

Neuregulin1 Downregulates Postsynaptic GABA_A Receptors at the Hippocampal Inhibitory Synapse

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ABSTRACT: The growth factor neuregulin 1 (NRG1) has been proposed to contribute to the formation and maturation of neuromuscular and interneuronal synapses by upregulating the expression of specific neurotransmitter receptor subunits. In the present report, we show that, in the hippocampus, NRG1 is expressed in a pattern suggesting that it regulates synapse development in the CA1 region. However, in contrast to what has been shown in other synapses, NRG1 reduces the expression of γ -aminobutyric acid (GABA)_A receptors α subunits in hippocampal slices, and the mean amplitude of GABAergic miniature inhibitory postsynaptic currents (IPSCs) in hippocampal CA1 pyramidal neurons, without affecting IPSC kinetics or frequency. These effects of NRG1 occur without concomitant changes in glutamate receptors and other synaptic proteins. We propose that the role of NRG1 in the formation and maturation in the hippocampal inhibitory synapse is downregulation, rather than upregulation, of receptor subunit expression. These results suggest that NRG1 may contribute to the reduction in GABAergic synaptic activity in hippocampal CA1 pyramidal neurons that normally occurs during early postnatal development, and that alterations in NRG1 signaling in the hippocampus may contribute to schizophrenia and epilepsy. © 2004 Wiley-Liss, Inc.

KEY WORDS: neuregulin; GABA_A receptor; hippocampal CA1; downregulation; mRNA; mIPSC

INTRODUCTION

Changes in the level of expression and distribution of postsynaptic neurotransmitter receptors are critical steps in synapse development (for review, see Hall and Sanes, 1993; Sanes et al., 2000). During synapse maturation, certain neurotransmitter receptors, e.g., N-methyl-D-aspartate (NMDA) and γ -aminobutyric acid (GABA)_A receptors, change in their subunit composition due to increments or decrements in the levels of expression of specific receptor subunits (Mishina et al., 1986; Laurie et al., 1992; Takahashi et al., 1992; Monyer et al., 1994). These changes in expression of

receptor subunits occur in normal development and abnormalities in these processes may be responsible for neural diseases such as epilepsy (McDonald et al., 1991; Poulter et al., 1999) and schizophrenia (Benes et al., 1996; Gao et al., 2000). However, the mechanisms underlying neurotransmitter receptor expression remain poorly understood.

Neuregulins (NRGs) comprise a family of structurally related proteins, each containing an epidermal growth factor (EGF)-like domain that binds to and activates receptor tyrosine kinases of the erbB family (for review, see Lemke, 1996; Fischbach and Rosen, 1997). NRG1 was originally identified as a factor that induces expression of acetylcholine receptors at the neuromuscular junction (Jessell et al., 1979; Usdin and Fischbach, 1986). Even through the brain is the site of the highest expression of NRG1 (Holmes et al., 1992; Corfas et al., 1995) and of erbB4, one of the NRG1 receptors (Plowman et al., 1993; Gerecke et al., 2001), the role of these molecules in the central nervous system (CNS) synapses remains unclear. NRG1 has been shown to induce expression of the GABA_A receptor β 2 subunit (Rieff et al., 1999) and NMDA receptor 2C subunit (NR2C) (Ozaki et al., 1997) in cerebellar granule cells, as well as the nicotinic acetylcholine receptor (nAChR) α 7 subunit in hippocampal GABAergic interneurons (Liu et al., 2001). Thus, NRG1 may play a key role in the normal regulation of neurotransmitter receptors in the CNS.

We found that NRG1 leads to a specific reduction in the expression of GABA_A receptor α subunit mRNA in the CA1 region of the hippocampus without affecting the expression of NMDA and α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate receptors. NRG1 also reduces the mean amplitude of GABAergic miniature inhibitory postsynaptic currents (mIPSCs) recorded from hippocampal CA1 pyramidal neurons. These results suggest that NRG1-mediated regulation of GABA_A receptor expression may be involved in diseases such as epilepsy and schizophrenia.

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MATERIALS AND METHODS

In Situ Hybridization

In situ hybridization was performed as previously described (Corfas et al., 1995). Tissues from postnatal day 6 (P6) rats were fixed in 4% paraformaldehyde in phos-

phate-buffered solution for overnight at 4°C. After dehydration, tissues were embedded in paraffin, and coronal sections were collected. Sections were treated with proteinase K and triethanolamine/acetic acid anhydride and then dehydrated. Hybridization was carried out at 52°C for 18 h in 50% deionized formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM NaPO₄ (pH 8.0), 10% dextran sulfate, 1× Denhardt's solution, and 50 mg/ml total yeast RNA with 3.5 × 10⁴ cpm/μl ³⁵S-labeled RNA probe. Slides were then rinsed in 5× SSC, 10 mM dithiothreitol (DTT) at 50°C, and in 50% formamide, 2× SSC, 10 mM DTT at 65°C. Sections were then treated with RNase A (20 μg/ml; Sigma) and washed at 37°C for 15 min in 2× SSC, and then for 15 min in 0.1 × SSC. Sections were dehydrated rapidly, processed for autoradiography using NTB-2 Kodak emulsion, exposed for 2 weeks at 4°C, and examined under a darkfield microscope. The templates for probe transcription were the Ig-like domain of rat ARIA (Corfas et al., 1995) and nucleotides 978–1902 of the rat erbB4 coding sequence (GenBank accession no. AF041838).

Immunostaining

P10 rat pups were anesthetized with pentobarbital and fixed by intracardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The tissue was cryoprotected in 20% sucrose; 50-μm sections were cut with a freezing microtome (Karl Zeiss). Floating sections were incubated in blocking solution (5% normal goat serum, 0.1% Triton X-100 in PBS) for 1 h at room temperature, followed by incubation with rabbit polyclonal anti-erbB4 0615 (Zhu et al., 1995) and monoclonal anti-GABA_A receptor β2/3 (clone 62-3G1; Upstate Biotechnology) antibodies in blocking solution at 4°C overnight. Sections were then washed with PBS and incubated with Cy3- or FITC-labeled secondary antibodies for 1 h at room temperature. Sections were washed in PBS and analyzed using a fluorescence microscope.

Hippocampal Slice Culture and NRG1 Application

Organotypic cultures were carried out according to the method described previously (Stoppini et al., 1991). Hippocampal slices (350 μm thick) of P3 Wistar rats were kept on Millicell-CM membranes (Millipore) in culture in a CO₂ incubator at 32°C. The form of NRG1 used in this study was the one containing an Ig-like domain (Ig-NRG1=NDF β1_(14–246)) (Amgen, Thousand Oaks, CA) and was applied at 1 nM. For mRNA experiments, Ig-NRG1 was applied to hippocampal slice cultures at days in vitro (DIV) 0, and slices were incubated with it for 6 days. For electrophysiological analysis, NRG treatment was started at DIV 3 and the recordings were carried out at DIV 9–12, because we could not make stable recordings from neurons at DIV 6–8.

Semiquantitative RT-PCR

The CA1 region of the hippocampus was trimmed from five or six slices at DIV 6, and the mRNAs were assayed by reverse transcription-polymerase chain reaction (RT-PCR) (Okada et al.,

2000). Total RNAs extracted by the acid-guanidine-phenol-chloroform method were reverse-transcribed using SuperScript II (Life Technologies, Gaithersburg, MD). The RT products were amplified by PCR using Taq DNA polymerase (Promega, Madison, WI). The PCR program was common to all genes except for the glutamic acid decarboxylase (GAD) 65/67. It consisted of a 5-min initial denaturation at 94°C, followed by indicated cycles of amplification (95°C, 42 s; 61°C, 42 s; 72°C, 42 s), and 5-min final elongation at 72°C. The PCR program for GAD was different at the annealing temperature (58°C). The number of PCR cycles for α1/α2, β2, γ2 subunits of GABA_A receptor, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), calcium-calmodulin-dependent protein kinase II (CaMKII), GAD, and NR2A/2B/2C were 23, 24, 24, 21, 26, 28, and 24, respectively. The PCR primers used was as follows: GABA_A α1/α2, CTGGATGGTTA(T/C)GA(C/T)AATCGTCT and ATAA(C/A)CCAGTCCATGGC(C/A)GT; GABA_A β2, ACTGGAAAGCTCAATGGCATGGGC and CTGTCCCAACTGCGTGCCAACCTT; GABA_A γ2, AAGGGGCATCATTAGCCTTTGATTC and CACAACGTACCCCAAGCGAACGT; CaMKII, GGCCTGGCCATAGAGGTTGAG and ACCAGCTTTGATCTGCTGGTAC; GAD65/67, GCATGTGGATGCTGC(C/T)TGGGGTG and GATGACCAT(C/G)CGGAAGAAGTTG; and NR2A/NR2B/NR2C GTGTGGGCCTTCTT(C/T)GCTGTCAT and CTCATCACC-TCATTCCTTCTC. The primers for G3PDH were purchased from Clontech (Palo Alto, CA). The amount of each mRNA was then measured with an acrylamide gel stained with SYBRGreen I (Molecular Probes, Eugene, OR), using a FluoroImager 595 (Molecular Dynamics, Sunnyvale, CA). The validity of PCR assays of GABA_A α1/α2, β2, γ2 subunits, NR2A/2B/2C subunits, and GAD65/67 was described previously (Sakaguchi et al., 1997; Rieff et al., 1999; Okada et al., 2000; Sudweeks and Yakel, 2000). Otherwise, the validity of PCR for CaMKII was confirmed with specificity of PCR and with linear amplification depending on PCR cycles and the amount of template (data not shown).

Relative amounts of GABA_A receptor α1 and α2 subunit and those of NR2A, NR2B, and NR2C were measured by digestion of the RT-PCR products with subunit specific restriction enzymes, as described in our previous study (Sakaguchi et al., 1997; Okada et al., 2000). The PCR products of GABA_A receptor were cut with *Nsi*I (α1) and *Mfe*I (α2). Similarly, PCR products of NR2 subunits were digested with restriction enzymes specific for NR2A (*Ssp*I), NR2B (*Sph*I), and NR2C (*Eco*47III) and were subjected to acrylamide gel electrophoresis and SYBRGreen staining.

Electrophysiology

Slices were cultivated for 9–12 days. NRG1 was applied for 6–9 days from DIV 3. Slices attached to the Millicell membranes were transferred into a superfusing chamber on a stage of upright microscope (Olympus, Tokyo, Japan). The superfusing artificial cerebrospinal fluid (aCSF) had the following composition (in mM): NaCl, 126; KCl, 2.7; NaH₂PO₄, 1.1, NaHCO₃, 2.8; glucose 12; CaCl₂, 2.5; and MgCl₂ 1.3. The patch pipettes were filled with the solution of the following composition (in mM): CsCl, 140; MgCl₂, 1; Na₂ATP, 2; HEPES, 10; and EGTA, 10. The pH was

adjusted to 7.2 with CsOH. Whole-cell recordings of mIPSCs were made from CA1 pyramidal cells at a holding potential of -70 mV under voltage-clamp, using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at $27 \pm 0.2^\circ\text{C}$. When the access resistance was >30 M Ω , the data were discarded. To isolate the GABAergic mIPSCs, tetrodotoxin (TTX) ($1 \mu\text{M}$), D(-)-2-amino-5-phosphonopentanoic acid (AP-5) ($50 \mu\text{M}$), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) ($10 \mu\text{M}$) were added to the aCSF. Under this condition, bicuculline, a GABA_A receptor antagonist, abolished virtually all remaining spontaneous currents. Records were low-pass filtered at 5 kHz, digitized at 10 kHz, and analyzed on a personal computer. The amplitude and the frequency of mIPSCs were analyzed from 300 events in each neuron using Axograph (Axon Instruments). The statistical significance was evaluated by Student's *t*-test unless otherwise noted.

Animal Procedures

All animal experiments were carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and the guiding principle of Physiological Society of Japan.

RESULTS

Neuregulin and erbB4 Expression in the Hippocampus

In juvenile rats, NRG1 mRNA was found in pyramidal cells in the CA3 region, in granule cells in the dentate gyrus, and at the hippocampal fissure, but its expression was relatively low in the CA1 region (Fig. 1A). In contrast, the mRNA for erbB4 was found predominantly in the CA1 region. Immunofluorescence staining confirmed the expression of erbB4 in CA1 pyramidal cells and showed coexpression of erbB4 and GABA_A receptor $\beta 2/3$ subunits in these cells (Fig. 1B). Nearly all neurons in the CA1 pyramidal layer expressed erbB4, but expression was particularly higher in a subset of neurons (data not shown), which presumably represents interneurons (Gerecke et al., 2001). These results suggest that NRG1 synthesized by CA3 neurons may be released from their axon terminals and activate the erbB4 receptors in the CA1 region, thereby changing the level of expression of postsynaptic neurotransmitter receptor subunits, similar to the action of NRG1 at the neuromuscular junction.

Neuregulin Reduces the Expression of GABA_A Receptor α Subunits in the Hippocampus in Organotypic Culture

The NRG1 gene generates several isoforms via alternative splicing (Lemke, 1996; Fischbach and Rosen, 1997). Chronic treatment with the NRG1 isoform containing an immunoglobulin-like domain, Ig-NRG1, is known to enhance neurotransmitter receptor expression in cerebellar neurons (Ozaki et al., 1997; Rieff et al., 1999). Because this isoform is highly expressed in CA3 (Corfas et al., 1995), we tested the effects of Ig-NRG1 on the expression of GABA and glutamate receptors

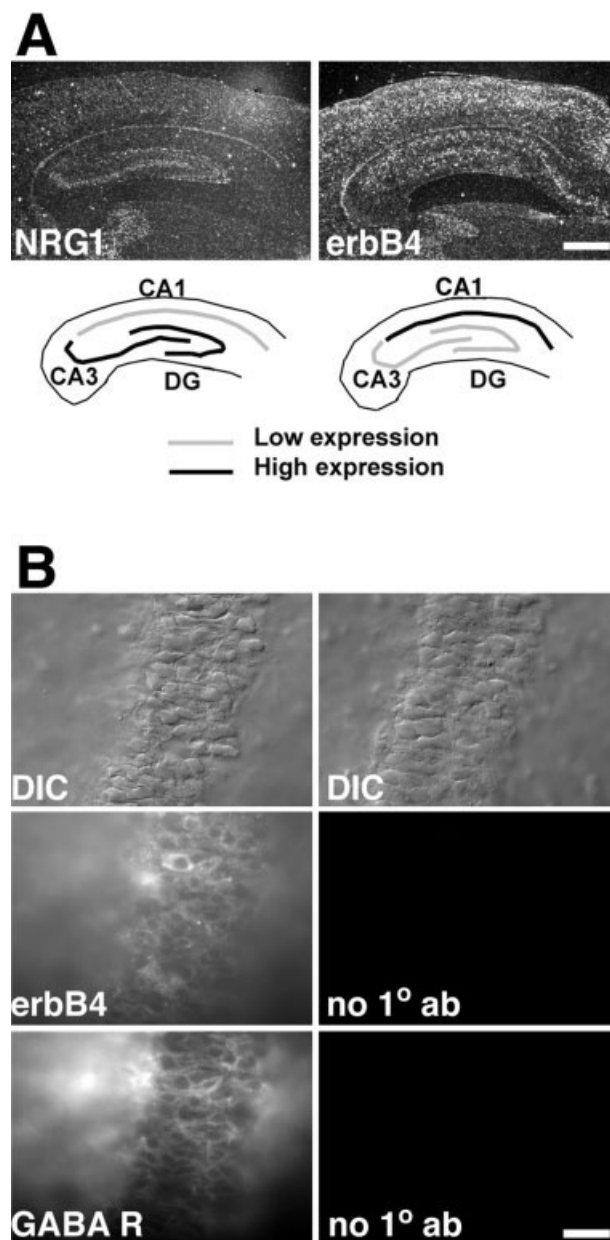


FIGURE 1. Expression of neuregulin, erbB4, and γ -aminobutyric acid (GABA)_A receptors in the hippocampus. **A:** Coronal sections of postnatal day 6 (P6) rat forebrains were hybridized with neuregulin and erbB4 probes, respectively. Darkfield views indicate expression of neuregulin 1 (NRG1) in the dentate gyrus and the CA3 regions, and expression of the NRG1 receptor erbB4 in the CA1 pyramidal layer. **B:** Section from P10 rat hippocampus immunostained with antibodies against erbB4 and GABA_A receptor $\beta 2/3$ subunits. Top panels are differential interference contrast photographs. Note that the immunoreactivities colocalize in the pyramidal cell somata. Scale bars = 1 mm in A; 100 μm in B.

subunits known to be abundantly expressed in the CA1 region of the hippocampus (Monyer et al., 1994; McKernan and Whiting, 1996; Sperk et al., 1997). Surprisingly, chronic treatment of hippocampal slices with NRG1 for 6 days specifically decreased the expression of mRNAs encoding GABA_A receptor

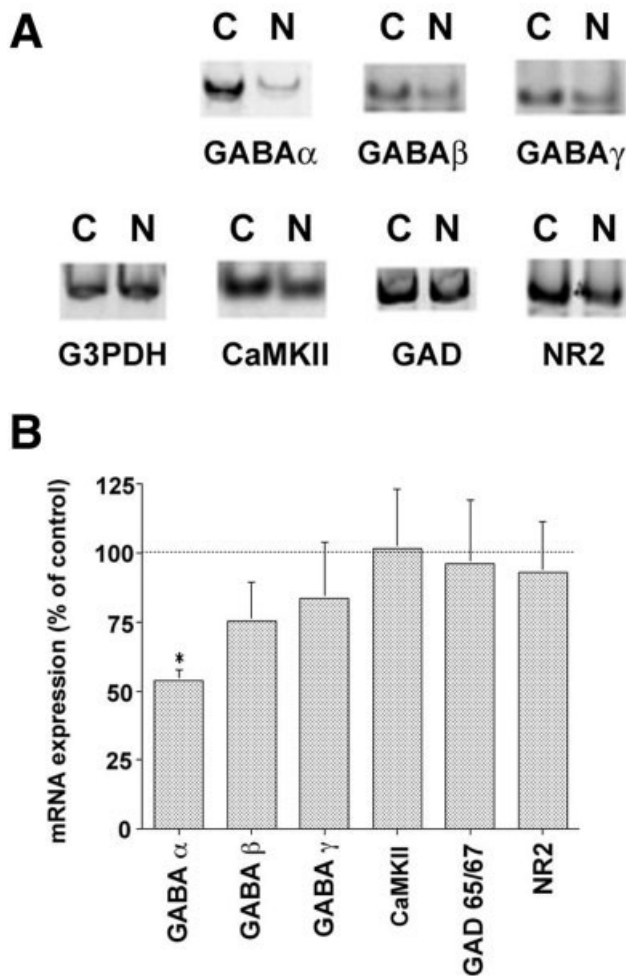


FIGURE 2. Neuregulin 1 (NRG1) treatment selectively reduces the mRNA for γ -aminobutyric acid (GABA)_A receptor α subunits. **A:** mRNA samples from control slices (C) and slices treated with 1 nM NRG1 (N) were subjected to reverse transcription-polymerase chain reaction (RT-PCR) for GABA_A receptor α 1/ α 2, β 2, and γ 2 subunits, glyceraldehyde 3-phosphate dehydrogenase (G3PDH), calcium-calmodulin-dependent protein kinase II (CaMKII), glutamic acid decarboxylase (GAD), and NR2. **B:** Semiquantitative analysis of RT-PCR. The level of expression of each molecule of interest in NRG-treated slices was compared to those of control slices. Levels of GABA_A receptor α 1/ α 2 subunits mRNAs were selectively and significantly reduced after chronic NRG1 treatment. The levels of mRNAs for β 2 and γ 2 subunits were also decreased, but the changes were statistically insignificant. No changes were found in mRNAs encoding CaMKII, GAD, and NR2 ($n = 4$, $*P < 0.01$, paired t -test).

α subunits (Fig. 2). The expression of GABA_A β 2 and γ 2 subunit mRNAs also decreased, but this decrease was not statistically significant. Importantly, NRG1 had no effect on mRNAs for the GABA synthesizing enzymes GAD65/67. In contrast to GABA_A receptors, mRNAs for NR2 subunits, which determine the levels of functional NMDA receptors, were not altered by the treatment. Expression of AMPA receptor proteins (GluR1, GluR2, GluR3) were also unaffected by the NRG1 (data not shown). Similarly, NRG1 had no effect on the expression of CaMKII, the most abundant protein in CA1 neurons. Thus,

the downregulatory effect of NRG1 seems specific to the GABA_A receptor α 1/ α 2 subunits in the hippocampal CA1 region in culture. Similar results were obtained with shorter (3 day) NRG treatments (not shown).

Neuregulin Does Not Affect the Relative Levels of GABA_A Receptor α Subunits or NMDA NR2 Subunits

During postnatal development in the forebrain, the α 2 subunit of GABA_A receptors is replaced by the α 1 subunit (Laurie et al., 1992; Fritschy et al., 1994). A similar subunit switch occurs in the intact developing hippocampus, where mRNAs for the α 2 subunit decrease; in contrast, those for the α 1 subunit increase, their expression eventually reaching a similar level by P15 (Fig. 3A). In contrast, the α 1-to- α 2 ratio remained unchanged in hippocampal organotypic culture, similar to what has been reported for thalamic cultures (Okada et al., 2000). These results suggest that some factor(s) involved in the α 2-to- α 1 switch of GABA_A receptor subunits in situ might be missing in organotypic culture.

NRG1 has been shown to contribute to the nAChR and NMDA receptor subunit switch by preferentially inducing the expression specific subunits (Martinou et al., 1991; Ozaki et al., 1997). To test whether NRG1 does the same for GABA_A receptor α subunits, hippocampal slices were treated with Ig-NRG1 for 6 days; the ratio of α 1 or α 2 subunit mRNAs was then measured. As shown in Figure 3B, NRG1 had no effect on the α 1-to- α 2 mRNA ratio. Similarly, NR2B in the rat hippocampus is replaced by NR2A during early postnatal development (Monyer et al., 1994), but this developmental change cannot be reproduced in hippocampal slice cultures (Sakaguchi et al., 1997). It has been reported that NRG1 induces the expression of NR2C mRNAs in cultured cerebellar slices (Ozaki et al., 1997). Therefore, we also examined whether NRG1 might affect the relative ratio of NR2 subunit mRNAs in hippocampal slice cultures (Fig. 3C). Again, NRG1 had no effect on the relative ratio of NR2 subunit mRNAs.

Neuregulin Downregulates Functional GABA_A Receptors in Cultured Hippocampal Slices

The α subunits of GABA_A receptors are essential for ligand binding and channel opening. Since GABA_A receptor α subunit mRNAs were downregulated by NRG1, we examined whether NRG1 treatment affected the function of GABA_A synapses. The mean mIPSCs amplitude recorded from CA1 pyramidal neurons (60 ± 5.8 pA, $n = 6$) was significantly ($P < 0.05$) reduced by treatment with NRG1 (44.6 ± 2.7 pA) (Fig. 4A). The difference between NRG1-treated neurons and controls could be easily detected in the cumulative histograms of mIPSCs ($P < 0.001$, Kolmogorov-Smirnov-test; Fig. 4B). When the amplitudes of averaged mIPSCs with or without NRG1 were normalized, they overlapped almost completely (Fig. 4A), suggesting that NRG1 has no effect on their kinetics. Importantly, the mean frequency of mIPSCs in NRG1 treated slices (1.17 ± 0.35 Hz, $n = 6$) was not significantly different from control (0.84 ± 0.16 Hz, $n = 6$, $P = 0.35$). These results suggest that the NRG1 treatment reduces the density of functional subsynaptic GABA_A receptors in CA1 pyramidal neurons.

DISCUSSION

The anatomical localization of NRG1 and its erbB receptors can provide important clues to the potential biological actions of this ligand-receptor system. Our finding that NRG1 is highly ex-

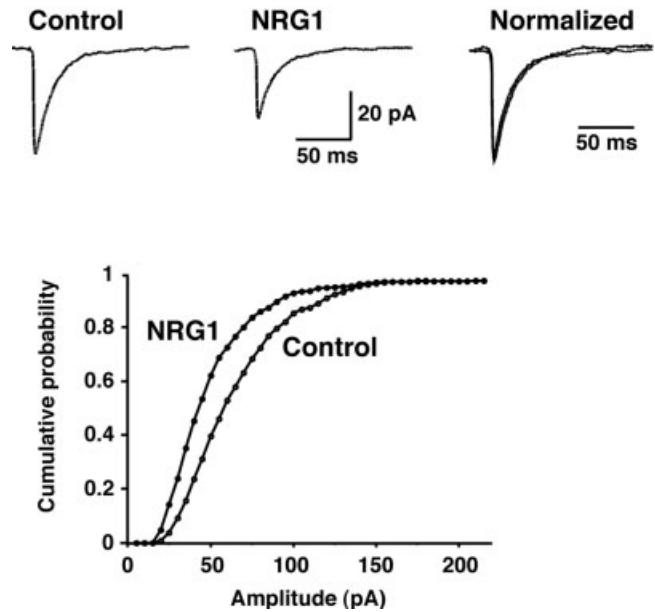
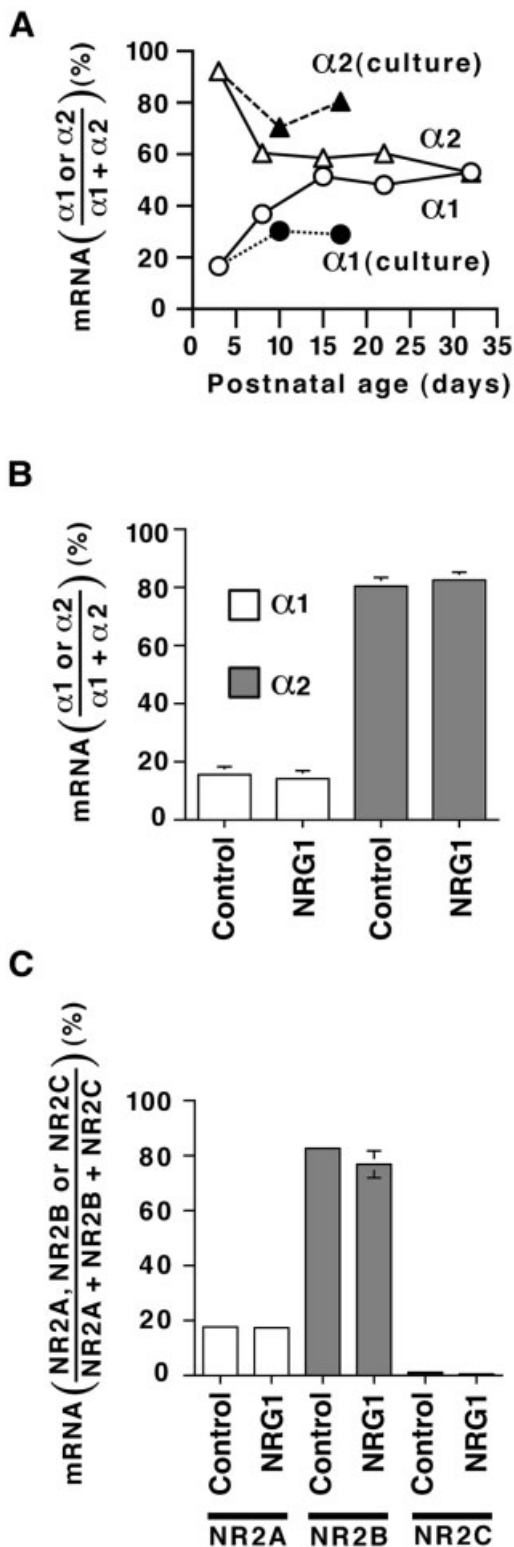


FIGURE 4. Neuregulin 1 (NRG1) reduces the amplitude of γ -aminobutyric acid (GABA)_A ergic miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal CA1 pyramidal neurons. Top: Averaged mIPSCs (of 100 events, aligned at the peak) in control or NRG1-treated slices show a reduction in amplitude in the treated slices. Superimposition after normalization (Normalized) shows no difference in current kinetics between the two conditions. Bottom: The cumulative amplitude histogram of mIPSCs (a total of 600 events from six cells) with or without NRG1 treatment shows the reduction in the mIPSCs after NRG1 treatment.

pressed in the CA3 region, as compared with erbB4, which is highly expressed by CA1 neurons, is a strong indication that these molecules participate in the CA3-CA1 synapses. Even though the pattern of expression of NRG and erbB4 suggests that the effects can be direct, the possibility that they are indirect, e.g., through NRG effects on glia (Prevot et al., 2003), should be considered. While expression of erbB4 is sufficient to make a cell responsive to NRG1 stimulation (Carraway and Cantley, 1994), erbB2 and

FIGURE 3. Neuregulin has no effect on the composition of γ -aminobutyric acid (GABA)_A α subunits or N-methyl-D-aspartate (NMDA) NR2 subunits. A: Developmental changes in the expression of mRNA for GABA_A receptor $\alpha 1$ and $\alpha 2$ subunits in the hippocampal CA1 region of rats at various postnatal days were subjected to reverse transcription-polymerase chain reaction (RT-PCR) assays. The expression of the GABA_A receptor $\alpha 1$ subunit in situ (\circ) increased as the animal matured, whereas that of the $\alpha 2$ subunit (Δ) decreased. In a cultured slice, however, GABA_A receptor α subunits failed to change significantly ($\alpha 1$, \bullet , $\alpha 2$, \blacktriangle). B: Treatment of cultured slices with NRG1 for 6 days had no effect on the α subunit composition of GABA_A receptor mRNAs. Ordinate indicates the percentage in the amount of GABA_A receptor $\alpha 1$ or $\alpha 2$ subunit mRNA relative to their sum. Neuregulin had no effect on the ratio (n = 5). C: Neuregulin had also no effect on the expressions of NMDA NR2 subunit mRNAs. Ordinate indicates the percentage of mRNAs encoding NR2A, NR2B, or NR2C subunits relative to their sum (n = 4).

erbB3 are also expressed in CA1 pyramidal neurons (Gerecke et al., 2001). Thus, it is possible that the effects of NRG1 on CA1 neurons are mediated through the signaling of more than one erbB receptor.

Chronic treatment of cultured hippocampal slices with Ig-NRG1 significantly decreased the expression of GABA_A receptors α 1 and α 2 subunit mRNAs in the CA1 region without affecting the expression levels of CaMKII, NR2A, NR2B, NR2C, or GAD. Hence, the downregulatory effect of NRG1 is specific to GABA_A receptor subunits. Concomitant with the reduction in mRNA of GABA_A receptor α 1/2 subunits, the mean amplitude of GABAergic mIPSCs in CA1 pyramidal neurons was significantly reduced by treatment with Ig-NRG. Assembly of functional GABA_A receptors depends on the adequate expression of several subunits (Connolly et al., 1996), and subunits that fail to form pentameric hetero-oligomers are retained at the endoplasmic reticulum and degraded (Gorrie et al., 1997). Thus, upon NRG treatment, the levels of GABA_A receptor α subunits could become a rate-limiting factor in the assembly of receptors, resulting in the degradation of other subunits and decreased number of functional receptors in the postsynaptic membranes. This would ultimately lead to the observed physiological changes. However, the possibility that the decrease in GABA-IPSCs is due to the posttranscriptional changes of GABA_A receptors, such as receptor phosphorylation, cannot be excluded.

The GABA_A receptor agonist muscimol can decrease the expression of the GABA_A receptor α subunit (Hirouchi et al., 1992). Thus, it could be argued that NRG1 might downregulate GABA_A receptors by stimulating the activity of GABAergic interneurons. However, the predominant expression of the NRG1 receptor erbB4 in CA1 pyramidal neurons and the lack of effect of Ig-NRG1 on the expression of GAD65/67 argue against this possibility. NRG1 was recently demonstrated to increase the postsynaptic expression of α 7 nAChRs and to enhance GABAergic transmission in dissociated hippocampal cultures (Liu et al., 2001). However, since our culture system has no cholinergic innervation, it is unlikely that our present results are affected by an enhancement of cholinergic transmission.

The present study demonstrates that Ig-NRG1 treatment reduces GABAergic synaptic currents in the hippocampus. In contrast, in primary cultured cerebellar granule cells, Ig-NRG1 increases the expression of the GABA_A receptor β 2 subunit and the amplitude of GABA-induced currents (Rieff et al., 1999). Therefore, NRG1 appears to affect GABA_A receptors differentially, depending on the cell type. Consistent with this notion, NRG1 has no effect on NR2 subunits in CA1 pyramidal cells, whereas it upregulates NR2 subunits in cerebellar granule cells (Ozaki et al., 1997). The presence of splice variants of erbB4 might underlie the differential effect of NRG1 (Kainulainen et al., 2000; Rio et al., 2000).

The developmental switch in the subunit composition of postsynaptic receptors is a common and important phenomenon in synaptic maturation. At the mammalian neuromuscular junction, the γ subunit of nAChR is replaced by the ϵ subunit in the early development (Mishina et al., 1986). Similarly, in the developing CNS, the GABA_A receptor α 2 subunit is replaced by α 1

subunit (Fritschy et al., 1994; Okada et al., 2000), and the NMDA receptor NR2B subunit is replaced by NR2A and NR2C subunits (Monyer et al., 1994). At the neuromuscular junction, NRG1 preferentially induces the expression of the ϵ subunit of nAChR compared with the γ subunit, suggesting that it regulates the developmental switch of nAChR subunits (Martinou et al., 1991). In cerebellar neurons, NRG1 specifically increases the NR2C subunit mRNAs (Ozaki et al., 1997). Our present results, however, do not indicate a similar preferential effect of NRG1 on the expression of GABA_A receptor α 1 subunits or NR2A subunits in hippocampal neurons. Thus, NRG1 may not be a ubiquitous developmental switching factor for GABA_A receptor α subunits or NR2 subunits. Alternatively, additional factors might be required for this process in the hippocampal CA1 region, e.g., the synergistic effects of multiple trophic factors (Cameron et al., 1998) and/or neuronal activity (Futai et al., 2001).

The finding that Ig-NRG1 downregulates expression of GABA_A receptor subunits provides insights into the possible developmental and pathological significance of this factor. GABAergic synaptic activity in hippocampal CA1 pyramidal neurons diminishes in amplitude during postnatal development (Cohen et al., 2000), the period during which hippocampal NRG1 expression is highest (Chen et al., 1994). Our present results suggest that NRG1 may play a role in the developmental downregulation of GABA_A receptors. Pathologically, failure in NRG1 signaling could result in increased expression of GABA_A receptors in the hippocampus, such as that observed in the hippocampus of schizophrenics (Benes et al., 1996). Interestingly, the NRG1 gene was recently mapped to a schizophrenia locus (Stefansson et al., 2002, 2003). Thus, defects in the regulation of GABA_A receptor expression by NRG1 may be a key event in the mechanisms by which this factor contributes to schizophrenia. NRG1-mediated regulation of hippocampal GABA_A receptor expression may also play a role in epilepsy. It has been shown that postsynaptic GABA_A receptor function (Kamphuis et al., 1991; Gibbs et al., 1997) and ³H-muscimol binding (Titular et al., 1994) in rat hippocampal CA1 neurons are reduced by seizures, and that GABA_A and benzodiazepine binding are reduced in the hippocampal CA1 of patients with temporal lobe epilepsy (McDonald et al., 1991). Since NRG1 expression in the hippocampus is increased by kainate-induced seizures (Eilam et al., 1998), it is possible that pathologic increases in NRG1 expression due to seizures may lead to reduced GABA_A receptor expression in hippocampus, thereby contributing to increased excitability and further epileptic seizures.

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REFERENCES

- Benes FM, Khan Y, Vincent ST, Wickramasinghe R. 1996. Differences in the subregional and cellular distribution of GABA_A receptor binding in the hippocampal formation of schizophrenic brain. *Synapse* 22:338–349.
- Cameron JS, Lhuillier L, Subramony P, Dryer SE. 1998. Developmental regulation of neuronal K⁺-channels by target-derived TGF β in vivo and in vitro. *Neuron* 21:1045–1053.
- Carraway KL III, Cantley LC. 1994. A new acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* 78:5–8.
- Chen MS, Bermingham-McDonogh O, Danehy FT Jr, Nolan C, Scherer SS, Lucas J, Gwynne D, Marchionni MA. 1994. Expression of multiple neuregulin transcripts in postnatal rat brains. *J Comp Neurol* 349:389–400.
- Cohen AS, Lin DD, Coulter DA. 2000. Protracted postnatal development of inhibitory synaptic transmission in rat hippocampal area CA1 neurons. *J Neurophysiol* 84:2465–2476.
- Connolly CN, Wooltorton JR, Smart TG, Moss SJ. 1996. Subcellular localization of gamma-aminobutyric acid type A receptors is determined by receptor β subunits. *Proc Natl Acad Sci USA* 93:9899–9904.
- Corfas G, Rosen KM, Aratake H, Krauss R, Fischbach GD. 1995. Differential expression of ARIA isoforms in the rat brain. *Neuron* 14:103–115.
- Eilam R, Pinkas-Kramarski R, Ratzkin BJ, Segal M, Yarden Y. 1998. Activity-dependent regulation of Neu differentiation factor/neuregulin expression in rat brain. *Proc Natl Acad Sci USA* 95:1888–1893.
- Fischbach GD, Rosen KM. 1997. ARIA: a neuromuscular junction neuregulin. *Annu Rev Neurosci* 20:429–458.
- Fritschy JM, Paysan J, Enna A, Mohler H. 1994. Switch in the expression of rat GABA_A-receptor subtypes during postnatal development: an immunohistochemical study. *J Neurosci* 14:5302–5324.
- Futai K, Okada M, Matsuyama K, Takahashi T. 2001. High-fidelity transmission acquired via a developmental decrease in NMDA receptor expression at an auditory synapse. *J Neurosci* 21:3342–3349.
- Gao XM, Sakai K, Roberts RC, Conley RR, Dean B, Tamminga CA. 2000. Ionotropic glutamate receptors and expression of N-methyl-D-aspartate receptor subunits in subregions of human hippocampus; effect of schizophrenia. *Am J Psychiatry* 157:1141–1149.
- Gerecke KM, Wyss JM, Karavanova I, Bunanno A, Carrol SL. 2001. ErbB transmembrane kinase receptors are differentially expressed throughout the adult rat central nervous system. *J Comp Neurol* 433:86–100.
- Gibbs JW III, Shumate MD, Coulter DA. 1997. Differential epilepsy-associated alterations in postsynaptic GABA_A receptor function in dentate granule and CA1 neurons. *J Neurophysiol* 77:1924–1938.
- Gorrie GH, Vallis Y, Stephenson A, Whitfield J, Browning B, Smart TG, Moss SJ. 1997. Assembly of GABA_A receptors composed of α1 and β2 subunits in both cultured neurons and fibroblasts. *J Neurosci* 17:6587–6596.
- Hall ZW, Sanes JR. 1993. Synaptic structure and development: the neuromuscular junction. *Cell/Neuron* 72(suppl):99–121.
- Hirouchi M, Ohkuma S, Kuriyama K. 1992. Muscimol-induced reduction of GABA_A receptor α1-subunit mRNA in primary cultured cerebral cortical neurons. *Brain Res Mol Brain Res* 15:327–331.
- Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, Shepard HM, Kuang W-J, Wood WI, Goeddel DV, Vandlen RL. 1992. Identification of heregulin, a specific activator of p185erbB2. *Science* 256:1205–1210.
- Jessell TM, Siegel RE, Fischbach GD. 1979. Induction of acetylcholine receptors on cultured skeletal muscle by a factor extracted from brain and spinal cord. *Proc Natl Acad Sci USA* 76:5397–5401.
- Kainulainen V, Sudvall M, Maatta JA, Santiestevan E, Klagsbrun M, Elenius K. 2000. A natural erbB4 isoform that does not activate phosphoinositide 3-kinase mediate proliferation but not survival or chemotaxis. *J Biol Chem* 275:8641–8649.
- Kamphuis W, Gorter JA, da Silva FL. 1991. A long-lasting decrease in the inhibitory effect of GABA on glutamate responses of hippocampal pyramidal neurons induced by kindling epileptogenesis. *Neuroscience* 41:425–431.
- Laurie DJ, Wisden W, Seeburg PH. 1992. The distribution of thirteen GABA_A receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* 12:4151–4172.
- Lemke G. 1996. Neuregulins in development. *Mol Cell Neurosci* 7:247–262.
- Liu Y, Ford B, Mann MA, Fischbach GD. 2001. Neuregulins increase α7 nicotinic acetylcholine receptors and enhance excitatory synaptic transmission in GABAergic interneurons of the hippocampus. *J Neurosci* 21:5660–5669.
- Martinou J-C, Falls DL, Fischbach GD, Merlie JP. 1991. Acetylcholine receptor-inducing activity stimulates expression of the ε-subunit gene of the muscle acetylcholine receptor. *Proc Natl Acad Sci USA* 88:7669–7673.
- McDonald JW, Garofalo EA, Hood T, Sackellares JC, Gilman S, McKeever PE, Troncoso JC, Johnston MV. 1991. Altered excitatory and inhibitory amino acid receptor binding in hippocampus of patients with temporal lobe epilepsy. *Ann Neurol* 29:529–541.
- McKernan RM, Whiting PJ. 1996. Which GABA_A-receptor subtypes really occur in the brain? *Trends Neurosci* 19:139–143.
- Mishina M, Takai T, Imoto K, Noda M, Takahashi T, Numa S, Methfessel C, Sakmann B. 1986. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature* 321:406–411.
- Monyer H, Burnashev N, Laurie DJ, Sakman B, Seeburg PH. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529–540.
- Okada M, Onodera K, Renterghem CV, Sieghart W, Takahashi T. 2000. Functional correlation of GABA_A receptor α subunits expression with the properties of IPSCs in the developing thalamus. *J Neurosci* 20:2202–2208.
- Ozaki M, Sasner M, Yano R, Lu HS, Buonanno A. 1997. Neuregulin-β induces expression of an NMDA-receptor subunit. *Nature* 390:691–694.
- Plowman GD, Culouscou J, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG, Shoyab M. 1993. Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. *Proc Natl Acad Sci USA* 90:1746–1750.
- Poulter MO, Brown LA, Tynan S, Willick G, William R, McIntyre DC. 1999. Differential expression of α1, α2, α3, and α5 GABA_A receptor subunits in seizure-prone and seizure-resistant rat models of temporal lobe epilepsy. *J Neurosci* 19:4654–4661.
- Prevot V, Rio C, Cho GJ, Lomniczi A, Heger S, Neville CM, Rosenthal NA, Ojeda SR, Corfas G. 2003. Normal female sexual development requires neuregulin-erbB receptor signaling in hypothalamic astrocytes. *J Neurosci* 23:230–239.
- Rieff HI, Raetzman LT, Sapp DW, Yeh HH, Siegel RE, Corfas G. 1999. Neuregulin induces GABA_A receptor subunit expression and neurite outgrowth in cerebellar granule cells. *J Neurosci* 19:10757–10766.
- Rio C, Buxbaum JD, Peschon JJ, Corfas G. 2000. Tumor necrosis factor α-converting enzyme is required for cleavage of erbB4/HER4. *J Biol Chem* 275:10379–10387.
- Sakaguchi T, Okada M, Kuno M, Kawasaki K. 1997. Dual mode of N-methyl-D-aspartate-induced neuronal death in hippocampal slice cultures in relation to N-methyl-D-aspartate receptor properties. *Neuroscience* 76:411–423.
- Sanes DH, Reh TA, Harris WA. 2000. In: *Development of the nervous system*. San Diego, CA: Academic Press. p 288–348.
- Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W. 1997. GABA_A receptor subunits in the rat hippocampus. I. Immunocytochemical distribution of 13 subunits. *Neuroscience* 80:987–1000.
- Stefansson H, Sigurdsson E, Steinthorsdottir V, Bjornsdottir S, Sigmundsson T, Ghosh S, Brynjolfsson J, Gunnarsdottir S, Ivarsson O, Chou TT, Hjaltason O, Birgisdottir B, Jonsson H, Gudnadottir VG, Gudmundsdottir E, Bjornsson A, Ingvarsson B, Ingason A, Sigfusson

- S, Hardardottir H, Harvey RP, Lai D, Zhou M, Brunner D, Mutel V, Gonzalo A, Lemke, G, Sainz, J, Johannesson, G, Andresson, T, Gudbjartsson, D, Manolescu, A, Frigge ML, Gurney ME, Kong A, Gulcher JR, Petursson H, Stefansson K. 2002. Neuregulin 1 and susceptibility to schizophrenia. *Am J Hum Genet* 71:877–892.
- Stefansson H, Sarginson J, Kong A, Yates P, Steinthorsdottir V, Gudfinnsson E, Gunnarsdottir S, Walker N, Petursson H, Crombie C, Ingason A, Gulcher JR, Stefansson K, St Clair D. 2003. Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. *Am J Hum Genet* 72:83–87.
- Stoppini L, Buchs PA, Muller D. 1991. A simple method for organotypic cultures of nerves tissue. *J Neurosci Methods* 37:173–182.
- Sudweeks SN, Yakel JL. 2000. Functional and molecular characterization of neuronal nicotinic ACh receptors in rat CA1 hippocampal neurons. *J Physiol* 527:515–528.
- Takahashi T, Momiyama A, Hirai K, Hishinuma F, Akagi H. 1992. Functional correlation of fetal and adult forms of glycine receptors with developmental changes in inhibitory synaptic receptor channels. *Neuron* 9:1155–1161.
- Titular MN, Kamphuis W, Pool CW, van Heerikhuizen JJ, Lopes Da Silva FH. 1994. Kindling induces time-dependent and regional specific changes in the [³H]muscimol binding in the rat hippocampus: a quantitative autoradiographic study. *Neuroscience* 59:817–826.
- Usdin TB, Fischbach GD. 1986. Purification and characterization of a polypeptide from chick brain that promotes the accumulation of acetylcholine receptors in chick myotubes. *J Cell Biol* 103:493–507.
- Zhu X, Lai C, Thomas S, Burden SJ. 1995. Neuregulin receptors, erbB3 and erbB4, are localized at neuromuscular synapses. *EMBO J* 14:5842–5848.