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Nicotine-induced neuroplasticity counteracts the effect of schizophrenia-linked neuregulin 1 signaling on NMDAR function in the rat hippocampus

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Abstract

A high rate of heavy tobacco smoking among people with schizophrenia has been suggested to reflect self-medication and amelioration of cognitive dysfunction, a core feature of schizophrenia. NMDAR hypofunction is hypothesized to be a mechanism of cognitive dysfunction, and excessive schizophrenia-linked neuregulin 1 (NRG1) signaling through its receptor ErbB4 can suppress NMDAR function by preventing Src-mediated enhancement of NMDAR responses. Here we investigated whether chronic nicotine exposure in rats by subcutaneous injection of nicotine (0.5–1 mg/kg, twice daily for 10 to 15 days) counteracts the suppressive effect of NRG1β on NMDAR-mediated responses recorded from CA1 pyramidal cells in acute hippocampal slices. We found that NRG1β, which prevents the enhancement of NMDAR responses by the Src-family-kinase-activating peptide pYEEI in naive rats, failed to block the effect of pYEEI in nicotine-exposed rats. In naive rats, NRG1β acts only on GluN2B-NMDARs by blocking their Src-mediated upregulation. Chronic nicotine exposure causes enhanced GluN2B-NMDAR responses via Src upregulation and recruits Fyn for the enhancement of GluN2A-NMDAR responses. NRG1β has no effect on both enhanced basal GluN2B-NMDAR responses and Fyn-mediated enhancement of GluN2A-NMDAR responses. Src-mediated enhancement of GluN2B-NMDAR responses and Fyn-mediated enhancement of GluN2A-NMDAR responses initiate long-term potentiation (LTP) of AMPAR synaptic responses in naive and nicotine-exposed CA1 pyramidal cells, respectively. These results suggest that NRG1β suppresses LTP by blocking Src-mediated enhancement of GluN2B-NMDAR responses, but has no effect on LTP in nicotine-exposed rats. These effects of chronic nicotine exposure may counteract the negative effect of increased NRG1-ErbB4 signaling on the cellular mechanisms of learning and memory in individuals with schizophrenia, and therefore may motivate heavy smoking.

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Keywords

nicotine; GluN2A; GluN2B; Src; Fyn; neuregulin 1

1. Introduction

Despite the fact that smoking tobacco causes serious health problems, many people continue to smoke, and individuals suffering from schizophrenia or other mental illnesses that can cause cognitive deficits are particularly likely to be heavy smokers (Ziedonis et al., 2008). Understanding the mechanistic basis of this association may provide novel insights into the etiology and treatment of cognitive dysfunction.

Chronic nicotine exposure, which improves hippocampal memory (Levin et al., 1992; Kenney and Gould, 2008), acts via Src tyrosine kinase signaling to enhance the response of *N*-methyl-D-aspartate receptors (NMDARs) in CA1 pyramidal cells (Yamazaki et al., 2006a, b). Interestingly, this effect of nicotine is mimicked by two acetylcholinesterase inhibitors, donepezil and galantamine (Ishibashi et al., 2014), which are currently used for treatment of cognitive deficits associated with Alzheimer's disease (AD). Furthermore, administration of these cholinergic drugs has also been shown to improve cognition in schizophrenic patients (Levin et al., 2006; Gray and Roth, 2007; Buchanan et al., 2008). Thus, the observed enhancement of NMDAR function might be responsible for the enhanced cognition in AD and schizophrenic patients.

The two major NMDAR subtypes, GluN2A-NMDAR and GluN2B-NMDAR, exist as a macromolecular complex (Husi et al., 2000) and their function can be affected by interactions among many proteins linked to various pathways (Salter and Kalia, 2004). This macromolecular complex appears to be a convergence point for schizophrenia susceptibility genes and pathways that may affect NMDAR function and signaling (Hahn, 2011; Snyder and Gao, 2013). Indeed, increasing evidence indicates that NMDAR hypofunction in the hippocampus-prefrontal cortex pathway is a potential mechanism underlying cognitive dysfunction in schizophrenia patients (Snyder and Gao, 2013).

Among schizophrenia susceptibility genes and pathways, excessive neuregulin 1 (NRG1)-ErbB4 signaling has been shown to participate in NMDAR hypofunction (Hahn et al., 2006; Shamir et al., 2012) by preventing Src-mediated enhancement of NMDAR function (Pitcher et al., 2011; Salter and Pitcher, 2012). Thus, Src is a point of convergence for both nicotine-initiated and schizophrenia-linked signaling, and the effect of schizophrenia-linked signaling is the exact opposite of what occurs with chronic nicotine exposure. In this study, we investigated whether nicotine-induced neuroplasticity counteracts the suppressive effects of the schizophrenia-linked signaling on NMDAR function.

2. Materials and methods

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with protocols approved by the

Institutional Animal Care and Use Committee of the University of California at Irvine and the Animal Research Committees of Yamagata University.

2.1. Chronic nicotine treatment

The current study was designed based on our previous results demonstrating that chronic nicotine exposure in Sprague-Dawley rat pups (starting at around postnatal day 21) by subcutaneous injection of nicotine (0.5–1 mg/kg nicotine base, twice daily for 10 to 15 days) causes the enhancement of NMDAR-mediated responses via Src upregulation in adolescence (Yamazaki et al., 2006a, b; Ishibashi et al., 2014). Therefore, to achieve the same effect of nicotine in adolescence, the same treatment regimen was used in the present study. Smoking several cigarettes delivers an acute dose of 60–300 nM nicotine in the venous blood (Benowitz et al., 1990) and approximately 600 nM nicotine in the arterial blood (Henningfield et al., 1993), which better represents the level of nicotine in the brain. Plasma concentrations of nicotine reach peak values (2.2 μM) within 5–10 min after nicotine (1 mg/kg) administration into the femoral vein of rats, decrease to 0.9 μM 20 min after administration and are maintained at this level for the next 40 min (Sastry et al., 1995). Because the plasma half-life of nicotine in rats is about 45 minutes and it is about 2 hr in humans (Matta et al., 2006), the dose used for subcutaneous injection (intended to be absorbed slowly) in the current study should produce blood levels of nicotine similar to those found in heavy smokers. Nicotine injection induced seizures in most of the pups, which were usually brief, and the effect of nicotine became progressively weaker by continuing daily injection of nicotine. Nicotine-induced enhancement of NMDAR-mediated responses was mimicked by a muscarinic ACh receptor agonist and acetylcholinesterase inhibitors without inducing seizures, and co-administration of the m1 antagonist pirenzepine prevents the effect of nicotine without blocking nicotine-induced seizures (Ishibashi et al., 2014). These observations suggest that the effect of nicotine is mediated by increased release of ACh via the activation of nicotinic acetylcholine receptors (nAChRs) and involves m1 muscarinic receptor activation through ACh, but not nicotine-induced seizures.

2.2. Electrophysiological recording

Ninety minutes after the last injection of phosphate-buffered saline (PBS) or nicotine, transverse hippocampal slices were prepared and maintained at 30–32°C in artificial cerebrospinal fluid (ACSF) containing (in μM): 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2.5 $CaCl_2$, 22 $NaHCO_3$, and 10 glucose, and oxygenated with 95% O_2 and 5% CO_2. Although in the present study we harvested brains approximately 90 minutes after the last nicotine injection, the enhanced NMDAR-mediated responses were also observed in hippocampal slices from brains harvested 12 hours after the last nicotine injection, suggesting that the observed change in NMDAR function is not an acute effect of nicotine. Furthermore, the enhanced NMDAR responses were also observed in slices kept for 6 hours in ACSF (Yamazaki et al., 2006b; Ishibashi et al., 2014), suggesting that the effects elicited in vivo are maintained in slices. We made whole-cell patch-clamp recordings from CA1 pyramidal cells from control (naive or PBS-treated) and nicotine-exposed rats. Pyramidal cells were visualized for whole-cell recording with an infrared differential interference contrast microscope (Axioskop, Zeiss, Germany), using a 40× water-immersion objective. Patch electrodes were pulled from borosilicate glass (World Precision Instruments, Sarasota,

FL, USA) using a micropipette puller (P-97, Sutter Instrument, Novato, CA, USA). The electrodes had a resistance of 5–7 MΩ after being filled with pipette solution containing (in μM) 117 Cs-methanesulfonate, 10 HEPES, 0.5 EGTA, 2.8 NaCl, 5 TEA-Cl, 5 QX-314, 2.5 Mg-ATP, and 0.3 Na-GTP, adjusted to pH 7.3 with CsOH. For current-clamp recordings, pipette solution contained (in μM) 140 K-gluconate, 10 HEPES, 0.5 EGTA, 10 NaCl, 1 $MgCl_2$, 2 Mg-ATP, 0.2 Na-GTP, and 5 QX-314, adjusted to pH 7.3 with KOH. Excitatory synaptic responses were evoked by stimulation of the Schaffer collateral input to CA1 pyramidal cells. NMDAR-mediated excitatory postsynaptic currents (EPSCs) were recorded from CA1 pyramidal cells voltage-clamped at −30 to −40 mV in the presence of the non-NMDAR antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM), and the $GABA_A$ receptor antagonist, bicuculline (10 μM) as described previously (Yamazaki et al., 2006a, b). The peptides [Src ($p60^{c\text{-}Src}$, Upstate, Charlottesville, VA, USA), Src-family activating phosphopeptide (pYEEI; Invitrogen-Biosource, Camarillo, CA, USA), Src inhibitor peptide (40–58) (custom synthesis), Fyn (Millipore, Dundee, UK and Signalchem, Richmond, Canada), Yes (Signalchem, Richmond, Canada), and Lyn (Signalchem)] were directly applied into the pyramidal cells by diffusional exchange through the patch pipettes and neuregulin 1β (NRG1β) (Shenandoah Biotechnology, Warwick, PA, USA) was bath-applied. To monitor the change of the synaptic NMDAR-mediated EPSCs, the mean amplitudes recorded 30–35 min after establishment of whole-cell configuration in the absence or presence of peptide were calculated and expressed as a percentage of the mean amplitudes during the first 5–10 min (baseline responses). When current responses did not stabilize within 10 min after achievement of whole-cell configuration, the experiments were stopped and the data were discarded.

AMPAR-mediated excitatory postsynaptic potentials (EPSPs) were recorded from current-clamped pyramidal cells to monitor LTP induction in the presence of 10 μM bicuculline using hippocampal slices with a surgical cut made between the CA1 and CA3 regions as described previously (Yamazaki et al., 2006b), and the intensity of stimulation was set to produce about 30% of maximum responses without epileptic burst. To evaluate the magnitude of Src- or Fyn-induced LTP, the mean values of the slopes of EPSPs from 25–30 min were calculated and expressed as a percentage of the mean baseline EPSP slopes from 0–5 min. To evaluate the magnitude of tetanus-induced LTP, the mean values of the slopes of EPSPs from 55–60 min post-tetanus were calculated and expressed as a percentage of the mean EPSP slopes during 5 min before delivery of the tetanus. To induce LTP, a tetanus consisting of 100 pulses at 100 Hz was delivered. Series resistances were monitored throughout the experiments by application of hyperpolarizing pulses through the patch pipette; if the series resistances changed more than 20%, the experiment was stopped and the data were discarded. Responses of currents and potentials were recorded using Axopatch-200B or Axoclamp-2B (Axon Instruments, Union City, CA, USA), and stored in a computer after conversion by an analog–digital converter (PCI-6023E; National Instruments, Austin, TX, USA, or Digidata 1200; Axon Instruments).

2.3. Statistical analysis

Because initial data obtained from electrophysiological recordings were indistinguishable between male and female rats, all data were combined for statistical analysis. Also, naive

and PBS-treated control rats yielded equivalent results, thus data obtained from those groups were combined for statistical analysis. Data were analyzed off-line using a wave-analyzing program developed by ourselves and Origin (OriginLab, Northampton, MA, USA). Data are expressed as the mean ± SEM. Sample size n refers to the number of CA1 pyramidal cells recorded in hippocampal slices prepared from at least three different rats. For statistical analysis, differences between means were compared using Student's *t*-test. A comparison was considered statistically significant if $P < 0.05$. Sample size *n* refers to the number of neurons analyzed in electrophysiological recordings from hippocampal slices from different rats.

2.4. Drugs

Most chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). NVP-AAM077 was a generous gift from Novartis Pharma AG (Basel, Switzerland).

3. Results

3.1. NRG1β prevents Src-family-kinase-mediated enhancement of NMDAR responses in naive, but not nicotine-exposed, rats

At the hippocampal CA3-CA1 pathway, activity-dependent release of NRG1 from presynaptic CA3 pyramidal cells activates postsynaptic ErbB4 receptors (Law et al., 2004; Okada and Corfas, 2004). NRG1β, a soluble form of NRG1, has been used to mimic the increased NRG1-ErbB4 signaling induced by schizophrenia (Pitcher et al., 2011). NRG1β application was shown to have no effect on basal NMDAR responses in CA1 pyramidal cells, but prevented the Src-mediated enhancement of NMDAR function (Pitcher et al., 2011). Thus, we compared the impact of NRG1β on Src-mediated enhancement of NMDAR function in CA1 pyramidal cells of naive and nicotine-exposed rats. We activated endogenous Src via intracellular application of Src-family-kinase (SFK)-activating pYEEI peptides (100 μM) through a patch pipette, and simultaneously recorded the pYEEI-mediated potentiation of NMDAR EPSCs in the absence and presence of NRG1β (2 nM). In naive pyramidal cells, we confirmed the previous finding (Lu et al., 1998; Yamazaki et al., 2006b; Pitcher et al., 2011) that pYEEI caused the enhancement of NMDAR-mediated EPSCs, which reached a maximum within 30 min (Fig. 1A; 152.5 ± 11.7%, n = 5, $t_4 = 4.58$, p = 0.010). This effect was prevented in the presence of NRG1β (Fig. 1B; 108.5 ± 4.6%, n = 9, $t_8 = 1.84$, p = 0.11). However, in nicotine-exposed cells, although pYEEI-mediated enhancement of NMDAR responses similarly occurred (Fig. 1C; 128.4 ± 7.1%, n = 5, $t_4 = 7.29$, p = 0.0019), NRG1β failed to suppress the effect (Fig. 1D; 123.9 ± 4.9%, n = 7, $t_6 = 5.36$, p = 0.0017). These results suggest that chronic nicotine exposure counteracts the effect of increased NRG1-ErbB4 signaling.

3.2. Src selectively enhances GluN2B-NMDAR responses in naïve rats

The SFKs - Src, Fyn, Yes, Lyn, and Lck - share a common domain structure and thus are all activated by the SH2 domain ligand pYEEI (Salter and Kalia, 2004). Thus, NRG1β's inability to block pYEEI-mediated enhancement of NMDAR responses in nicotine-exposed hippocampi could be due to the pYEEI-mediated activation of other SFKs that enhances NMDAR-mediated responses. Therefore, we examined whether Src and other Src family

kinases specifically modulate the two predominant NMDAR subtypes, GluN2A-NMDAR and GluN2B-NMDAR. To begin, we pharmacologically isolated GluN2A- and GluN2B-NMDAR responses in pyramidal cells of naïve rats using the GluN2B-selective antagonist ifenprodil (3 μM) and the GluN2A-selective antagonist NVP-AAM077 (50 nM), respectively, then tested whether Src enhanced the isolated responses. We found that intracellular application of exogenous Src (30 units/ml) potentiated GluN2B-NMDAR EPSCs (Fig. 2A; 148.5 ± 12.9%, n = 7, t_6 = 4.07, p = 0.0066), but not GluN2A-NMDAR EPSCs (Fig. 2B; 99.8 ± 2.6%, n = 7, t_6 = 0.062, p = 0.95). There is a unique domain (amino acids 40–70) that is poorly conserved among SFKs (Salter and Kalia, 2004). In Src, this domain is important in anchoring the kinase to the NMDAR complex through the adaptor protein NADH dehydrogenase subunit 2 (Gingrich et al., 2004), and a Src-interfering peptide corresponding to this region, Src (40–58), prevents Src-mediated enhancement of NMDAR function (Lu et al., 1998). Thus, to test whether other SFKs enhance the function of GluN2A- and GluN2B-NMDARs, we co-applied pYEEI with Src-interfering peptides (25 μg/ml) and found that co-application had no effect on either GluN2A- (Fig. 2D; 102.3 ± 5.2%, n = 6, t_5 = 0.63, p = 0.55) or GluN2B-NMDAR responses (Fig. 2C; 99.2 ± 7.9%, n = 5, t_4 = 0.23, p = 0.83). These observations demonstrate that in naive pyramidal cells, Src selectively enhances GluN2B-NMDAR-mediated responses and other SFKs have no effect on either GluN2A- or GluN2B-NMDAR responses.

3.3. Fyn selectively enhances GluN2A-NMDAR responses in nicotine-exposed rats

To understand how chronic nicotine treatment might alter the impact of NRG1β, we next investigated the role of Src and other SFKs in modulating GluN2A- and GluN2B-NMDAR function. Unlike in naive hippocampi, in nicotine-exposed hippocampi when exogenous Src (30 units/ml) was applied directly into the pyramidal cells while NMDAR EPSC were monitored simultaneously, Src had no effect on either GluN2B- (Fig. 3A; 96.4 ± 6.7%, n = 4, t_3 = 0.71, p = 0.58) or GluN2A-NMDAR responses (Fig. 3B; 107.4 ± 5.3%, n = 5, t_4 = 1.29, p = 0.27). Because nicotine treatment enhances GluN2B-NMDAR function by upregulating Src (Yamazaki et al., 2006b; Ishibashi et al., 2014), the lack of exogenous Src effect on GluN2B-NMDAR responses is likely due to saturated stimulation of endogenous Src by nicotine exposure, occluding further enhancement of GluN2B-NMDAR responses by exogenous Src.

To investigate the involvement of other SFKs, we then examined the effect of co-application of pYEEI with Src-interfering peptides on GluN2A- and GluN2B-NMDAR responses. We found that the co-application had no effect on GluN2B-NMDAR responses (Fig. 3C; 112.7 ± 10.2%, n = 6, t_5 = 1.80, p = 0.15), but enhanced GluN2A-NMDAR responses (Fig. 3D; 138.9 ± 14.4%, n = 5, t_4 = 2.88, p = 0.044).

Thus, our results indicate that chronic nicotine exposure recruits other SFKs to the enhancement of GluN2A-NMDAR responses.

To identify the SFK that is recruited by nicotine exposure, we examined whether intracellular application of active Fyn, Yes or Lyn potentiated GluN2A-NMDAR responses in nicotine-exposed pyramidal cells. We found that applying Fyn (5–10 μg/ml), which had no effect on GluN2B-NMDAR function (Fig. 4A; 110.2 ± 5.0%, n = 4, t_3 = 1.48, p = 0.23),

enhanced GluN2A-NMDAR responses (Fig. 4B; 174.1 ± 15.4%, n = 5, t_4 = 4.77, p = 0.0089), whereas applying Yes (1 μg/ml; Fig. 4C; 105.4 ± 9.2%, n = 3, t_2 = 0.63 p = 0.59) or Lyn (1 μg/ml; Fig. 4D; 104.6 ± 9.0%, n = 4, t_3 = 0.70, p = 0.53) had no effect on GluN2A-NMDAR responses. Thus, in nicotine-exposed pyramidal cells, a Fyn signaling pathway becomes operational for the selective enhancement of GluN2A-NMDAR responses. This strongly suggests that the pYEEI-induced potentiation of NMDAR responses that is observed in nicotine-exposed hippocampi (Fig. 1C) is due to the enhancement of GluN2A-NMDAR responses via Fyn activation. Under the conditions used, Yes and Lyn had no effect on GluN2A-NMDAR responses. Thus, higher concentrations of Yes and Lyn were also applied. However, adding more enzyme-containing solution to the intracellular recording solution altered membrane properties of cells being recorded. Thus, we were unable to exclude the possibility that higher concentrations of Yes and Lyn also enhance GluN2A-NMDAR responses.

3.4. NRG1β blocks Src-mediated GluN2B-NMDAR upregulation in naive rats, but has no effect on both Fyn-mediated GluN2A-NMDAR upregulation and enhanced basal GluN2B-NMDAR responses in nicotine-exposed rats

The above results suggest that NRG1β selectively blocks Src-mediated enhancement of GluN2B-NMDAR responses in naive rats, but has no effect on Fyn-mediated enhancement of GluN2A-NMDAR responses in chronic nicotine-exposed rats. To confirm selectivity of NRG1β's effect, we recorded GluN2B-NMDAR EPSCs in naive rats and examined whether NRG1β blocks pYEEI-induced enhancement of GluN2B-NMDAR responses. We found that in the presence of NRG1β, pYEEI, which activates endogenous Src, failed to enhance GluN2B-NMDAR EPSCs (Fig. 5A; 108.3 ± 5.7%, n = 7, t_6 = 1.54, p = 0.17). We then tested whether in nicotine-exposed rats NRG1β blocks Fyn-induced enhancement of GluN2A-NMDAR responses and found that in the presence of NRG1β, Fyn still enhanced GluN2A-NMDAR responses (Fig. 5B; 181.3 ± 14.9%, n = 8, t_7 = 4.58, p = 0.0026).

Chronic nicotine-exposed pyramidal cells show enhanced basal GluN2B-NMDAR EPSCs as compared to control pyramidal cells (Yamazaki et al., 2006a, b; Ishibashi et al., 2014). This effect of chronic nicotine is mediated by Src upregulation (Yamazaki et al., 2006a, b). Because NRG1β blocks Src activity (Pitcher et al., 2011), we also examined whether NRG1β affects enhanced basal GluN2B-NMDAR EPSCs. We found that NRG1β had no effect on basal GluN2B-NMDAR responses (Fig. 5C; 101.4 ± 2.5%, n = 7, t_6 = 0.34, p = 0.55), suggesting that the enhanced basal GluN2B-NMDAR responses by Src upregulation is insensitive to NRG1β. Thus, our results indicate that nicotine exposure likely counteracts NMDAR hypofunction by the two different mechanisms, enhancing GluN2B-NMDAR function and recruiting Fyn for GluN2A-NMDAR upregulation.

3.5. Src induces LTP via the enhancement of GluN2B-NMDAR responses in naive rats

LTP-inducing stimulation increases Src activity and blocking Src activity suppresses LTP in CA1 pyramidal cells (Lu et al., 1998). Src-enhanced NMDAR currents initiate Ca^{2+}-mediated potentiation of AMPAR synaptic responses in CA1 pyramidal cells (Lu et al., 1998; Ali and Salter, 2001; Salter and Kalia, 2004), occluding stimulation-induced LTP. This LTP is blocked by NRG1β (Pitcher et al., 2011). Based on the above findings, we predict

that Src-mediated potentiation of GluN2B-NMDAR function contributes to LTP induction in naive rats. This could be important to understanding the mechanism of NRG1β-mediated suppression of LTP, because NRG1β inhibits Src-mediated enhancement of GluN2B-NMDAR function. Therefore, we recorded AMPAR-mediated EPSPs in the presence of the GluN2A-selective antagonist NVP-AAM077 (50 nM) in hippocampal slices from PBS-treated control rats. We found that during recordings in which exogenous Src (30 units/ml) was delivered into pyramidal cells, the slope of AMPAR-EPSPs gradually increased to the maximum level within 25–30 min (Fig. 6A; 190.2 ± 27.2%, n = 6, t_5 = 3.39, p = 0.019). We then delivered LTP-inducing tetanic stimulation and found no further potentiation (Fig. 6A; 115.6 ± 45.9%, n = 6; t_5 = 1.61, p = 0.17). Thus, exogenous Src-induced LTP occludes further potentiation by tetanus. To determine whether Src contributes to LTP induction exclusively through the enhancement of GluN2B-NMDAR responses, we repeated experiments in the presence of the GluN2B-selective antagonist ifenprodil (3 μM). We found that intracellular application of Src had no effect on AMPAR-EPSPs (Fig. 6B; 98.1 ± 5.9%, n = 6, t_5 = 0.77, p = 0.48). However, when tetanic stimulation was delivered, LTP was induced (Fig. 6B; 148.6 ± 8.5%, n = 6, t_5 = 5.32, p = 0.0031). These findings suggest that Src contributes to LTP exclusively via enhancement of GluN2B-NMDAR responses, and therefore that the suppressive effect of NRG1β on LTP (Pitcher et al., 2011) is due to its prevention of Src-mediated enhancement of GluN2B-NMDAR responses.

3.6. Fyn induces LTP via the enhancement of GluN2A-NMDAR responses in nicotine-exposed hippocampi

We next examined whether Fyn-mediated enhancement of GluN2A-MDAR function induces LTP in nicotine-exposed hippocampi. We delivered Fyn into pyramidal cells while recording AMPAR-EPSPs in the presence of ifenprodil. We found that AMPAR-EPSPs gradually increased to the maximum level within 20–30 min (Fig. 6D; 163.7 ± 9.8%, n = 7, t_6 = 5.64, p = 0.0013). We then delivered titanic stimulation and found that there was no further potentiation (Fig. 6D; 96.7 ± 12.3%, n=7, t_6 = 0.92, p = 0.39). To determine whether Fyn contributes to LTP via the enhancement of GluN2B-NMDAR responses, we repeated experiments in the presence of NVP-AAM077 (50 nM) and found that delivery of Fyn had no effect on AMPAR-EPSPs (Fig. 6C; 109.7 ± 7.2%, n = 6, t_5 = 1.48, p = 0.20). However, when tetanic stimulation was delivered, LTP was induced (Fig. 6C; 118.2 ± 10.1%, n = 6, t_5 = 3.28, p = 0.022). Our findings indicate that in chronic-nicotine-exposed pyramidal cells Fyn contributes to LTP exclusively via enhancement of GluN2A-NMDAR responses and, in addition, tetanus-induced GluN2B-NMDAR activation alone induces LTP. Because NRG1β has no effect on basal GluN2B-NMDAR responses and Fyn-mediated potentiation of GluN2A-NMDAR responses, these observations suggest that NRG1 signaling in CA1 pyramidal cells has no effect on LTP in nicotine-exposed hippocampi.

4. Discussion

Src and Fyn are a crucial point of convergence for signaling pathways that regulate NMDAR-mediated responses (Ali and Salter, 2001; Trepanier et al., 2012; Salter and Kalia, 2004). Previous studies show that Src and Fyn selectively upregulate GluN2A-NMDAR and GluN2B-NMDAR responses in mice, respectively (Yang et al., 2012). However, the present

study demonstrates that Src enhances GluN2B-NMDAR responses in CA1 pyramidal cells of naive rats, and that NRG1β application prevents it. These findings are consistent with the previous observations that NRG1-ErbB4 signaling inhibits Src kinase activity and Src-mediated GluN2B tyrosine phosphorylation (Pitcher et al., 2011). Our current study does not provide any evidence that ErbB4 is the downstream NRG1 receptor mediating the effect. However, it was previously demonstrated that the effect of NRG1β is absent in ErbB4 knockout mice and is blocked by the ErbB4 inhibitor AG1478 (Pitcher et al., 2011), suggesting that direct stimulation of ErbB4 on pyramidal cells mediates the effect. This is paradoxical in light of compelling evidence that ErbB4 expression is very low or absent in pyramidal cells and is largely restricted to GABAergic interneurons (Vullhorst et al., 2009; Neddens and Buonanno, 2010; Fazzari et al., 2010). However, because NMDAR-EPSCs were recorded in the presence of the $GABA_A$ receptor antagonist bicuculline, it is unlikely that the observed effect is mediated indirectly by NRG1β-stimulated ErbB4 signaling in GABAergic neurons. Perhaps, very low levels of synaptic ErbB4 in pyramidal cells play a role in the effect. Alternatively, because it is known that NRG1 acutely increases extracellular dopamine levels in the hippocampus via ErbB4 activation (Kwon et al., 2008), it is possible that the effect of NRG1β is mediated by a novel dopamine-dependent mechanism without requiring pyramidal cell ErbB4.

NRG1β has no effect on basal NMDAR-mediated synaptic responses in naive CA1 pyramidal cells, but prevents endogenous Src activation-induced potentiation of NMDAR EPSCs (Pitcher et al., 2011), suggesting that Src is a downstream target of ErbB4. The interaction of post-synaptic density protein 95 kD (PSD-95) and Src SH2 domain inhibits Src kinase activity (Kalia et al., 2006) and NRG1-ErbB4 signaling depends on ErbB4 interaction with PSD-95 (Huang et al., 2000; Xie et al., 2007; Murphy and Bielby-Clarke, 2008) that increases in schizophrenia subjects (Hahn et al., 2006). Thus, ErbB4 might interact with the Src SH2 domain via PSD-95, causing the inhibition of Src and preventing the binding of pYEEI to the Src SH2 domain. While the interaction of Fyn SH2 domain with PSD-95 does not inhibit kinase activity (Tezuka et al., 1999). This might be why NRG1β has no effect on Fyn-induced enhancement of GluN2A-NMDAR function in nicotine-exposed pyramidal cells, although Fyn is a downstream target of ErbB4 (Bjarnadottir et. al., 2007).

Chronic nicotine exposure causes the sustained activation of Src, leading to enhanced GluN2B-NMDAR function (Yamazaki et al., 2006b). It is currently unknown which nAChR subtype mediates the effect of nicotine. As discussed in our previous paper (Ishibashi et al., 2014), the effect of nicotine is secondary to increased release of ACh via the activation of nAChRs and involves m1 muscarinic receptor activation by ACh. This activation of m1 muscarinic receptors is required for the sustained enhancement of GluN2B-NMDAR responses. Thus, any nAChR subtype that triggers the release of ACh from cholinergic terminals would initiate the signaling cascade. Our current study shows that NRG1 signaling has no effect on enhanced basal GluN2B-NMDAR-mediated responses in nicotine-exposed rats. Because Src needs to be anchored within the NMDAR complex through the adaptor protein NADH dehydrogenase subunit 2 for upregulation of NMDAR activity (Gingrich et al., 2004), nicotine-activated Src is most likely associated with GluN2B-NMDAR complexes. Thus, the current observation suggests that Src that is associated with the GluN2B-NMDAR complex is inaccessible to acute inhibition by NRG1-ErbB4 signaling,

providing the mechanism by which nicotine exposure counteracts the effect of NRG1-ErbB4 signaling on GluN2B-NMDAR responses.

Our current study also demonstrates that chronic nicotine exposure recruits Fyn for the enhancement of GluN2A-NMDAR responses. Although Fyn has been shown to phosphorylate both GluN2A and GluN2B (Tezuka et al., 1999; Yang et al., 2012; Trepanier et al., 2012), it remains unknown how nicotine exposure recruits Fyn for the selective enhancement of GluN2A-NMDAR responses. α7 nAChRs can interact with both GluN2A-NMDARs (Li et al., 2012) and Fyn (Kihara et al., 2001), and α7 nAChR signaling converges with GluN2A-NMDAR signaling after chronic nicotine exposure (Guan et al., 2006; Galvez et al., 2016). Thus, activation of α7 nAChRs during nicotine exposure might recruit Fyn in the postsynaptic density. Because the interaction of Fyn with PSD-95 can induce tyrosine phosphorylation of GluN2A (Tezuka et al., 1999), it is possible that PSD-95 serves as an activator scaffold protein in the GluN2A-NMDAR complex, allowing Fyn to enhance GluN2A-NMDARresponses via GluN2A phosphorylation.

NRG1-ErbB4 signaling has been found to suppress or reverse stimulation-induced LTP at CA3-CA1 synapses (Pitcher et al., 2008, 2011; Huang et al., 2000; Kwon et al., 2005, 2008). Accumulating evidence suggests that NRG1β-stimulated ErbB4 signaling in GABAergic and/or dopaminergic neurons indirectly contributes to the effect (Chen et al., 2010; Shamir et al., 2012; Kwon et al., 2008). However, it has been shown that Src-enhanced NMDAR currents initiate Ca^{2+}-mediated potentiation of AMPAR synaptic responses in CA1 pyramidal cells (Lu et al., 1998; Ali and Salter, 2001; Salter and Kalia, 2004) and this LTP is blocked by NRG1β in the presence of the $GABA_A$ receptor antagonist bicuculline (Pitcher et al., 2011). This suggests that the suppressive effect of NRG1β on Src-induced LTP is not mediated by ErbB4 activation in GABAergic neurons. Although the relative contributions of GluN2A- and GluN2B-NMDAR subtypes to LTP induction have been highly controversial, either subtype is capable of supporting LTP induction (Shipton and Paulsen, 2013). Our current results suggest that Src-induced enhancement of GluN2B-NMDAR responses contributes to LTP in naive CA1 pyramidal cells and, thus, NRG1-ErbB4 signaling likely suppresses LTP by blocking Src-mediated enhancement of GluN2B-NMDAR responses in the naive hippocampus. There is evidence that increased synaptic GluN2B-NMDARs facilitate LTP and improve hippocampus-dependent memory (Tang et al., 1999; Cao et al., 2007; Wang et al., 2009; Shipton and Paulsen, 2013). Indeed, chronic nicotine exposure, which results in increased GluN2B-NMDAR function and LTP facilitation (Yamazaki et al., 2006a), improves hippocampus-dependent memory (Levin et al., 1992; Kenney and Gould, 2008). Interestingly, LTP induced in nicotine-exposed hippocampi is long-lasting that is GluN2B-NMDAR-dependent and protein synthesis-independent (Yamazaki et al., 2006a). The long-lasting nature of LTP might imply a highly selective role of GluN2B-NMDARs in long-term memory. Therefore, it is possible that the suppressive effects of NRG1-ErbB4 signaling on LTP via blocking Src-mediated enhancement of GluN2B-NMDAR responses is linked to cognitive impairment in schizophrenia. Our present study also demonstrates that Fyn induces LTP via GluN2A-NMDAR activation in chronic-nicotine-exposed rats. Thus, our results demonstrate that Src and Fyn stimulate distinct signaling pathways to induce LTP, and suggest that LTP induction mediated by these pathways in nicotine-exposed hippocampi is not affected by NRG1-ErbB4 signaling.

Because the NMDAR and LTP are a critical factor for learning and memory, the effects of nicotine exposure observed might explain why individuals suffering from schizophrenia have a particularly high rate of heavy smoking. Nicotine exposure may also have impact on negative affective symptoms of schizophrenia, as the SFK-induced upregulation of GluN2B-NMDAR function is implicated in the regulation of depression-like behavior in animals (Ohnishi et al., 2010). Thus, it is possible that the nicotine-initiated signaling cascade also converges with the schizophrenia-linked signaling for mood regulation, counteracting negative affective symptoms of schizophrenia.

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Abbreviations

ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor
AP5	2-amino-5-phosphonovaleric acid
DNQX	6,7-dinitroquinoxaline-2,3-dione
EPSCs	excitatory postsynaptic currents
EPSPs	excitatory postsynaptic potentials
LTP	long-term potentiation
nAChR	nicotinic acetylcholine receptors
NMDAR	*N*-methyl-D-aspartate receptor
NRG1	neuregulin 1
NRG1β	neuregulin 1β
PBS	phosphate-buffered saline
PSD-95	post-synaptic density protein 95 kD
SFK	Src-family kinase

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- Src selectively enhances GluN2B-NMDAR responses in na◆ve rats
- NRG1βblocks Src-mediated enhancement of GluN2B-NMDAR responses in naive rats
- NRG1βhas no effect on basal GluN2B-NMDAR responses in nicotine-exposed rats
- Fyn selectively enhances GluN2A-NMDAR responses in nicotine-exposed rats
- NRG1βhas no effect on Fyn-mediated enhancement of GluN2A-NMDAR responses

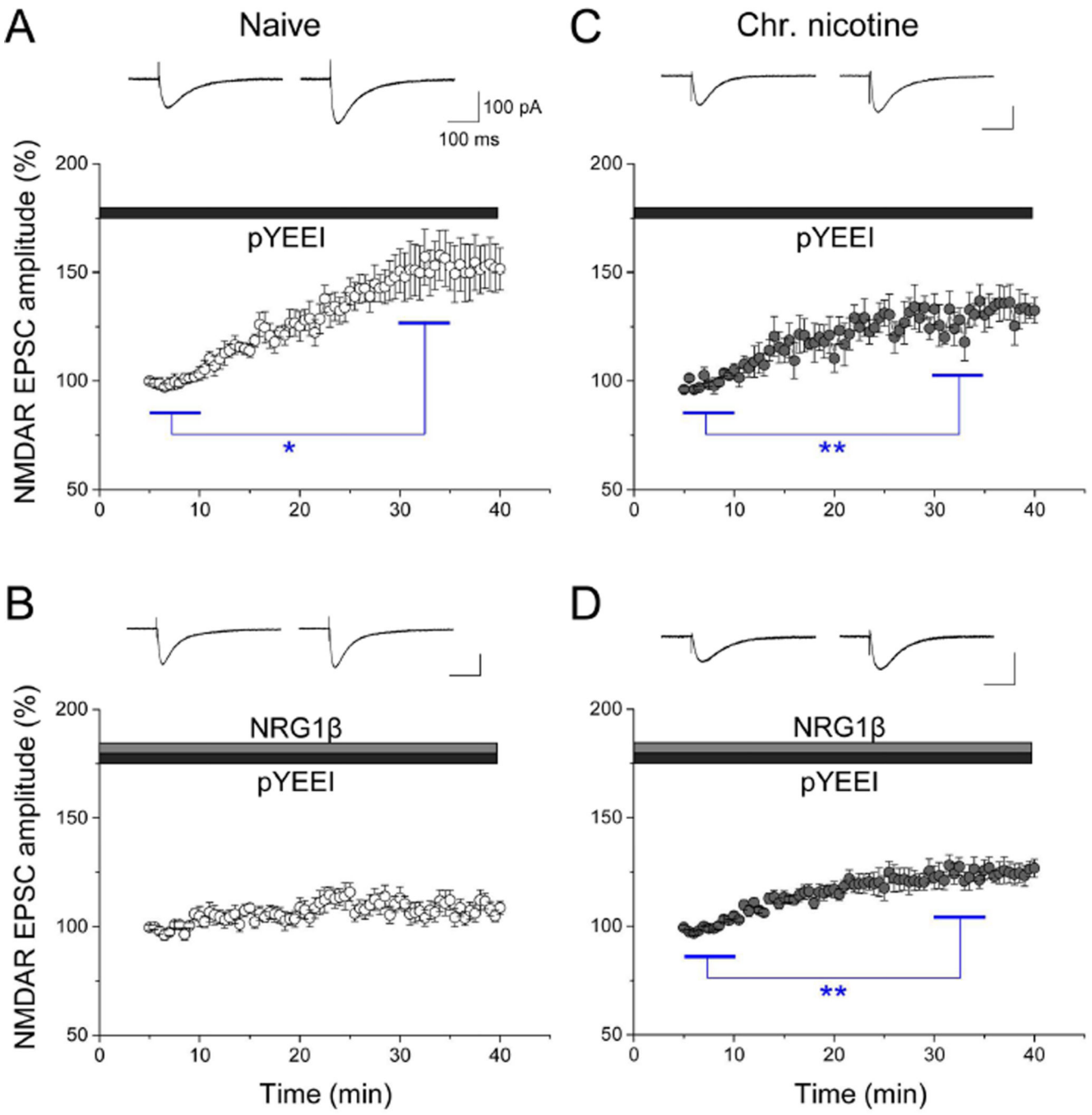

Figure 1. Chronic nicotine exposure prevents the suppressive effect of NRG1βon pYEEI-mediated enhancement of NMDAR function

NMDAR EPSCs evoked by stimulation of the Schaffer collateral afferent were recorded in CA1 pyramidal cells from naive (A, B) and chronic-nicotine-exposed (C, D) rats. Time course of the effect of intracellular application of pYEEI through a patch pipette on NMDAR EPSCs in the absence (A, C) or presence (B, D) of NRG1β (2 nM) is shown as a percent change in amplitude (mean ± SEM). Amplitudes of NMDA EPSCs during the first 5–10 min and the period from 30 to 35 min were used to calculate a percent change for statistical analysis. In naive pyramidal cells, pYEEI enhances NMDAR EPSCs (A) and

NRG1β blocks the effect of pYEEI (B). In chronic-nicotine-exposed pyramidal cells, pYEEI enhances NMDAR EPSCs (C), but NRG1β has no effect on this enhancement (D). In this and the following figures, traces above the graph show representative NMDA EPSCs recorded during the first 5 to 10-min period (left) and 30 to 35-min period (right) after whole-cell configuration was established. Each trace is the average of four NMDAR EPSCs. *$p < 0.05$, ** $p < 0.01$

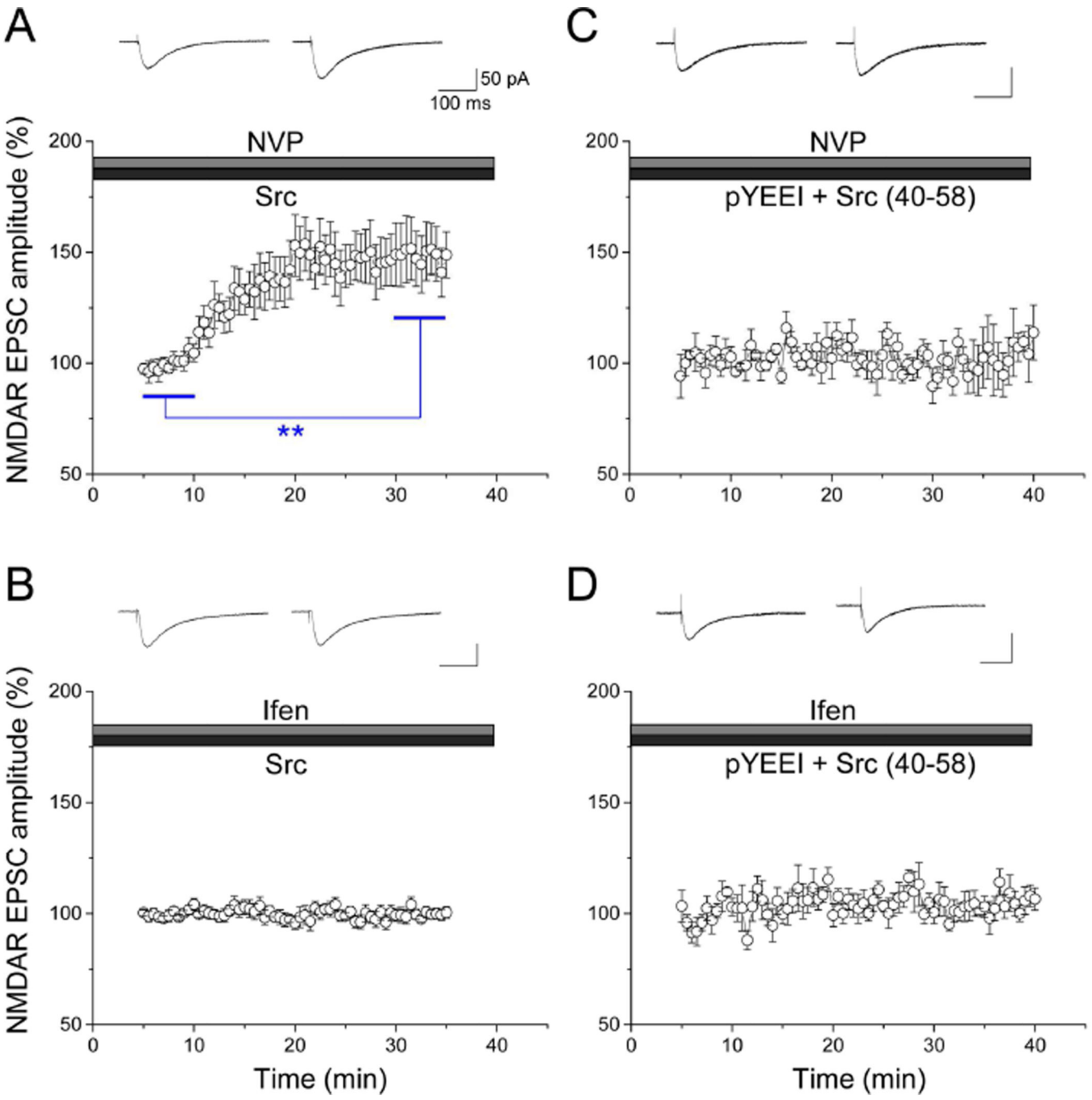

Figure 2. Src selectively enhances GluN2B-NMDAR EPSCs in CA1 pyramidal cells of naive rats

Pharmacologically isolated GluN2B-NMDAR EPSCs (A, C) and GluN2A-NMDAR EPSCs (B, D) were recorded in CA1 pyramidal cells of naive rats using the GluN2A-selective antagonist NVP-AAM077 (NVP; 50 nM) and the GluN2B-selective antagonist ifenprodil (Ifen; 3 μM), respectively. Time course of the effect of intracellular application of Src (A, B) or pYEEI + Src (40–58) peptide (C, D) through a patch pipette on GluN2B-NMDAR EPSCs (A, C) or GluN2A-NMDAR EPSCs (B, D) is shown as a percent change in amplitude (mean ± SEM). Amplitudes of GluN2B-NMDAR EPSCs (A, C) or GluN2A-NMDAR EPSCs (B,

D) during the first 5–10 min and the period from 30 to 35 min were used to calculate a percent change for statistical analysis. Src enhances GluN2B-NMDAR EPSCs (A), but not GluN2A-NMDAR EPSCs (B). Co-application of pYEEI with Src (40–58) has no effect on both GluN2B-NMDAR EPSCs (C) and GluN2A-NMDAR EPSCs (D). **$p < 0.01$

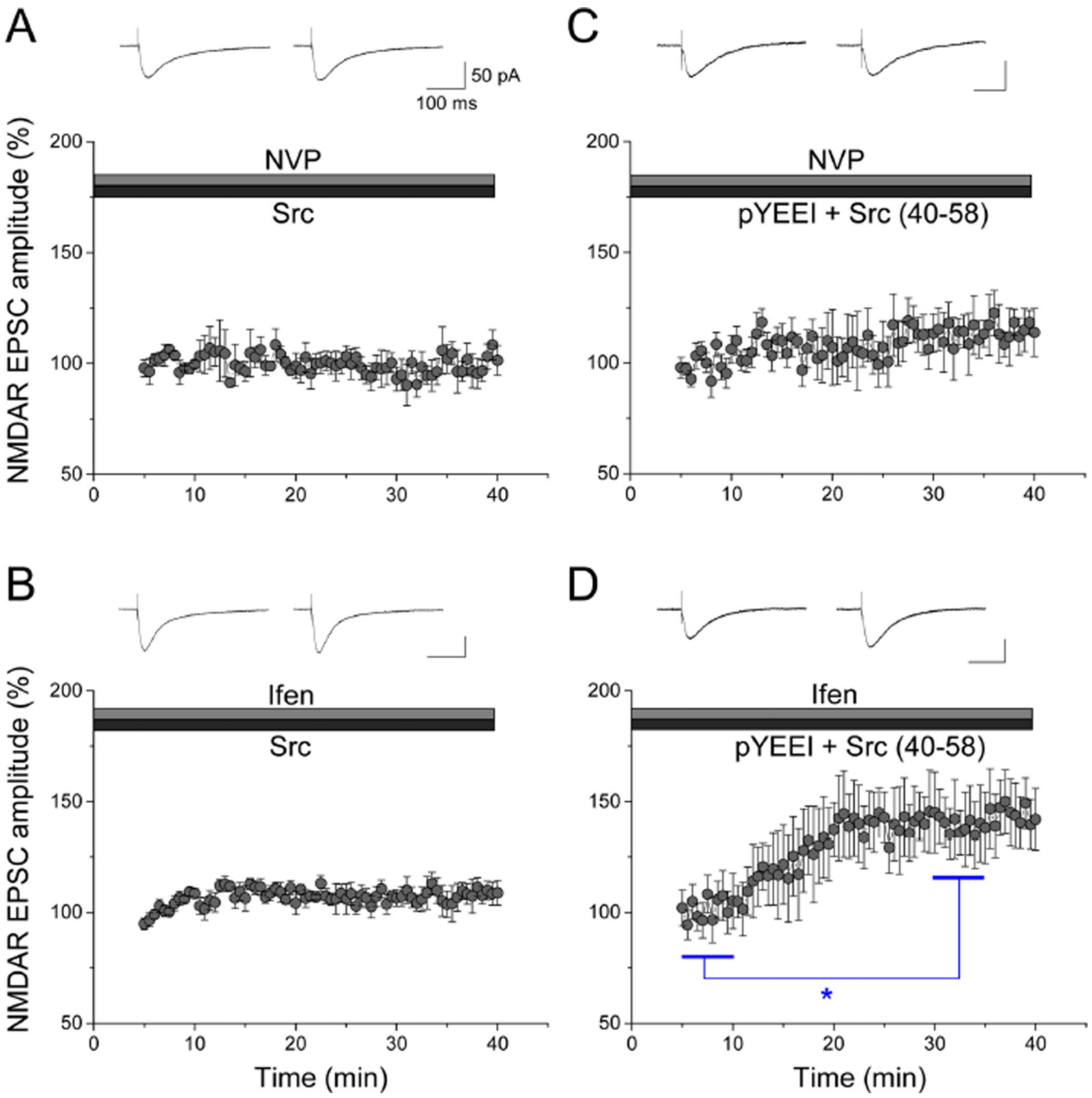

Figure 3. A Src family kinase other than Src enhances GluN2A-NMDAR EPSCs in CA1 pyramidal cells of chronic-nicotine-exposed rats

Pharmacologically isolated GluN2B-NMDAR EPSCs (A, C) and GluN2A-NMDAR EPSCs (B, D) were recorded in CA1 pyramidal cells of chronic-nicotine-exposed rats. Time course of the effect of intracellular application of Src (A, B) or pYEEI + Src (40–58) peptide (C, D) on GluN2B-NMDAR EPSCs (A, C) or GluN2A-NMDAR EPSCs (B, D) is shown as a percent change in amplitude (mean ± SEM). Amplitudes of GluN2B-NMDAR EPSCs (A, C) or GluN2A-NMDAR EPSCs (B, D) during the first 5–10 min and the period from 30 to 35 min were used to calculate a percent change for statistical analysis. Src has no effect on

both GluN2B-NMDAR EPSCs (A) and GluN2A-NMDAR EPSCs (B). Co-application of pYEEI with Src (40–58) has no effect on GluN2B-NMDAR EPSCs (C), but enhances GluN2A-NMDAR EPSCs (D). *$p < 0.05$

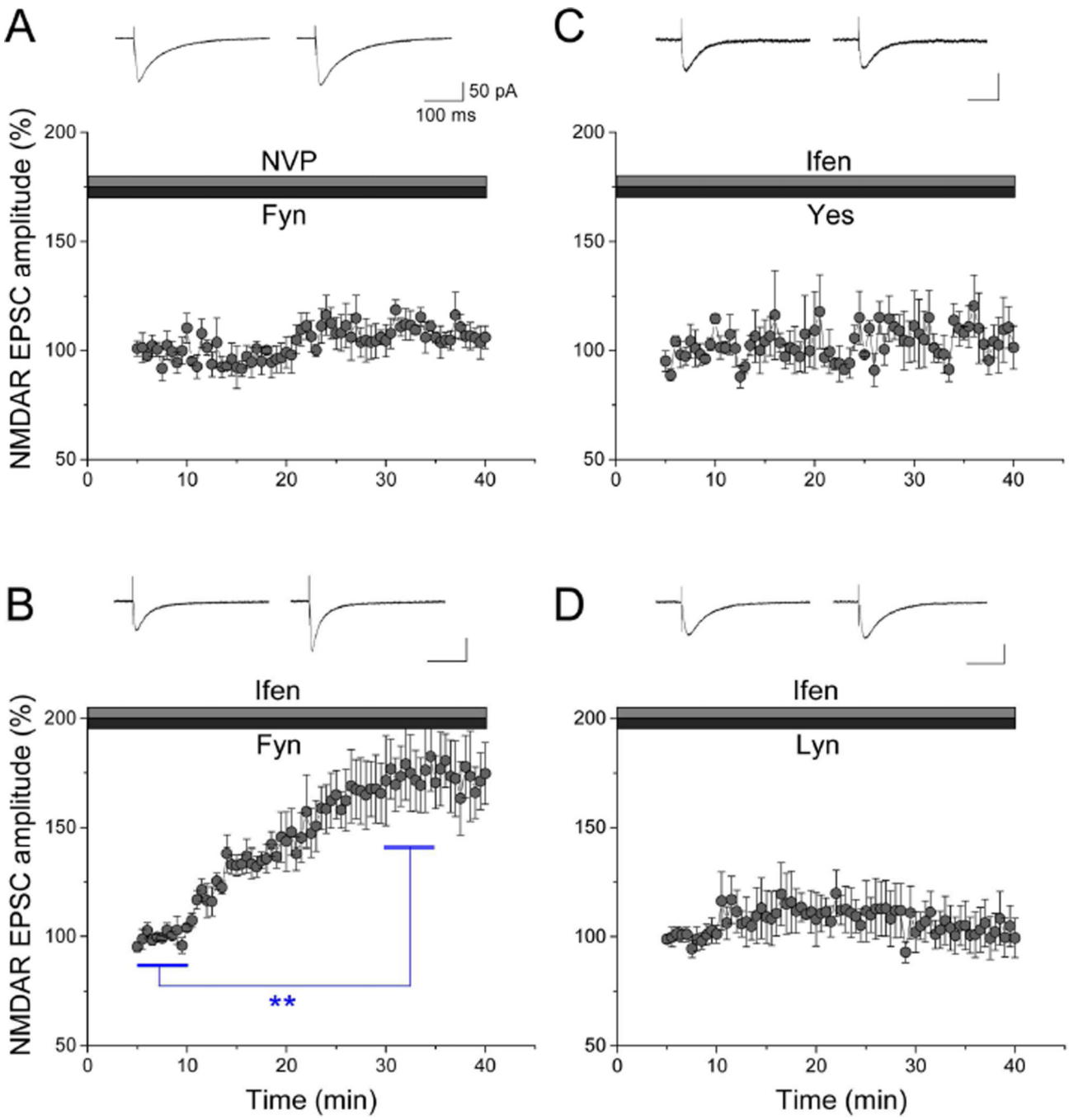

Figure 4. Fyn enhances GluN2A-NMDAR EPSCs in CA1 pyramidal cells of chronic-nicotine-exposed rats

Pharmacologically isolated GluN2B-NMDAR EPSCs (A) and GluN2A-NMDAR EPSCs (B–D) were recorded in CA1 pyramidal cells of chronic-nicotine-exposed rats. Time course of the effect of intracellular application of Fyn (A, B), Yes (C), or Lyn (D) on GluN2B-NMDAR EPSCs (A) or GluN2A-NMDAR EPSCs (B–D) is shown as a percent change in amplitude (mean ± SEM). Amplitudes of GluN2B-NMDAR EPSCs (A) or GluN2A-NMDAR EPSCs (B–D) during the first 5–10 min and the period from 30 to 35 min were used to calculate a percent change for statistical analysis. Fyn has no effect on GluN2B-

NMDAR EPSCs (A), but enhances GluN2A-NMDAR EPSCs (B). Yes (C) and Lyn (D) have no significant effect on GluN2A-NMDAR EPSCs. **p < 0.01

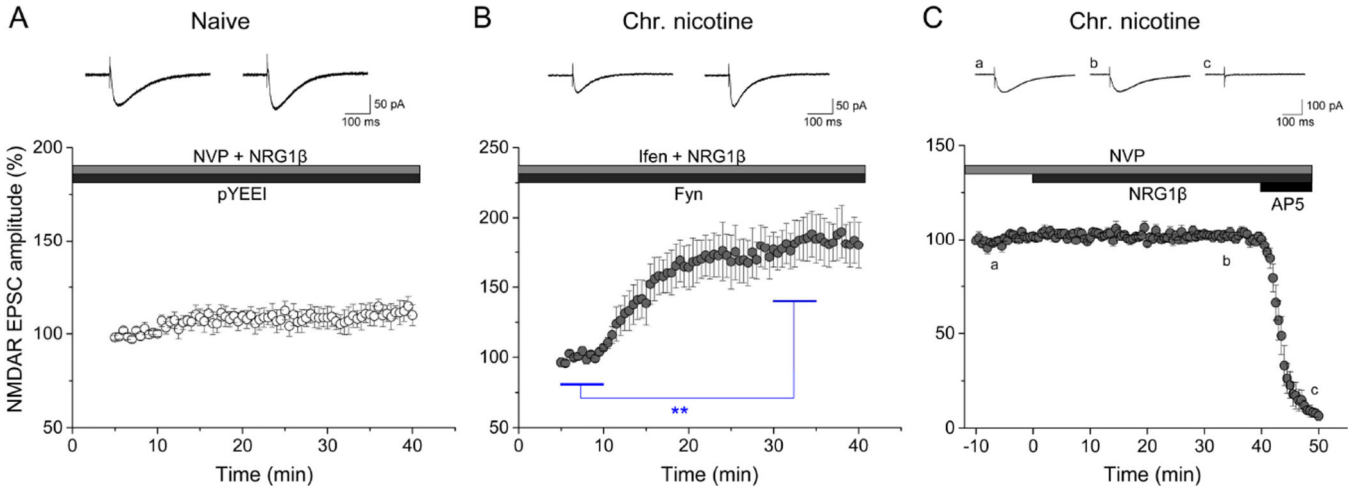

Figure 5. NRG1β blocks pYEEI-mediated enhancement of GluN2B-NMDAR EPSCs in naive rats, but has no effect on Fyn-mediated enhancement of GluN2A-NMDAR EPSCs and basal GluN2B-NMDAR-mediated EPSCs in chronic-nicotine-exposed rats

Pharmacologically isolated GluN2B-NMDAR EPSCs (A, C) and GluN2A-NMDAR EPSCs (B) were recorded in CA1 pyramidal cells of naive (A) or chronic-nicotine-exposed (B, C) rats. Time course of the effect of intracellular application of pYEEI (A) or Fyn (B) in the presence of NRG1β (2 nM) on GluN2B-NMDAR EPSCs (A) or GluN2A-NMDAR EPSCs (B) is shown as a percent change in amplitude (mean ± SEM). (C) Time course of the effect of application of NRG1β and AP5 on GluN2B-NMDAR EPSCs in chronic-nicotine-exposed pyramidal cells is shown as a percent change in amplitude (mean ± SEM). Amplitudes of GluN2B-NMDAR EPSCs (A) or GluN2A-NMDAR EPSCs (B) during the first 5–10 min and the period from 30 to 35 min were used to calculate a percent change for statistical analysis. In (C), amplitudes of GluN2B-NMDAR EPSCs during the first −10 – −5 min and the period from 30 to 35 min were used to calculate a percent change for statistical analysis. In naive rats, NRG1β blocks the pYEEI-mediated enhancement of GluN2B-NMDAR EPSCs (A). In chronic-nicotine-exposed rats, NRG1β has no effect on Fyn-mediated enhancement of GluN2A-NMDAR EPSCs (B) and basal GluN2B-NMDAR EPSCs (C), which are completely blocked by the NMDAR antagonist AP5. **$p < 0.01$

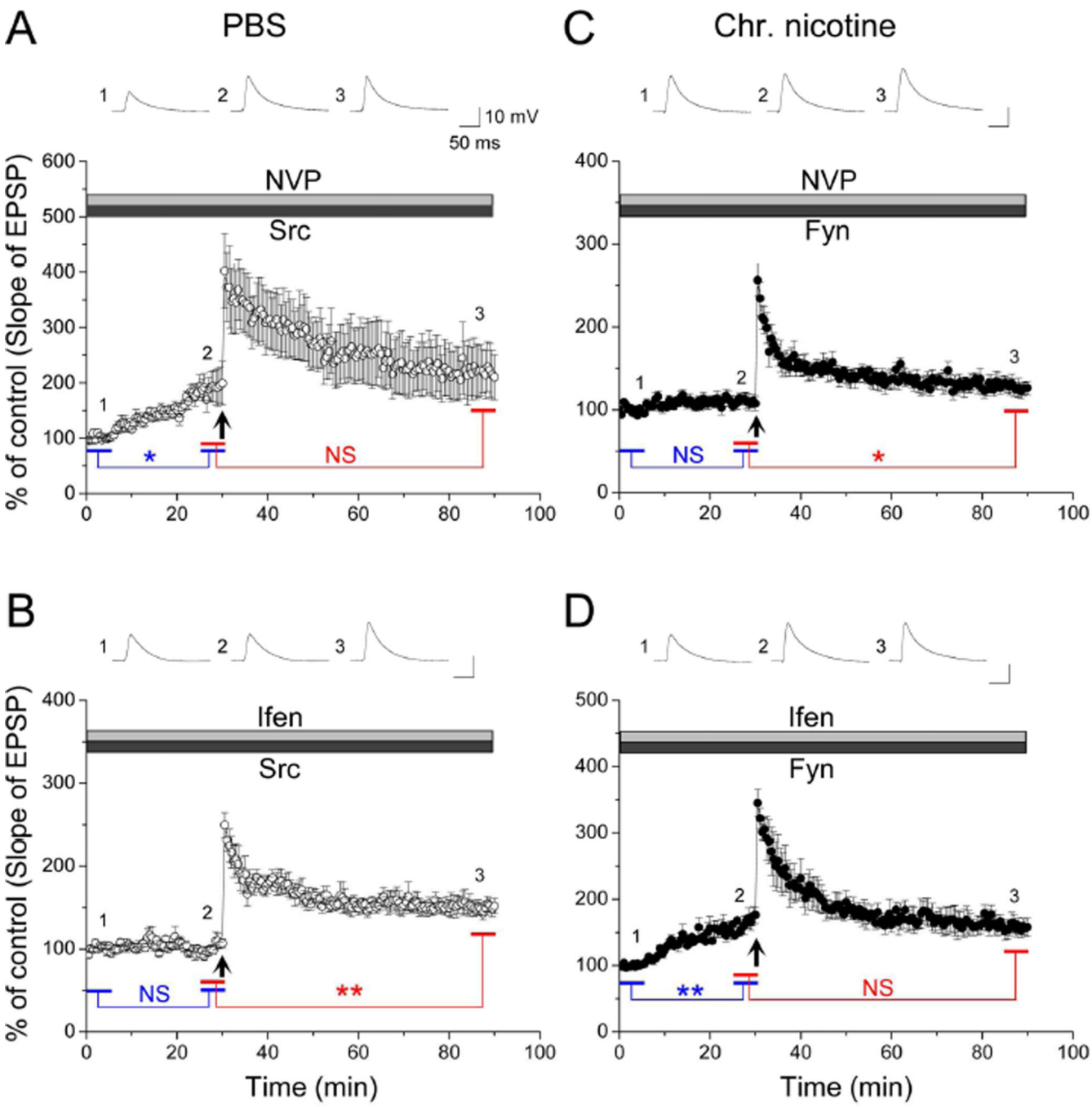

Figure 6. Src-mediated enhancement of GluN2B-NMDAR responses in PBS-treated rats and Fyn-mediated enhancement of GluN2A-NMDAR responses in chronic-nicotine-exposed rats induce LTP

AMPAR-mediated EPSPs were evoked by Schaffer collateral stimulation in the presence of bicuculline (10 μM) and recorded in current-clamped pyramidal cells from PBS- (A, B) and chronic-nicotine-treated rats (C, D). Time course of the effect of intracellular application of Src (A, B) or Fyn (C, D) in the presence of the GluN2A-selective antagonist NVP-AAM077 (NVP; A, C) or the GluN2B-selective antagonist ifenprodil (Ifen; B, D) is shown as a percent change in the slope of AMPAR-mediated EPSPs (mean ± SEM). To evaluate the

magnitude of Src-induced LTP (A, B) or Fyn-induced LTP (C, D), slopes of AMPAR-mediated EPSPs during the first 0–5 min and the period from 25 to 30 min were used to calculate a percent change for statistical analysis (blue line). Tetanic stimulation was delivered at the time indicated by the arrow. To evaluate the magnitude of tetanus-induced LTP, slopes of AMPAR-mediated EPSPs during 5 min before delivery of the tetanus and the period from 85–90 min (i.e., 55–60 min post-tetanus) were used to calculate a percent change for statistical analysis (red line). Each trace above the graph in (A–D) is the average of four EPSPs recorded at the time indicated by the numbers. In PBS-treated pyramidal cells, Src induced the enhancement of AMPAR EPSPs in the presence of NVP that occluded tetanus-induced LTP (A) and Src had no effect on AMPAR EPSPs in the presence of Ifen, but tetanus induced LTP (B). In chronic-nicotine-exposed pyramidal cells, Fyn induced the enhancement of AMPAR EPSPs in the presence of Ifen that occluded tetanus-induced LTP (D) and Fyn had no effect on AMPAR EPSPs in the presence of NVP, but tetanus induced LTP (C). *$p < 0.05$, **$p < 0.01$, NS (no significance, $p > 0.05$).