Regulation of NMDA Receptors by Neuregulin Signaling in Prefrontal Cortex

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Recent linkage studies have identified a significant association of the neuregulin gene with schizophrenia, but how neuregulin is involved in schizophrenia is primarily unknown. Aberrant NMDA receptor functions have been implicated in the pathophysiology of schizophrenia. Therefore, we hypothesize that neuregulin, which is present in glutamatergic synaptic vesicles, may affect NMDA receptor functions via actions on its ErbB receptors enriched in postsynaptic densities, hence participating in emotional regulation and cognitive processes that are impaired in schizophrenia. To test this, we examined the regulation of NMDA receptor currents by neuregulin signaling pathways in prefrontal cortex (PFC), a prominent area affected in schizophrenia. We found that bath perfusion of neuregulin significantly reduced whole-cell NMDA receptor currents in acutely isolated and cultured PFC pyramidal neurons and decreased NMDA receptor-mediated EPSCs in PFC slices. The effect of neuregulin was mainly blocked by application of the ErbB receptor tyrosine kinase inhibitor, phospho-protein kinase kinase 1. Moreover, the neuregulin effect was prevented by agents that stabilize or disrupt actin polymerization but not by agents that interfere with microtubule assembly. Furthermore, neuregulin treatment increased the abundance of internalized NMDA receptors in cultured PFC neurons, which was also sensitive to agents affecting actin cytoskeleton. Together, our study suggests that both PLC/IP3R/Ca2+ and Ras/MEK/ERK (extracellular signal-regulated kinase) signaling pathways are involved in the neuregulin-induced reduction of NMDA receptor currents, which is likely through enhancing NR1 internalization via an actin-dependent mechanism.

Key words: neuregulin; ErbB receptors; NMDA receptors; schizophrenia; internalization; actin

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Introduction

Schizophrenia is a devastating psychiatric disease with high heritability. Recent genome-wide linkage analysis and large-scale profiling of gene expression have implicated the neuregulins (NRGs) and their receptors as the potential susceptibility genes for schizophrenia (Harrison and Owen, 2003; Corfas et al., 2004). The neuregulins constitute a family of proteins coded by four distinct genes (NRG-1 to NRG-4), each of which contains an epidermal growth factor (EGF)-like domain (Holmes et al., 1992). The neuregulins signal through three receptors, ErbB2, ErbB3, and ErbB4, that are members of the EGF receptor-related family of tyrosine kinases (Fischbach and Rosen, 1997). Although NRG-1 has been associated with schizophrenia in diverse populations (Stefansson et al., 2002, 2003; Williams et al., 2003), and a significant reduction in the level of ErbB3 expression has been found in the prefrontal cortex (PFC) of schizophrenics (Hakak et al., 2001; Tkachev et al., 2003), the mechanisms underlying the involvement of NRG/ErbB in schizophrenia are unclear. One mechanism may be associated with the key role of NRG/ErbB signaling in a spectrum of neurodevelopmental processes in the CNS, including neuronal migration (Anton et al., 1997; Rio et al., 1997), regulation of transmitter receptor expression (Ozaki et al., 1997; Yang et al., 1998; Rieff et al., 1999; Liu et al., 2001), oligodendrocyte development, and myelination (Vartanian et al., 1999; Fernandez et al., 2000). The role of NRG/ErbB signaling in the adult CNS, which could also contribute to the pathophysiology of schizophrenia, is primarily unknown.

NRGs and their ErbB receptors are continually expressed in mature brain and accumulate at synapse-rich regions (Pinkas-Kramarski et al., 1994; Corfas et al., 1995; Gerecke et al., 2001), suggesting that NRGs are involved in the maintenance and/or regulation of synaptic structure and function in adult brain. Moreover, it has been found that ErbB4 associates with postsynaptic density-95 (PSD-95) and NMDA receptors (NMDARs) at PSDs of excitatory terminals (Garcià et al., 2000), and PSD-95 enhances NRG signaling by facilitating ErbB4 dimerization (Huang et al., 2000). The colocalization of NMDA and ErbB receptors at PSDs offers the potential for their interactions.

The NMDA glutamate receptor (GluR), which contains an intrinsic ligand-gated ion channel, plays a central role in the regulation of synaptic plasticity. Mounting evidence has suggested...
that glutamatergic transmission via NMDA receptors is especially involved in schizophrenia (Tsai and Coyle, 2002). Systemic administration of noncompetitive NMDA receptor antagonists produces schizophrenia-like behavioral symptoms (Jentsch and Roth, 1999). The expression of NMDA receptor subunits is altered in prefrontal cortex of schizophrenia patients (Akbarian et al., 1996). Therefore, the NMDA receptor is potentially an important target of the NRG/ErbB signaling involved in schizophrenia. In this paper, we demonstrate that NRG, by activating ErbB receptor-mediated signaling pathways, inhibits NMDAR-mediated currents through a mechanism involving the internalization of NMDA receptors that is dependent on actin cytoskeleton.

Materials and Methods


Acute-dissociation procedure and primary neuronal culture. PFC neurons from young adult (3–5 weeks postnatal) Sprague Dawley rats were acutely dissociated using procedures similar to those described previously (Wang et al., 2003; Chen et al., 2004). All experiments were performed with the approval of State University of New York at Buffalo Animal Care Committee. After incubation of brain slices in a NaHCO3-buffered saline, PFC was dissected and placed in an oxygenated chamber containing papan (0.8 mg/ml; Sigma, St. Louis, MO) in HEPS-buffered HBSS (Sigma) at room temperature. After 40 min of enzyme digestion, tissue was rinsed three times in the low-Ca2+2.2–4 M


To study the regulation of NMDAR-mediated EPSCs by NRG in PFC slices, the whole-cell voltage-clamp recording technique (Wang et al., 2003; Zhong et al., 2003) was used. Electrodes were filled with the following internal solution (in mM): 180 NaCl, 20 CsCl, 10 HEPES, 1 MgCl2, 5 EGTA, 2.2 Na2-ethyl bromide quaternary salt, 12 phosphocreatine, 5 MgATP, 0.5 NaGTP, 0.1 leupeptin, pH 7.2–7.3, 265–270 mOsm/L. The slice (300 mm) was placed in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus, Melville, NY) and submerged in continuously flowing oxygenated artificial CSF (ACSF). Cells were visualized using a 40× water-immersion lens and illuminated with near-infrared (IR) light, and the image was detected with an IR-sensitive CCD camera. A Multiclamp 700A amplifier was used for these recordings. Tight seals (2–10 GΩ) from visualized pyramidal neurons were obtained by applying negative pressure. The membrane was disrupted with additional suction, and the whole-cell configuration was obtained. The access resistance ranged from 13 to 18 MΩ and were compensated 50–70%.

For the recording of NMDAR-mediated EPSCs, cells were bathed in ACSF containing CNQX (20 μM) and bicuculline (10 μM) to block AMPA/kainate receptors and GABA(A) receptors. Evoked currents were generated with a 50 μs pulse from a stimulation isolation unit controlled by a S48 pulse generator (Astro-Med, West Warwick, RI). A bipolar stimulating electrode (FHC, Bowdoinham, ME) was positioned 60 μm from the neuron under recording. Before stimulation, cells (voltage clamped at ~70 mV) were depolarized to ~60 mV for 3 s to fully relieve the voltage-dependent Mg2+2 block of NMDAR channels. Application of the NMDAR antagonist APV (10 μM) blocked the EPSCs, indicating that these synaptic currents were indeed mediated by NMDA receptors. The Clampfit program (Molecular Devices) was used to analyze evoked synaptic currents. The amplitude of EPSC was calculated by taking the mean of a 2–4 ms window around the peak and comparing with the mean of a 4–8 ms window immediately before the stimulation artifact.

Transfection and small interfering RNA. Cultured PFC neurons [11 d in vitro (DIV)] were transfected with green fluorescent protein (GFP)-tagged dominant-negative (dn) Ras (S12→N) (Feig and Cooper, 1988) or constitutively active (ca) Ras (G12→V) (Sweet et al., 1984; Krengel et al., 1990) or cotransfected with a plasmid encoding enhanced GFP (EGFP) and a plasmid containing either the wild-type (wt) MEK1 [MAP (mitogen-activated protein) kinase kinase 1] or the dominant-negative MEK1 (carrying M substitution at K97) construct (Kim et al., 2004). Transfection was conducted with the Lipofectamine 2000 method according to the manual (Invitrogen, San Diego, CA). Two to 3 d after transfection, electrophysiological recordings were performed on the GFP-positive neurons.

To suppress the expression of calcium-calmodulin-dependent protein kinase II (CaMII) in cultured neurons, we used the small interfering RNA (siRNA), a potent agent for sequence-specific gene silencing (McManus and Sharp, 2002). The siRNA oligonucleotide sequences selected from α-CaMII mRNA were 5′-GGGAGUGAUUGCCCGAAGAUUU-3′ (sense) and 5′-AAUUCUUGGCGCAUCAUCUGUG-3′ (antisense). siRNA was synthesized by Ambion (Austin, TX) and cotransfected with EGFP into cultured PFC neurons (11 DIV) using the Lipofectamine 2000 method. Cultures were used 2–3 d after transfection.
Immunocytochemical staining. After treatment, neurons cultured on coverslips were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and washed three times with PBS. Neurons were then permeabilized with 0.1% Triton X-100 in PBS for 5 min, followed by 1 h incubation with 5% bovine serum albumin to block nonspecific staining. Next, neurons were incubated with anti-CaMKII α subunit antibody (1:200; Upstate Biotechnology, Lake Placid, NY) or anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (1:100; Cell Signaling Technology, Beverly, MA) at 4°C overnight. After washing off the primary antibodies, the cells were incubated with a rhodamine (or FITC)-conjugated secondary antibody (1:200; Sigma) for 1 h at room temperature. After washing in PBS for three times, the coverslips were mounted on slides with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Fluorescent images were obtained using a 100× objective with a cooled CCD camera mounted on a Nikon microscope.

The internalized NR1 subunit of NMDA receptors was detected as described previously (Wang et al., 2003). Briefly, surface NR1 was labeled with a polyclonal anti-NR1 extracellular domain antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) in living cells for 20 min at 37°C in the culture medium. After washing, neurons were treated with neuregulin for 5 min at 37°C. In some experiments, neurons were preincubated with various agents (20 or 60 min before adding neuregulin). Immediately after neuregulin treatment, the antibody that binds to the remaining surface NR1 was stripped off with an acid solution (0.5 M NaCl, 0.2% acetic acid) at 4°C for 4 min. Cells were then washed, fixed, permeabilized, and incubated with a monoclonal anti-NR1 antibody (1:200; Upstate Biotechnology) for 2 h at room temperature. The internalized NR1 (labeled with a polyclonal NR1 antibody) was detected with a rhodamine-conjugated anti-rabbit secondary antibody, whereas the total NR1 (labeled with a polyclonal NR1 antibody) was detected with a FITC-conjugated anti-mouse secondary antibody. Staining of internalized and total GluR1 was done in the same way. Surface GluR1 was labeled with a polyclonal anti-GluR1 extracellular domain antibody (1:50; Oncogene Sciences, Uniondale, NY) in living cells for 20 min. The total GluR1 was labeled with a monoclonal anti-GluR1 antibody (1:200; Santa Cruz Biotechnology).

Labeled cells were imaged using a 100× objective with a cooled CCD camera mounted on a Nikon microscope. All specimens were imaged under identical conditions and analyzed using identical parameters. Levels of internalized and total NR1 or GluR1 immunoreactivity on the same length of dendrites and the same area of somas in cells treated with various agents were compared using Image J software and normalized to the immunoreactivity of untreated neurons. The internalized-to-total ratio was determined by dividing the computed red fluorescence (internalized) by the green fluorescence (total). Two controls were done for measuring the levels of internalized receptors, namely: a 0 min control (cells were subjected to acid stripping immediately after antibody labeling) and 5 min control (cells were subjected to acid stripping 5 min after antibody labeling). Three to four independent experiments for each of the treatments were performed. On each coverslip, the immunofluorescence intensity of four to six neurons was quantified. For each neuron, the immunoreactivity of soma (within a 15 × 15 μm area) and three to four neurites (50 μm each) were measured. Quantitative analyses were conducted blindly (without knowledge of experimental treatment).

Immunoprecipitation and Western blotting. Cultured PFC neurons (2 × 10^6 cells) were treated with neuregulin or vehicle for 5 min and then collected and homogenized in 500 μl of lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM NaPO4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodiumorthovanadate, 1 mM PMSF, and 1 mg/ml leupeptin). Lysates were centrifuged at 4°C at 16,000 × g, and supernatant fractions were incubated with antibodies against NMDA receptor subunits (4 μl each; Upstate Biotechnology) for 1 h at 4°C, followed by incubation with protein A/G plus agarose (Santa Cruz Biotechnology) at the same condition. Immunoprecipitates were washed for three times with the lysis buffer and then boiled in 2× SDS loading buffer for 5 min and separated on 7.5% SDS-polyacrylamide gels. Western blotting was performed using the ECL method according to the manufacturer’s protocol (Amersham Biosciences, Piscataway, NJ). Antibodies used for blotting include the following: anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (1:1000), anti-NR1 (1:5000), anti-NR2A (1:1000), anti-NR2B (1:1000), and anti-phospho-tyrosine (1:1000; Santa Cruz Biotechnology). Quantitation was obtained from densitometric measurements of immunoreactive bands on films.

Results

Activation of neuregulin signaling reduces NMDAR-mediated currents in PFC pyramidal neurons

To test whether NMDA receptor is a potential target of the neuregulin signaling, we examined the effect of NRG on NMDA receptor-mediated currents in PFC pyramidal neurons. Because the structure and function of NRG-1 have been characterized extensively (Holmes et al., 1992; Ozaki et al., 1997; Yang et al., 1998), and the gene encoding NRG-1 has been identified as a potential susceptibility gene for schizophrenia (Corfas et al., 2004), we used a recombinant polypeptide containing the EGF domain of the β-type NRG-1 (hereafter referred to as NRG) in this study. Whole-cell recordings of NMDA (100 μM)-evoked ionic currents in dissociated or cultured PFC pyramidal neurons (Wang et al., 2003, Tyszkiewicz et al., 2004) were first performed. As shown in Figure 1, A and B, bath application of NRG (4 nM) reversibly reduced the amplitude of NMDAR currents. After recovery, the second application of NRG produced a similar reduction of NMDAR currents. Dose–response experiments (Fig. 1C) show that different concentrations of NRG inhibited NMDR currents to different extents (1 nM: 10.3 ± 1.2%; n = 8; 4 nM: 18.8 ± 1.0%; n = 10; 10 nM: 22.4 ± 2.7%; n = 6; 100 nM: 26.2 ± 2.0%; n = 5), and the EC50 was ∼1.2 nM. The full-length NRG-β1 protein (5 nM) gave a similar effect, inhibiting NMDAR currents by 17.3 ± 0.5% (n = 7).

Because NRG receptors (ErbB2–4) are tyrosine kinases, we then examined whether the broad-spectrum tyrosine kinase inhibitor genistein or the more specific ErbB inhibitor 4-[3-(bromophenyl)amino]-6-(methylamino)-pyrido[3,4-d]pyrimidine (PD158780) (Fry et al., 1997) was able to block the NRG effect on NMDAR currents. As shown in Figure 1D, in the presence of genistein (20 μM) or PD158780 (1 μM), NRG (4 nM) failed to suppress NMDAR currents. In a sample of freshly isolated and cultured PFC neurons (Fig. 1E), NRG produced a significant inhibitory effect on NMDAR currents (18.7 ± 1.4%; n = 80; data pooled together), and this effect of NRG was markedly attenuated by genistein (2.8 ± 0.7%; n = 10) or PD158780 (4.1 ± 0.9%; n = 7).

To determine whether NRG affects synaptic NMDA receptors, we examined the effect of NRG on NMDAR EPSCs in PFC slices. Application of NRG (4 nM) potently reduced the amplitude of NMDAR EPSCs in a reversible manner. The time course and current traces from a representative cell is shown in Figure 2, A and B. In a sample of PFC pyramidal neurons we examined, NRG induced a significant reduction of the mean amplitude of NMDAR EPSCs (36.7 ± 3.7%; n = 8) (Fig. 2E). The bigger effect of NRG found in slices than in dissociated neurons suggests that NRG signaling may preferentially affect synaptic NMDA receptors. Thus, NRG had a larger impact on NMDAR EPSCs that were evoked by stimulation of synaptic NMDA receptors than on NMDAR currents in isolated neurons in which both extrasynaptic and synaptic NMDA receptors were stimulated.

We further examined the effect of NRG on NMDAR EPSCs evoked by paired pulses, a measure that is sensitive to changes in the probability of transmitter release (Manabe et al., 1993). Consistent with our previous results (Wang et al., 2003), when double pulses with 100 ms intervals were delivered to PFC neurons, the second NMDAR EPSC showed larger amplitude than the first
one (a phenomenon called paired-pulse facilitation). Application of NRG reduced the amplitudes of NMDAR EPSC triggered by both pulses (Fig. 2C,D) but did not cause a significant change in the ratio of this paired-pulse facilitation [paired-pulse ratio (PPR); control, 1.52 ± 0.1; NRG, 1.53 ± 0.1; n = 7] (Fig. 2E). This result suggests that activation of NRG signaling is likely to induce a change in postsynaptic NMDA receptors rather than glutamate release.

**Activation of the phospholipase C/inositol-1,4,5-triphosphate/Ca^{2+} pathway is required for the neuregulin modulation of NMDA receptor currents**

We next examined the signaling mechanism underlying the reduction of NMDAR currents by NRG. Because one of the common intracellular signaling molecules that can be activated by receptor tyrosine kinases is the γ-isozymes of phospholipase C (PLC) (Exton 1996), we first tested the role of PLC pathway in the NRG modulation of NMDAR currents. As shown in Figure 3A, application of the PLC inhibitor U73122 (1 μM) significantly blocked the NRG-induced decrease of NMDAR currents. As summarized in supplemental Figure 1D (available at www.jneurosci.org as supplemental material), the transfected CaMKII siRNA markedly abolished the expression of CaMKII (top), whereas without CaMKII siRNA, the expression of CaMKII was normal (bottom). Then, we examined the NRG effect on NMDAR currents in CaMKII siRNA-transfected neurons. As shown in supplemental Figure 1B (available at www.jneurosci.org as supplemental material), NRG decreased NMDAR currents in the GFP-positive neuron transfected with CaMKII siRNA to the same degree as in the control neuron transfected with GFP alone. To examine the role of PKC, we inhibited PKC activity by application of the selective PKC inhibitor Go66850. As shown in supplemental Figure 1C (available at www.jneurosci.org as supplemental material), Go66850 (1 μM) failed to block the NRG-induced decrease of NMDAR currents. As summarized in supplemental Figure 1D (available at www.jneurosci.org as supplemental material), NRG reduced NMDAR currents by 16.9 ± 1.4% (n = 10) in cultured neurons transfected with CaMKII siRNA, 17.2 ± 1.3% (n = 10) in the presence of Go66850, neither of which was significantly different from the effect of NRG in control neurons (18.1 ± 0.8%; n = 8). These results suggest that CaMKII or PKC are not involved in the NRG modulation of NMDAR currents.

**The Ras/MEK/ERK pathway is involved in the neuregulin modulation of NMDA receptor currents**

Previous studies have shown that activation of p44/42 MAP kinase (ERK) is essential for NRG-mediated expression of acetylcholine receptors at the neuromuscular junction (Si et al., 1996; Tansey et al., 1996; Altio et al., 1997), we then examined the involvement of ERK signaling in shown in Figure 3B, 2APB (30 μM), a membrane-permeable IP3,R antagonist, substantially blocked the NRG-induced decrease of NMDAR currents. Dialysis with the Ca^{2+} chelator BAPTA (10 mM) also mostly prevented NRG from decreasing NMDAR currents (Fig. 3C). As summarized in Figure 3D, the effect of NRG on NMDAR currents was significantly (p < 0.01; ANOVA) attenuated in the presence of U73122 (3.9 ± 0.5%; n = 8), 2APB (3.4 ± 0.3%; n = 8), or BAPTA (3.2 ± 0.6%; n = 8). These results suggest that the PLC/IP3/Ca^{2+} signaling pathway is involved in the NRG regulation of NMDAR currents.

CaMKII and PKC are two major downstream signaling molecules that can be activated by the PLC signaling pathway. Previous studies have shown that both kinases are able to modulate NMDA receptors (Leonard et al., 1999; Lan et al., 2001; Wang et al., 2003). We then tested whether they are involved in NRG regulation of NMDAR currents. To examine the role of CaMKII, we suppressed CaMKII protein expression in cultured PFC neurons by transfecting an siRNA directed against CaMKII. GFP was cotransfected with CaMKII siRNA, and the expression of CaMKII was detected with the immunocytochemical approach. We found that in GFP-positive neurons (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material), the transfected CaMKII siRNA markedly abolished the expression of CaMKII (top), whereas without CaMKII siRNA, the expression of CaMKII was normal (bottom). Then, we examined the NRG effect on NMDAR currents in CaMKII siRNA-transfected neurons. As shown in supplemental Figure 1B (available at www.jneurosci.org as supplemental material), NRG decreased NMDAR currents in the GFP-positive neuron transfected with CaMKII siRNA to the same degree as in the control neuron transfected with GFP alone. To examine the role of PKC, we inhibited PKC activity by application of the selective PKC inhibitor Go66850. As shown in supplemental Figure 1C (available at www.jneurosci.org as supplemental material), Go66850 (1 μM) failed to block the NRG-induced decrease of NMDAR currents. As summarized in supplemental Figure 1D (available at www.jneurosci.org as supplemental material), NRG reduced NMDAR currents by 16.9 ± 1.4% (n = 10) in cultured neurons transfected with CaMKII siRNA, 17.2 ± 1.3% (n = 10) in the presence of Go66850, neither of which was significantly different from the effect of NRG in control neurons (18.1 ± 0.8%; n = 8). These results suggest that CaMKII or PKC are not involved in the NRG modulation of NMDAR currents.
NRG modulation of NMDAR currents. Immunocytochemical staining and Western blotting analysis indicated that NRG treatment of cultured PFC neurons induced a strong increase in ERK phosphorylation (Thr202/Tyr 204) and activation (Fig. 4A), which was blocked by U0126 (20 μM), a specific inhibitor of MEK (the upstream kinase of ERK). Application of U0126 (20 μM) significantly blocked the NRG-induced decrease of NMDAR currents (Fig. 4B), suggesting the involvement of ERK. To further confirm it, we blocked the ERK signaling by overexpressing a dominant-negative mutant of MEK1 that is catalytically inactive (Mansour et al., 1994). As shown in Figure 4B, C and D, in the neuron transfected with dnMEK1, NRG showed little effect on NMDAR currents, whereas in the neuron transfected with wt-MEK1, NRG produced a significant reduction of NMDAR currents.

We then tested the involvement of Ras, the upstream molecule of MEK/ERK signaling, in the NRG regulation of NMDAR currents. We transfected neurons with a dominant-negative form of Ras (S17→N) or a constitutively active form of Ras (G12→V) in these neurons to prevent the potential activation of Ras by the NRG signaling. As shown in Figure 4E, the NRG effect on NMDAR currents was greatly attenuated in cultured neurons transfected with either dnRas or caRas. As summarized in Figure 4F, in the presence of U0126, NRG reduced NMDAR currents by 4.3 ± 0.9% (n = 12), which was significantly (p < 0.01; ANOVA) smaller than the effect of NRG in control neurons (18.7 ± 0.8%; n = 10). Moreover, NRG produced little effect on NMDAR currents in neurons transfected with dnMEK1 (4.3 ± 0.7%; n = 13), dnRas (4.5 ± 0.7%; n = 14), or caRas (5.4 ± 0.9%; n = 11), which was significantly (p < 0.01; ANOVA) different from the effect of NRG in neurons transfected with wild-type MEK (19.8 ± 1.0%; n = 10). These results suggest that the Ras/MEK/ERK pathway is involved in the NRG reduction of NMDAR currents.

We also examined the potential involvement of several other key molecules that are linked to NRG signaling and can potentially modulate NMDA receptors, including phosphatidylinositol 3-kinase (PI3K), Src, and cyclin-dependent kinase 5 (cdk5) (Tansey et al., 1996; Fu et al., 2001; Li et al., 2001; Salter and Kalia, 2004). As shown in supplemental Figure 2A (available at www.jneurosci.org as supplemental material), the NRG-induced reduction of NMDAR currents was intact in the presence of wortmannin (1 μM), a specific PI3K inhibitor. Application of the nonreceptor tyrosine kinase Src inhibitor PP2 (5 μM; supplemental Fig. 2B, available at www.jneurosci.org as supplemental material) or the specific Cdk5 inhibitor roscovitine (40 μM; supplemental Fig. 2C, available at www.jneurosci.org as supplemental material) also failed to block the NRG-induced reduction of NMDAR currents. The effect of NRG on NMDAR currents in the absence or presence of various inhibitors is summarized in supplemental Figure 2D (available at www.jneurosci.org as supplemental material). Comparing to the effect in control neurons (18.4 ± 1.1%; n = 8), NRG produced a similar reduction of NMDAR currents in the presence of wortmannin (16.4 ± 0.9%; n = 9), PP2 (18.4 ± 0.9%; n = 8), roscovitine (16.4 ± 2.2%; n = 8), or the PKA inhibitor PKI14–22 (0.1 μM; 18.6 ± 2.0%; n = 8), suggesting the lack of involvement of PI3K, Src, Cdk5, or PKA in the regulation of NMDAR currents by neuregulin.

The neuregulin modulation of NMDA receptors is dependent on the integrity of actin

Emerging evidence has suggested that the trafficking of NMDA receptors plays an important role in regulating the function of these channels at the cell membrane (Carroll and Zukin, 2002; Wenthold et al., 2003). Cytoskeleton proteins, such as actin and microtubule, are often critically involved in the trafficking of membrane receptors (Rogers and Gelfand, 2000). To determine whether the neuregulin modulation of NMDAR currents is af-
fected by the integrity of filamentous actin (F-actin) (Rosenmund and Westbrook, 1993), we examined the effect of NRG on NMDAR currents in the presence of agents that interfere with actin filaments. Application of the actin-depolymerizing agent latrunculin B (5 μM, 20 min) or cytochalasin D (5 μM, 20 min) reduced NMDAR currents (lat B: 21.8 ± 4.2%, n = 8; cyto D: 19.5 ± 3.7%, n = 8), consistent with previous results (Rosenmund and Westbrook, 1993). In the presence of latrunculin B or cytochalasin D, subsequent application of NRG had no additional effect on NMDAR currents (Fig. 5A, B). Dialysis with the F-actin stabilizer phalloidin (2 μM) also essentially blocked the effect of NRG (Fig. 5A, B). On the other hand, the NRG effect on NMDAR currents was not affected by the microtubule depolymerizer colchicine (30 μM) or the microtubule stabilizer Taxol (10 μM) (Fig. 5C). As summarized in Figure 5D, the effect of NRG on NMDAR currents was significantly (p < 0.01; ANOVA) attenuated in the presence of latrunculin B (4.2 ± 1.0%; n = 13), cytochalasin D (4.4 ± 0.8%; n = 8), or phalloidin (4.9 ± 1.1%; n = 9) but not colchicine (16.4 ± 1.2%; n = 10) or Taxol (15.8 ± 1.2%; n = 10). These results suggest that the neuregulin regulation of NMDAR currents is dependent on the integrity of actin cytoskeleton rather than microtubule network.

Neuregulin increases NMDA receptor internalization through an actin-dependent mechanism

To further confirm the involvement of NMDAR trafficking in the neuregulin regulation of NMDAR currents, we then examined the effect of NRG on NR1 internalization in cultured PFC neurons. As shown in Figure 6A, NRG treatment (5 min) induced a marked increase in internalized NR1 in both cell body and dendrites, compared with both 0 and 5 min control cells (which exhibited similar levels of internalized NR1), whereas the total NR1 was not altered by NRG treatment. Quantification analysis (Fig. 6C) indicated that the ratio of internalized NR1 to total NR1 was 14.1 ± 4.4% (n = 25) in control cells and was increased to 36.5 ± 7.2% (n = 25; p < 0.01; ANOVA) in NRG-treated cells. Application of the specific ErbB inhibitor PD158780 blocked the NRG-induced increase of NR1 internalization [16.2 ± 3.8% (ratio); n = 16] (Fig. 6A, C, PD158780 + NRG), suggesting the mediation through ErbB receptor tyrosine kinases.

We then tested whether the signaling pathways involved in NRG reduction of NMDAR currents were also involved in the NRG increase of NR1 internalization. As shown in Figure 6, A and C, the PLC inhibitor U73122 and MEK inhibitor U0126, both of which blocked NRG reduction of NMDAR currents, also abolished the NRG-induced increase of NR1 internalization [U73122 plus NRG: 15.9 ± 3.2% (ratio), n = 16; U0126 plus NRG: 17.3 ± 3.5% (ratio), n = 16]. U73122 or U0126 alone did not alter the levels of internalized NR1 protein (data not shown). None of these treatments caused any change on total NR1 (Fig. 6A, C). In contrast to the effect of NRG on NR1 internalization, NRG treatment did not alter the AMPA receptor GluR1 subunit internalization or the total level of GluR1 (Fig. 6B, C). The ratio of internalized GluR1 to total GluR1 was 24.8 ±
Next, we examined whether the NRG-induced increase of NR1 internalization was affected by agents that interfere with actin or microtubule. As shown in Figure 7, A and B, application of the membrane-permeable actin stabilizer phalloidin oleate (0.5 μM) blocked the enhancing effect of NRG on NR1 internalization [phalloidin: 13.9 ± 2.5% (ratio), n = 18; phalloidin plus NRG: 15.3 ± 3.8% (ratio), n = 18], whereas phalloidin itself did not alter the levels of internalized NR1 protein. The actin-depolymerizing agent latrunculin B itself increased NR1 internalization and occluded the effect of subsequently applied NRG [lat B: 34.1 ± 6.7% (ratio), n = 18; lat B plus NRG: 37.6 ± 7.3% (ratio), n = 18]. Moreover, the NRG effect on NR1 internalization was intact in the presence of the microtubule stabilizer Taxol [Taxol: 14.9 ± 3.1% (ratio), n = 18; Taxol plus NRG: 33.5 ± 6.8% (ratio), n = 18; p < 0.01; ANOVA], and Taxol alone did not alter NR1 internalization. The level of total NR1 was not changed by any of these treatments (Fig. 7A, B). These results suggest that activation of neuregulin signaling increases NR1 internalization through an actin-dependent mechanism.

Because tyrosine phosphorylation of NMDA receptors regulates the channel activity and trafficking (Wang and Salter, 1994; Vissel et al., 2001), we would like to know whether the NRG modulation of NMDAR currents is through the direct phosphorylation of NMDA receptors by the ErbB receptor tyrosine kinase. Thus, we examined whether NRG treatment altered the tyrosine phosphorylation of NMDA receptor subunits. As shown in supplemental Figure 3, A and B (available at www.jneurosci.org as supplemental material), the tyrosine phosphorylation of NR1, NR2A, and NR2B subunits was not significantly changed by NRG treatment. The total level of NR1, NR2A, and NR2B was also unchanged by NRG treatment. It suggests that the NRG reduction of NMDAR currents is not attributable to the change of NMDA receptor tyrosine phosphorylation.

Because NRG treatment induced NR1 internalization, we further tested whether the internalization is mediated via a clathrin/dynamin-dependent pathway (Vissel et al., 2001; Nong et al., 2003). We tested the effect of NRG on the NMDAR current in cells dialyzed with the dynamin inhibitory peptide QVPSRPNRAP, which competitively blocks binding of dynamin to amphiphysin (Gout et al., 1993) and thus prevents endocytosis when administered intracellularly (Lissin et al., 1998; Kittler et al., 2000). As shown in Figure 8A, when NMDAR endocytosis was inhibited by the dynamin inhibitory peptide (50 μM), NRG failed to suppress NMDAR currents, whereas the effect of NRG was intact in the presence of a scrambled control peptide (50 μM). As summarized in Figure 8B, NRG had significantly smaller effect in neurons dialyzed with the dynamin inhibitory peptide (3.9 ± 0.7%; n = 9), compared with the control peptide (18.9 ± 1.7%; n = 8). These results suggest that the mechanism underlying the NRG-induced downregulation of NMDAR currents is a decrease of functional surface NMDA receptors mediated by clathrin/dynamin-dependent endocytosis.

Discussion

Neuregulin is present in glutamatergic synaptic vesicles, and ErbB receptors are enriched in the PSD subcellular fraction (Gar- cia et al., 2000; Huang et al., 2000). Moreover, ErbB4 interacts with PSD-95 in the adult brain and colocalizes with NMDA receptors in neuronal synapses (Garcia et al., 2000; Huang et al., 2000). Therefore, it has been suggested that neuregulin may play a role in modulating NMDAR-dependent synaptic plasticity (Huang et al., 2000; Buonanno and Fischbach, 2001). The potential interactions between NRG and NMDA receptors are further supported by the finding that NRG-1 induces a large increase in the NMDA receptor NR2C subunit mRNA in cerebellar slices (Ozaki et al., 1997). In addition, NRG +/+ mice exhibit a significant decrease in the number of functional NMDA receptors in prefrontal cortex (Stefansson et al., 2002). Nevertheless, direct measurement of the impact of NRG/ErbB signaling on NMDA receptor functions has been lacking. In this study, we demon-
strate that there exists a functional interaction between NRG/ErbB signaling and NMDA receptors. Activation of the NRG signaling with a polypeptide containing the EGF domain of NRG-β1 produces a significant reduction of the NMDA receptor-mediated ionic currents and synaptic currents in PFC pyramidal neurons.

Because multiple signaling cascades could converge to regulate NMDA receptors, we have examined signaling mechanisms underlying the NRG action. After NRG stimulation, ErbB receptors are homodimerized or heterodimerized, and subsequently, tyrosine residues in the C termini become phosphorylated, which serve as binding sites for cytoplasmic signaling molecules such as the γ-isozymes of PLC (Exton, 1996). Activated PLC will catalyze the hydrolysis of phospholipid, releasing IP₃, which mobilizes Ca²⁺ from intracellular stores, and diacylglycerol, which activates PKC. Our data showed that inhibition of PLC, IP₃ receptor, or [Ca²⁺]ᵢ abolished the NRG regulation of NMDAR currents, suggesting the involvement of the PLC/IP₃/Ca²⁺ signaling pathway in the action of NRG. CaMKII and PKC, two downstream targets of PLC, appear to play no role in this process.

Another important downstream signaling molecule that can be activated by the ErbB receptor tyrosine kinase is the ERK subgroup of MAP kinases. Many studies have shown that activation of ERK is essential for NRG-mediated expression of ACh receptor (AChR) subunits at the neuromuscular junction (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997). Our data showed that suppression of Ras or ERK activation with dominant inhibitory mutants eliminated the ability of NRG to regulate NMDAR currents, suggesting the involvement of the Ras/MEK/ERK signaling pathway in the action of NRG. Interestingly, expression of a dominant-negative mutant of Ras or a constitutively active Ras mutant both interfered with NRG effects on NMDAR currents. The explanation is as follows. In a normal Ras-GTPase cycle, guanine-nucleotide-exchange factors (GEFs) activate Ras by promoting nucleotide exchange on Ras, and GTP-activated proteins inactivate Ras by promoting GTP hydrolysis by Ras. The dominant-negative RasS17N mutant blocks activation of endogenous Ras by binding more tightly to RasGEF than does normal Ras (Feig and Cooper, 1988). Because RasS17N cannot engage downstream targets even when bound to GTP, it prevents the formation of functional GDP-bound Ras in cells. On the other hand, the constitutively active RasG12V mutant has deficiency in the GTPase activity (Sweet et al., 1984; Krengel et al., 1990). Because RasG12V is locked at the active state, it prevents any additional activation of Ras in response to stimuli. Thus, if the NRG regulation of NMDAR currents requires the elevation of Ras activity, the effect will be lost in neurons transfected with either form of mutant Ras. Our data have confirmed this hypothesis.

Several other signaling molecules, such as PI3K and cdk5, have also been implicated in the NRG-induced AChR expression (Tansey et al., 1996; Fu et al., 2001). However, inhibition of these kinases did not affect the NRG regulation of NMDAR currents; thus, we ruled out the involvement of these molecules. Interestingly, PSD-95, the postsynaptic protein associated with NMDA receptors (Kornau et al., 1995), regulates NRG-mediated activation of ERK (Huang et al., 2000). It awaits to see whether PSD-95 also facilitates the NRG regulation of NMDA receptor currents.

To understand how the NRG-induced elevation of intracellular Ca²⁺ and activation of ERK lead to the downregulation of NMDA receptor currents, we examined the role of cytoskeleton dynamics that can be regulated by these intracellular second messengers. F-actin is a major component of the cytoskeleton in postsynaptic densities at glutamatergic synapses (Matus et al., 1982; Allison et al., 1998). It continuously undergoes dynamic remodeling (Fischer et al., 1998, 2000; Matus, 2000) and can be rapidly depolymerized by calcium (Pollard and Cooper, 1986) or activation of ERK (Kutsuna et al., 2004). NMDA receptors are associated with actin filaments via actin binding proteins, such as α-actinin (Wyszynski et al., 1997; Krupp et al., 1999) and spectrin (Wechsler and Teichberg, 1998). The activity of NMDA receptors is dependent on the integrity of F-actin, and actin depolymerization resulting from influx of Ca²⁺ causes a downregulation of NMDA channel activity (Rosenmund and Westbrook, 1993). Moreover, depolymerizing F-actin causes a 40% decrease of synaptic NMDA receptor clusters (Allison et al., 1998) and a selective depression of NMDAR-mediated synaptic transmission (Sattler et al., 2000).
In this study, we found that NRG induced a significant increase in NR1 subunit internalization in cultured PFC neurons but did not cause a significant change in the tyrosine phosphorylation of NMDA receptor subunits. Consistent with the actin dependence of the NRG effect on NMDAR currents, the NRG regulation of NR1 internalization was also sensitive to agents affecting actin cytoskeleton. Furthermore, electrophysiological data show that the NR1 internalization is mediated via a clathrin/dynamin-dependent pathway. These results suggest that the NRG-mediated downregulation of NMDAR currents could occur through the increased NR1 endocytosis through clathrin-coated pits via an actin-dependent mechanism.

Actin depolymerization has been shown to enhance the internalization of AMPA receptors (Zhou et al., 2001). NMDA receptors, however, are more closely linked to the actin cytoskeleton (Wyszynski et al., 1997; Wechsler and Teichberg, 1998; Krupp et al., 1999). Consistent with our physiological results, other functional studies have also shown that the NMDAR activity is dependent on the state of polymerization of F-actin (Rosenmund and Westbrook, 1993; Lei et al., 2001). In addition, our data provide direct evidence showing that NMDA receptor internalization can be enhanced by actin depolymerization, suggesting that the stability of surface NMDA receptors is dependent on an intact actin cytoskeleton.

In summary, this study has revealed a novel function of neuregulin in mature neurons (i.e., the regulation of NMDA receptor channel activity). Activation of the NRG/ErbB signaling causes the elevation of intracellular Ca$^{2+}$ and activation of ERK, which in turn enhance the actin depolymerization, leading to the increased NMDA receptor internalization and the reduced NMDAR-mediated currents. Given the key role of NMDA receptors in schizophrenia (Tsai and Coyle, 2002), the regulation of NMDAR function could be one of the mechanisms underlying the involvement of neuregulin in this neuropsychiatric disorder (Harrison and Owen, 2003).

References


