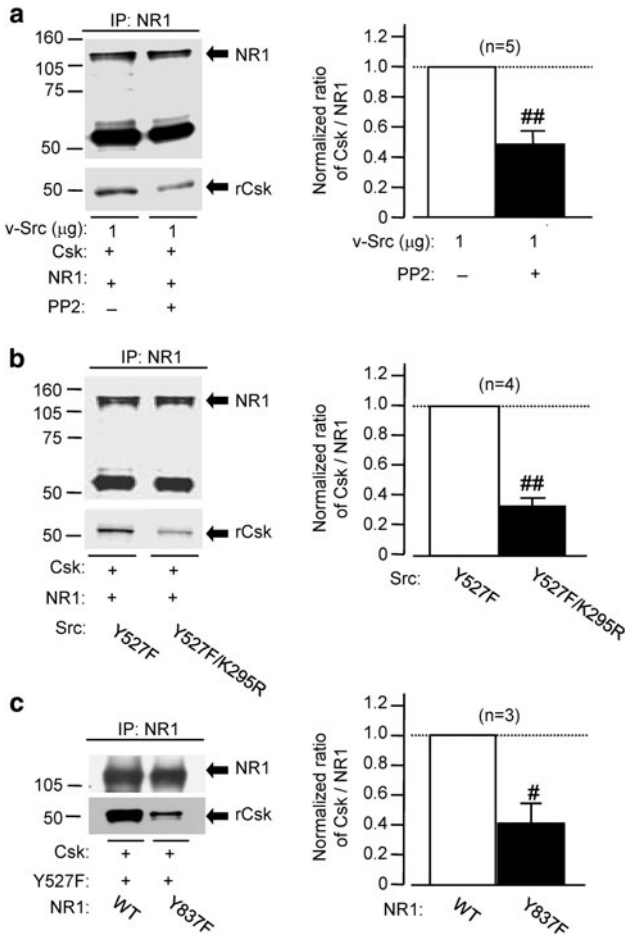

Fig. 4 The inhibition of Src kinase activity or the mutation of Y837 in the NR1-1a subunit reduces Csk association with the NR1 subunit. **a** The gel was loaded (from left to right) with NR1 immunoprecipitates from the cells without ("minus") and with ("plus") PP2 (10 μM) treatment. The *bar graph* shows ratios of the band intensities of Csk versus NR1 detected in the NR1 immunoprecipitates, which was normalized with that detected in cells without PP2 treatment (*dashed line*). **b** The gel was loaded (from left to right) with NR1 immunoprecipitates from cells co-transfacted with cDNAs of active (Y527F) and inactive Src (Y527/K295R). The *bar graph* shows ratios of Csk versus NR1, which were normalized with that detected in cells expressing active Src (*dashed line*). **c** The gel (from left to right) was loaded with NR1 immunoprecipitates from cells co-transfected with cDNAs of wild-type (WT) and mutated NR1-1a subunit in which Y837 was mutated to phenylalanine (Y837F). The *bar graph* shows ratios of Csk versus NR1, which were normalized with that detected in cells expressing the wild-type NR1-1a subunit (*dashed line*). $^{\#, \#\#}P < 0.05$, 0.01 (independent *t*-test)

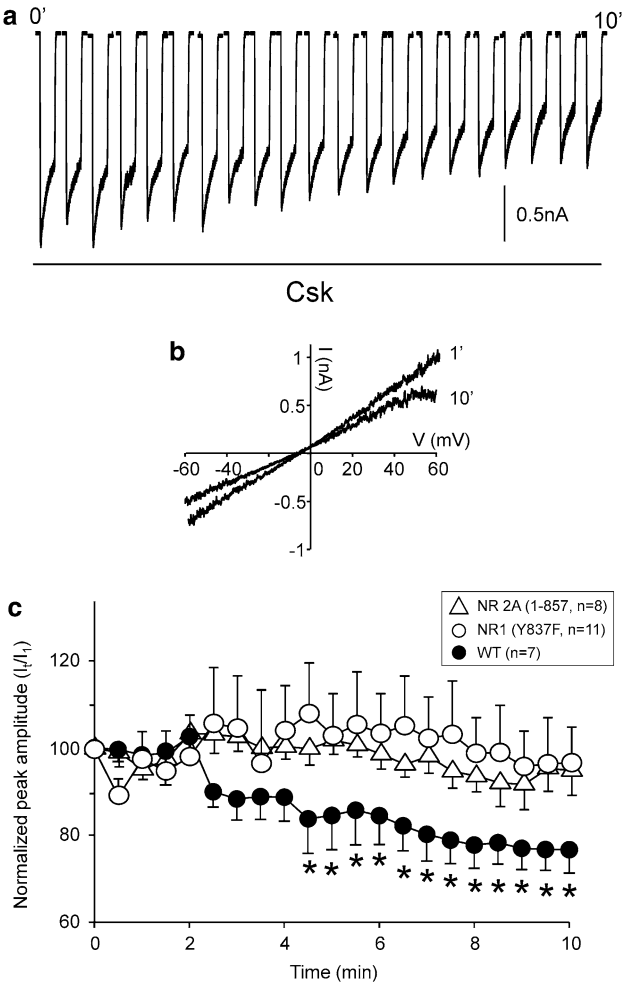

Fig. 5 The Y837F mutation in the NR1 subunit prevents the Csk-induced down-regulation of NMDAR activity. **a** An example of whole-cell recordings. NMDA-evoked whole-cell currents were recorded from a HEK-293 cell co-transfected with cDNAs encoding NR1-1a, NR2A, PSD-95 and Src. Csk (0.25 μg/ml) was included in the solution filling the recording electrode. **b** An example of current–voltage (I/V) relationship recorded immediately (0′) and 10′ after breakthrough from a cell with intracellular application of Csk. **c** Summary data showing normalized peak amplitudes of NMDA-evoked currents recorded in cells expressing NR1-1a/NR2A (WT), NR1-1a (Y837F)/NR2A or NR1-1a/NR2A (1-857) receptors. When compared with that recorded from wild-type NMDARs, either the truncation of the NR2A C-tail or mutation of Y837 to phenylalanine in the NR1 subunit significantly affected Csk-induced inhibition of NMDAR currents ($P < 0.001$, two-way ANOVA test). Compared with NMDAR responses recorded immediately after breakthrough a significant reduction was found in wild-type NMDARs at 4.5 min following intracellular Csk application ($^{*}P < 0.05$, Kruskal–Wallis *Z* Test)

it suggests that both the NR1 and NR2A subunits are critically involved in the regulation of NMDAR activity by Csk.

Discussion

Csk acts as a direct feedback mechanism negatively regulating the signaling by SFKs [1–4]. Our previous work has shown that the intracellular application of recombinant Csk depresses NMDAR-mediated responses in neurons and that this depression is dependent upon SFK activity as it is entirely occluded by their blockade [13].

In the CNS Csk has been found to associate with a post-synaptic density protein, post-synaptic density 93 (PSD-93)

[12]. Our data show that Csk is closely linked with the NMDAR complex along with Src and the Src activator, protein tyrosine phosphatase alpha (PTPα) [13]. As SFK activity is increased, both the phosphorylation of NR2A and NR2B subunits and the association of Csk with these subunits are augmented [13]. Csk is also found to bind to the NR2A C-tail protein in vitro while no direct binding to NR1 C-tail protein is detected [13]. Dephosphorylation of the NR2A C-tail protein, mutation of the SH2 domain of Csk, or application of an antibody targeting SH2-SH3 domains of Csk substantially reduces the Csk binding [13].

Our present data shows that a truncation of the NR1-1a C-tail significantly reduced the Csk association when compared to that detected in the NMDAR assembly consisting of the full length NR1-1a subunit and the C-tail truncated NR2A subunit. This association was enhanced by increases in the activity of Src and reduced by the mutation of Y837 to phenylalanine. Thus, this suggests that the NR1 subunit may also contribute to the association of Csk with NMDARs. Y837 of the NR1 subunit has been found to be involved in the Src-dependent regulation of NMDAR endocytosis [26]. The inability to detect tyrosine phosphorylation in the NR1 subunit in the brain [22, 25] might be due to the presence of too few tyrosine residues located in the intracellular domain [23, 24].

Csk can bind to NR2A and 2B subunits phosphorylated by SFKs [13, 31, 32]. Our present data obtained from an over-expression system shows that Csk associated with the NR1 subunit when Src activity was enhanced by over-expression of the kinase. This implies a possibility that Csk may also associate with the NR1 subunit when SFKs are activated.

The findings that Csk association could be reduced by truncation of either the NR1 or NR2A C-tail, and that further reductions in Csk association were noted when the C-tails of both NR1-1a and NR2A subunits were removed, suggest that Csk-NR1-1a and Csk-NR2A subunit associations may occur independently. Although it appeared that more Csk associated with the NR2A subunit than with NR1 subunit, the Csk application-induced down-regulation of NR1-1a/NR2A receptor activity was blocked by either the truncation of the NR2A C-tail or the mutation of Y837 in the NR1-1a subunit. Thus, both the Csk-NR1-1a and Csk-NR2A associations are critically involved in the regulation of NR1-1a/NR2A receptor activity by Csk.

Large amounts of data have shown that NMDARs are primarily important in the regulation of many physiological and pathophysiological processes such as neuronal development and death, glutamate-mediated plasticity and toxicity, in which SFKs and Csk are also found to be critical [6, 13, 14, 19, 32]. Our present findings have provided novel insights for understanding how Csk may act on NMDARs. This is essential for understanding the regulation of neuroplasticity in the CNS by NMDARs.

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