Journal of Neurochemistry, 2004, **90**, 1521–1529 doi:10.1111/j.1471-4159.2004.02685.x

Neuregulin induces $GABA_A$ receptor β_2 subunit expression in cultured rat cerebellar granule neurons by activating multiple signaling pathways

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Abstract

The $GABA_A$ receptor β subunit is required to confer sensitivity to γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the CNS. In previous studies we demonstrated that the growth and differentiation factor neuregulin 1 (NRG1) selectively induced expression of the β2 subunit mRNA and encoded protein in rat cerebellar granule neurons in culture. In the present report we examine the signaling pathways that mediate this effect. These studies demonstrate that the effects of NRG1 on β2 subunit polypeptide expression require activation of the ErbB4 receptor tyrosine kinase; its effects are inhibited by pharmacological blockade of ErbB4 phosphorylation or reduction of receptor level with an antisense oligodeoxynucleotide. The NRG1-induced activation of ErbB4 stimulates the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and cyclin-dependent kinase-5 (cdk5) pathways. Pharmacological blockade of any of these pathways inhibits increased β2 subunit expression, demonstrating that all three pathways are required to mediate the effects of NRG1 on $GABA_A$ receptor subunit expression in cerebellar granule neurons. These studies provide novel information concerning the actions of NRG1 on $GABA_A$ receptor expression in the CNS.

Keywords: cdk5 kinase, cerebellar granule neurons, ErbB4 receptor, $GABA_A$ receptor, MAP kinase, neuregulin.
J. Neurochem. (2004) **90,** 1521–1529.

The neuregulins (NRGs) are a family of growth and differentiation factors that play multiple tissue-specific roles in both the developing and postnatal nervous system (reviewed in Lemke 1996; Fischbach and Rosen 1997; Britsch *et al*. 1998; Buonanno and Fischbach 2001; Falls 2003). In neurons, NRGs promote neuronal migration and differentiation, and selectively regulate the expression of several ligand-gated neurotransmitter receptors (Rio *et al*. 1997; Sandrock *et al*. 1997; Vaskovsky *et al*. 2000; Sobeih and Corfas 2002). One of the NRGs, NRG1, stimulates expression of nicotinic acetylcholine receptor (nAChR) δ and ε subunits at the neuromuscular junction (Jo *et al*. 1995; Tansey *et al*. 1996; Si *et al*. 1999), induces expression of the *N*-methyl-D-aspartate (NMDA) receptor 2C subunit in maturing synapses in the brain (Ozaki *et al*. 1997), and increases the level of the $GABA_A$ receptor β2 subunit in cerebellar granule neurons in culture (Rieff *et al*. 1999).

The $GABA_A$ receptor mediates the actions of γ-aminobutyric acid, the major inhibitory neurotransmitter in the central nervous system. Of the 16 known $GABA_A$ receptor subunit genes in rodents (Mehta and Ticku 1999; Whiting *et al*. 1999), those encoding β subunits have been shown to play particularly important roles. These subunits confer sensitivity to GABA (Verdoorn *et al*. 1990) and are critical for targeting receptors to the cell surface (Connor *et al*. 1998; Taylor *et al*. 1999; Bollan *et al*. 2003). In previous studies, we demonstrated that NRG1, a growth factor expressed in the developing cerebellum (Rio *et al*. 1997; Ozaki *et al*. 2000), induces increases in β2 subunit mRNA and protein expression in granule neurons prepared for culture at postnatal day

Received April 8, 2004; revised manuscript received May 25, 2004; accepted May 31, 2004.

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Abbreviations used: Cdk5, cyclin-dependent kinase-5; CHX, cycloheximide; MAPK, mitogen-activated protein kinase; NRG, neuregulin; nAChR, nicotinic acetylcholine receptor; ODN, oligodeoxynucleotide; P, postnatal day; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase.

(P) 6. These increases are accompanied by a rise in the amplitude of GABA-evoked currents (Rieff *et al*. 1999). We also demonstrated that the β2 subunit is limiting for receptor assembly in cultured granule neurons, where reduction of its expression resulted in a decrease in the level of the α1 subunit (Kumar *et al*. 2001).

Although intracellular signaling pathways involved in mediating the effects of NRGs in many cell types have been identified, little is known about the events regulating $GABA_A$ receptor subunit expression in neurons in the CNS. In other regions, the NRGs initiate their effects by binding to ErbB receptors, tyrosine kinases that belong to the epidermal growth factor (EGF) receptor family (Britsch *et al*. 1998; Buonanno and Fischbach 2001; Carpenter 2003). Ligand binding induces receptor dimerization and activation. ErbB4 is active as a homodimer or as a heterodimer with ErbB2, which has an active kinase but does not bind NRG. In contrast, ErbB3 lacks an active kinase and must dimerize with ErbB2 to transduce a signal. NRG-induced receptor phosphorylation then triggers activation of different tissue-specific signaling pathways. Although we previously speculated that NRG1 activated ErbB4 (Rieff *et al*. 1999), the receptor was not identified and the pathway involved in inducing $GABA_A$ receptor β2 subunit expression was not investigated.

In the present study, signaling pathways that mediate the effects of NRG1 on $GABA_A$ receptor β2 subunit expression in cerebellar granule neurons were identified. These studies demonstrate that the NRG1-induced increase in subunit expression is dependent on cerebellar age and requires activation of the ErbB4 receptor. Activation stimulates the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and cyclin-dependent kinase-5 (cdk5) signaling pathways, all of which are required to induce the up-regulation of β2 subunit expression.

Experimental procedures

Materials

Recombinant neuregulin1-β1 (NRG1; residues 176–246) was from R & D Systems (Minneapolis, MN, USA). This form of NRG1 contains the EGF domain of NRG1-β1. The monoclonal antibody, bd17, which recognizes both the β2 and β3 (β2/3) $GABA_A$ receptor subunits (Haring *et al*. 1985), was from Roche Molecular Biochemicals (Indianapolis, IN, USA), and antibodies against ErbB2, ErbB3 and ErbB4 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against MAPK (p44/p42), phosphorylated MAPK (p44/42 MAPK Thr202/Tyr204; E10), phosphorylated Akt (Ser 473) and Akt were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The inhibitors U0126 and PD98059 were from Cell Signaling Technology, Inc., AG1478 was from Calbiochem (San Diego, CA, USA), and roscovitine and LY-294002 were from A.G. Scientific, Inc. (San Diego, CA, USA).

Tissue culture and drug treatments

Cerebellar granule cells were prepared for culture from Sprague-Dawley rats (Zivic Miller, Zelienople, PA, USA) at postnatal day 10 (P10) using previously described procedures (Behringer *et al*. 1996; Gault and Siegel 1997). The dissociated cells were plated at 2×10^3 cells/mm^2 onto 100 mm tissue culture plates (for western blots), 24-well culture dishes (for RT-PCR), or 24-well culture dishes containing 13 mm glass cover slips (for immunocytochemistry) coated with 0.1 mg/mL poly-L-lysine and 5 mg/mL laminin. The cells were maintained in a chemically defined medium composed of Neurobasal medium (Gibco-BRL, Grand Island, NY, USA) supplemented with B-27 (Gibco-BRL), 25 mM KCl, 6.0 g/L dextrose, 2 mM glutamine, 0.1 U/mL penicillin and 0.1 mg/mL streptomycin. Using this plating protocol, cultures contained approximately 95% granule neurons (Behringer *et al*. 1996).

Cells were treated with NRG1 (1–8 nM) beginning on the second day in culture. ErbB4 receptor activation was blocked by treatment with AG1478 (20 μM), and receptor expression was inhibited by addition of an ErbB4 antisense oligodeoxynucleotide (ODN; 3.5 μM) for 3 days beginning at plating. The antisense ODN (5′-CGTCGCCAGCTTCATTT-3′; Sigma/Genosys, The Woodlands, TX, USA) contained phosphorothioate bonds at both ends to enhance cell uptake (Pilowsky *et al*. 1994) and did not resemble any other sequence in the GenBank database. Sister cultures were treated with the corresponding sense ODN to confirm the specificity of the effects of the antisense ODN. Signaling through the MAPK, PI3K or cdk5 pathways was selectively blocked with U0126 (10 μM), LY-294002 (10 μM) or roscovitine (20 μM), respectively. Inhibitors were added to the cultures 30 min before NRG1 treatment and remained in the medium for the entire treatment period.

To confirm that cell survival and viability were not altered by any treatment, control and treated cultures were fixed with 4% formaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 7.9 mM Na_2HPO_4, pH 7.4) for 20 min at room temperature. The cells were stained with 0.4% cresyl violet, and total cell number was counted in 20 fields on two plates for each condition.

mRNA preparation and RT-PCR

Relative levels of $GABA_A$ receptor subunit mRNAs in control and NRG1-treated cultures were examined using a semi-quantitative RT-PCR protocol essentially as described previously (Behringer *et al*. 1996; Gault and Siegel 1997). In brief, total cellular RNA isolated from cells on the third day in culture was harvested in TRIzol (Gibco-BRL), reverse transcribed, and processed for PCR using α1, β2, β3 and γ2 subunit-specific primers in buffer containing [^{32}P]dCTP. The expression of 18S RNA was also quantified as an internal control. PCR products were separated on 8% non-denaturing polyacrylamide gels that were then dried and exposed to a Molecular Dynamics (Sunnyvale, CA, USA) Phosphor Screen. Band intensities were quantified using ImageQuant Software (Molecular Dynamics), and subunit mRNA levels were expressed as a ratio of the $GABA_A$ receptor PCR product to the 18S PCR product.

Western blot analysis

Cells were harvested by scraping in 25 mM Tris-HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, and centrifuged at 300 *g* for 7 min at 4°C. The cell pellet was homogenized in 0.2 mL homogenization

buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/mL leupeptin, 0.1% aprotinin, 1 mM iodoacetamide, 200 μg/mL bacitracin and 20 μg/mL soybean trypsin inhibitor) for 10 min on ice, followed by sonication for 15–20 s. The protein concentrations in the cell lysates were measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). For western blotting, samples (20 μg protein/lane) were separated on 10% sodium dodecyl sulfate (SDS) gels and then transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in PBS containing 0.05% Tween 20 for 1 h and then incubated overnight at 4°C with antibodies for $GABA_A$ receptor β2/3 subunit (bd17; 1 : 500), phospho-p44/42 and p44/42 MAPK (1 : 1000), phospho-Akt (1 : 1000) and anti-Akt (1 : 1000), or ErbB2, ErbB3 and ErbB4 (1 : 4000). After washing and incubation for 1 h at room temperature with a secondary antibody conjugated with horseradish peroxidase, the membranes were washed and immunoreactive bands were visualized by chemiluminescense (Renaissance Western Blot Chemiluminescense Reagent Plus; NEN Life Science Products, Boston, MA, USA). Relative levels of ErbB4 or $GABA_A$ receptor β2/3 subunit were compared by analyzing the scanned images using the NIH IMAGE program. Since bd17 recognizes both the β2 and β3 subunits of the $GABA_A$ receptor, and detects two bands on western blots, intensity was quantified in both bands. All studies were performed a minimum of four times using independent cultures.

Immunoprecipitation

For immunoprecipitation, cells in culture were harvested as described above. Cell pellets were solubilized in buffer containing 50 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM NaF, 1 mM Na_3VO_4, 1 mM EGTA, 1 mM EDTA and 1% NP40 for 10 min on ice, followed by sonication for 15–20 s. The pellet was then centrifuged at 14 000 *g* for 30 min at 4°C, and the supernatant fluid was saved as whole cell lysate. Protein A beads (50 μL) were added to each sample overnight to reduce non-specific adsorption. After centrifugation at 14 000 *g* for 20 s, primary antibody (anti-ErbB4, 1 : 1000) was added to the supernatant fluid and incubated for 1 h. Protein A beads were added and incubated for a minimum of 1 h at 4°C. The precipitate was collected after centrifugation at 14 000 *g* for 1 min, washed three times in lysis buffer and then suspended in gel loading buffer. Immunoprecipitated samples were then analyzed on western blots as described above. All experiments were performed a minimum of four times using independent cultures.

Immunocytochemistry

Control and NRG1-treated cultures were processed for MAPK and phosphorylated MAPK staining using previously described procedures (Kumar *et al*. 2001). The cells were fixed with 4% formaldhyde in PBS for 20 min at room temperature, rinsed three times in PBS, and permeabilized by incubation in PBS containing 5% bovine serum albumin (BSA) and 0.3% Triton X-100 (dilution buffer) for 1 h at room temperature. The cells were then incubated in dilution buffer containing primary antibody (1 : 200) for 1 h at room temperature. Controls were processed in dilution buffer lacking antibody. After three rinses in PBS, cells were incubated with a biotinylated species-specific secondary antibody (1 : 100; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature, and then incubated with streptavidin-conjugated horseradish peroxidase for 30 min at room temperature. To detect labeled cells, the samples were further processed as described previously (Raetzman and Siegel 1999) using the tyramide signal amplification (TSA) Fluorescein System (Perkin Elmer, Boston, MA, USA). Immunofluorescence was visualized using a FX-microphot microscope (Nikon, Melville, NY, USA).

Data analysis

All the numerical data are presented as the mean ± SEM. Statistical significance was calculated using the Student's *t*-test to determine whether compared groups are distinct. Differences were considered significant if $p < 0.05$.

Results

In previous studies, we demonstrated that of the six $GABA_A$ receptor subunits abundantly expressed by P6 cerebellar granule neurons in culture, only the β2 subunit was induced by treatment with NRG1 (Rieff *et al*. 1999). Whereas the β2 subunit transcript remains at a relatively low and constant level in control cultures, its expression and that of the encoded polypeptide increased at least twofold when P6 cells were treated for 2 days with NRG1. Because of its importance in conferring sensitivity to GABA, studies were performed to identify the signaling pathways used by NRG1 to trigger the up-regulation of β2 subunit expression.

Induction of β2/3 subunit polypeptide expression by neuregulin 1 is dependent on cell maturity

Since subunit expression in control cultures is age-dependent (Behringer *et al*. 1996; Raetzman and Siegel 1999), initial studies were performed to examine the effects of NRG1 on granule neurons prepared at different stages of cerebellar differentiation. In contrast to cells prepared at P6, β2 subunit expression increases over time in cultures prepared at P10. Although subunit expression is initially low at plating, it rises several fold by 4 days in culture. To test the effects of NRG1 on P10 cells, subunit expression was assessed by RT-PCR and western blots. These studies demonstrated that P10 neurons are responsive to NRG1, but its effects are dependent on time in culture. When NRG1 was added to P10 neurons on day 2, prior to the increase in subunit expression, the growth factor induced a transient increase in the β2 subunit mRNA (Fig. 1a). In contrast, NRG1 failed to induce a change in β3 subunit transcript expression (data not shown). The increase in β2 subunit mRNA was maximal at 6 h and began to decline following 12 h of treatment. NRG1 induced a more prolonged rise in expression of the encoded polypeptide (Fig. 1b). An increase was detectable within 6 h of treatment and was maximal at 12 h. In contrast, NRG1 failed to induce this increase if P10 cells were treated beginning on day 4 in culture, when subunit expression had already peaked. Because of the rapidity and stability of the

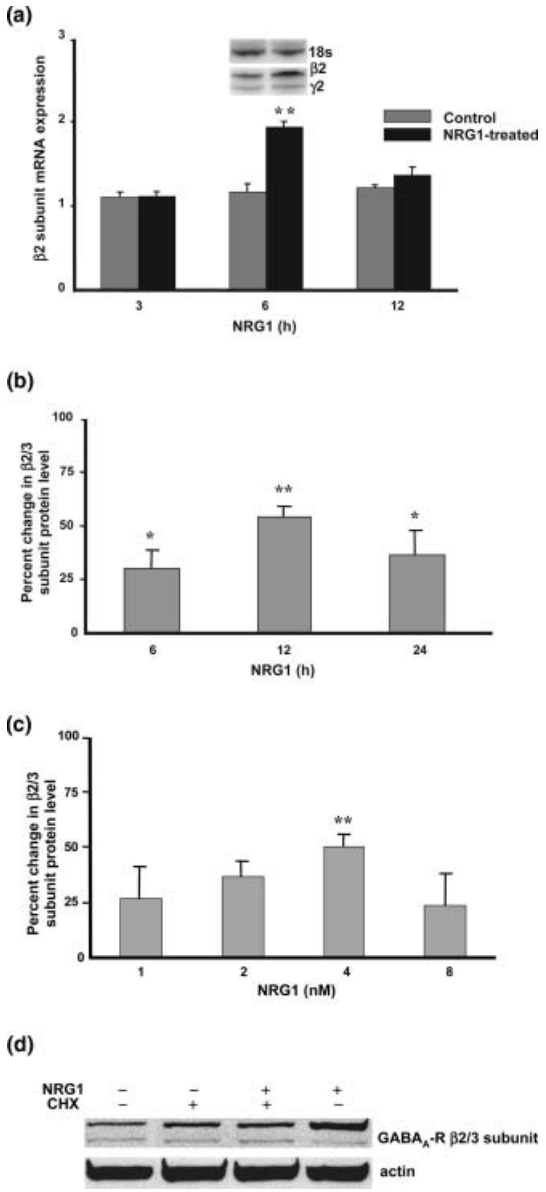

Fig. 1 NRG1 induces $GABA_A$ receptor β subunit mRNA and protein expression in P10 cerebellar granule neurons in culture. (a) Quantitative analysis of $GABA_A$ receptor subunit mRNA expression shows that NRG1 selectively induces the level of the β2 transcript. RT-PCR analysis of subunit mRNA expression in control and NRG1-treated (4 nM for 6 h) cells was performed as described in Experimental procedures and the data were plotted as a ratio of subunit PCR product to 18S PCR product. Inset: representative autoradiograph showing subunit mRNA expression in cultured granule neurons. Values in all histograms represent means ± SEM of at least 4 independent experiments; $^{**}p < 0.01$, $^{*}p < 0.05$ compared with control expression level. (b) $GABA_A$ receptor β2/3 subunit polypeptide expression in cultures treated with 4 nM NRG for 12 h. $GABA_A$ receptor β2/3 subunit expression was quantitated after western blot analysis as described in the Experimental procedures. (c) The effects of NRG1 are dose-dependent. Quantitative analysis of β2/3 subunit expression in cultures treated for 12 h with indicated amounts of NRG1. (d) The effects of NRG1 require protein synthesis. Western blot of β2/3 subunit expression in cells treated with cycloheximide (50 μg/mL) in the presence (+) or absence (–) of NRG (4 nM). After probing for β2/3 subunit expression (top), the membrane was stripped and reprobed with an anti-actin antibody (bottom). Similar results were observed in 3 independent experiments.

effects of NRG1, all subsequent studies examined polypeptide expression in P10 cells maintained in culture for 2 days.

The effects of neuregulin 1 on subunit polypeptide expression are dose-dependent

To investigate the dose dependence of NRG1 on β2/3 subunit polypeptide expression, cultures were treated with peptide concentrations ranging from 1 to 8 nM. Western blots revealed that 4 nM NRG1 induced a maximal increase in β2/3 expression of approximately 50% (Fig. 1c). Moreover, the effect of NRG1 required protein synthesis (Fig. 1d); treatment with the protein synthesis inhibitor, cycloheximide, for 12 h prevented the NRG1-induced increase in subunit expression. Cell number was not altered by this protocol, indicating that treatment with inhibitor did not affect cell viability.

ErbB4 receptor activation mediates the effect of neuregulin 1 on β2/3 subunit expression

Previous studies have shown that the NRGs mediate their effects by binding to members of the ErbB receptor family (Buonanno and Fischbach 2001; Carpenter 2003; Falls 2003) and that these receptors are expressed in the cerebellum (Ozaki *et al*. 1998; Rieff *et al*. 1999). To confirm that ErbB receptors are expressed in P10 granule neurons in culture, western blots were performed. These studies demonstrated that both ErbB3 and ErbB4 are present in P10 granule neurons maintained for 2 days in culture (Fig. 2a); in contrast, ErbB2 was virtually undetectable. Because ErbB3 must heterodimerize with ErbB2 to be activated and ErbB4 receptors can function as homodimers (Carpenter 2003), all subsequent studies focused on examining the subunit-inducing effects of NRG1 mediated by ErbB4 receptor activation.

To demonstrate that ErbB4 receptor activation was required for mediating the effects of NRG1, western blots were performed to examine the relationship between receptor phosphorylation and β2/3 subunit polypeptide expression. These studies demonstrated that 4 nM NRG1 induced ErbB4 phosphorylation in a time-dependent manner (Fig. 2b). The increase was maximal after 30 min of treatment, gradually declined over several hours, and returned to baseline by 10 h. To demonstrate the importance of ErbB4 receptor activity in mediating the effects of NRG1 on subunit expression, the cells were treated with AG1478, a potent and selective inhibitor of ErbB4 protein kinase (Fry 2003). This treatment inhibited NRG1-induced ErbB4 tyrosine phosphorylation (not shown) and blocked its effect on β2/3 subunit expression (Fig. 2c). In addition, AG1478 had no effect on cell viability.

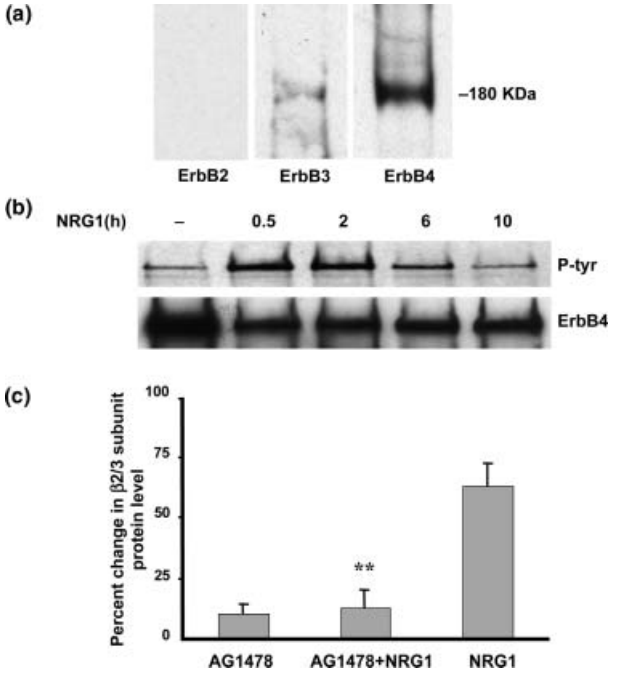

Fig. 2 ErbB4 receptor activation is required for NRG1-induced β2/3 subunit expression. (a) Cerebellar granule neurons express ErbB3 and ErbB4 receptors. Western blot analysis showing receptor expression in cells maintained for 2 days in culture. (b) NRG1 treatment activates the ErbB4 receptor. ErbB4 receptors were immunoprecipatated from cultures treated in the absence or presence of NRG1 for the indicated times as described under Experimental procedures. Immunoprecipitated samples were run on western blots probed with an anti-phosphotyrosine antibody (top) which were then stripped, and reprobed with anti-ErbB4 antibody (bottom). Similar results were observed in 3 independent experiments. (c) Quantitative analysis of β2/3 subunit expression in cells treated with AG1478 (20 μM) in the absence or presence of NRG1 (4 nM). Values represent the mean ± SEM of 4 independent experiments; $^{**}p < 0.01$.

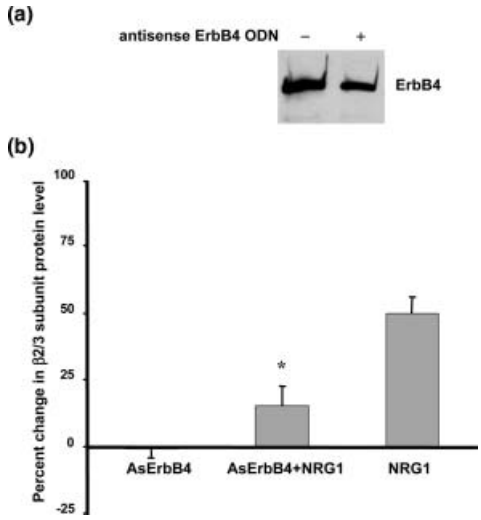

Fig. 3 ErbB4 mediates NRG1-induced β2/3 subunit expression. (a) Western blot of ErbB4 expression in cultures maintained in the absence (–) or presence (+) of the antisense ErbB4 oligodeoxynucleotide (3.5 μM) for 2 days. Similar results were observed in 5 experiments. (b) Quantitative analysis of β2/3 subunit expression in cells treated with antisense ErbB4 in the presence or absence of NRG1. The corresponding sense probe was used as control. Values represent mean ± SEM of 5 independent experiments; $^{*}p < 0.05$.

The importance of ErbB4 in mediating the effects of NRG1 was further tested by reducing its expression with an antisense ODN. After 2 days of treatment, the level of ErbB4 decreased by approximately 45% (Fig. 3a). Moreover, the treatment inhibited the NRG1-induced increase in $GABA_A$ receptor β2/3 subunit polypeptide expression (Fig. 3b). Cell viability and the level of β2/3 subunit expression were not affected by treatment with the ErbB4 ODN, or a corresponding sense probe (not shown), confirming that ODN treatment does not have toxic or proliferative effects.

Multiple intracellular signaling pathways are required for neuregulin 1-induced β2/3 subunit expression

NRG1 regulates expression of the nAChR subunits in the neuromuscular junction following ErbB receptor activation through multiple signaling pathways, including MAPK, PI3K and cdk5 (Altiok *et al*. 1995; Jo *et al*. 1995; Si *et al*. 1996; Tansey *et al*. 1996; Fu *et al*. 2001). Both the nAChR and $GABA_A$ receptor are pentameric in structure and are members of the ligand-gated ion channel family of receptors (Mehta and Ticku 1999; Schaeffer *et al*. 2001). Due to these similarities, studies were performed to explore the possibility that NRG1 acts in the same manner in cerebellar granule neurons to induce $GABA_A$ receptor β2/3 subunit polypeptide expression.

The effects of NRG1 on MAPK signaling were examined by western blotting and immuncytochemistry. Western blot analysis demonstrated that treatment of granule neurons with NRG1 for 1 min produced a marked phosphorylation of MAPK. This effect persisted for about 30 min and then declined (Fig. 4a). The NRG-induced phosphorylation of MAPK was blocked when NRG-induced activation of ErbB4 tyrosine phosphorylation was inhibited by treatment with AG1478 (data not shown). To identify the NRG1-responsive cells, immunocytochemical studies were performed. These studies demonstrated that NRG1 selectively induces the expression of P-MAPK in the granule neurons. Whereas few immunoreactive cells were observed in control cultures, staining was observed in virtually all of the neurons in the NRG1-treated cultures (Fig. 4b). In contrast, staining was not detected in the non-neuronal cells, which comprise approximately 10% of the cultured cell population. Finally, to test the importance of MAPK activation in inducing the effects of NRG, cells were treated with U0126, a potent and specific inhibitor of MEK1 and MEK2 kinases in the MAPK pathway (Favata *et al*. 1998). This inhibitor blocked the effects of

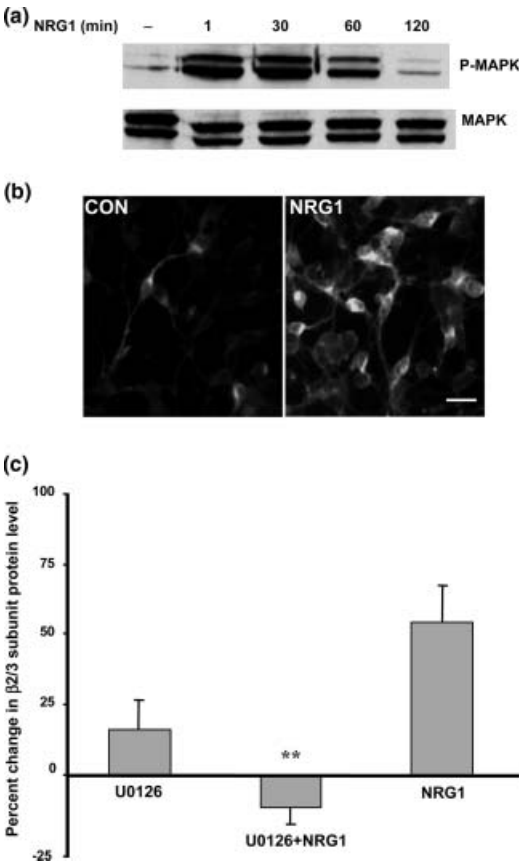

Fig. 4 NRG1 induces β2/3 subunit expression through the MAPK pathway. (a) Western blot of cultures treated with NRG1 (4 nM) for the indicated times probed with anti-pMAPK antibody (top) was stripped and reprobed with anti-MAPK antibody (bottom). Similar results were observed in 4 experiments. (b) Fluorescence micrographs of pMAPK staining in control and NRG1-treated cultures. Scale bar, 10 μm. (c) Quantitative analysis of $GABA_A$ receptor β2/3 subunit expression in cells treated with or without U0126 (10 μM) and NRG1. Values are means ± SEM of 7 independent experiments; $^{**}p < 0.01$ in comparison with NRG1-mediated increase in the absence of the MEK inhibitor.

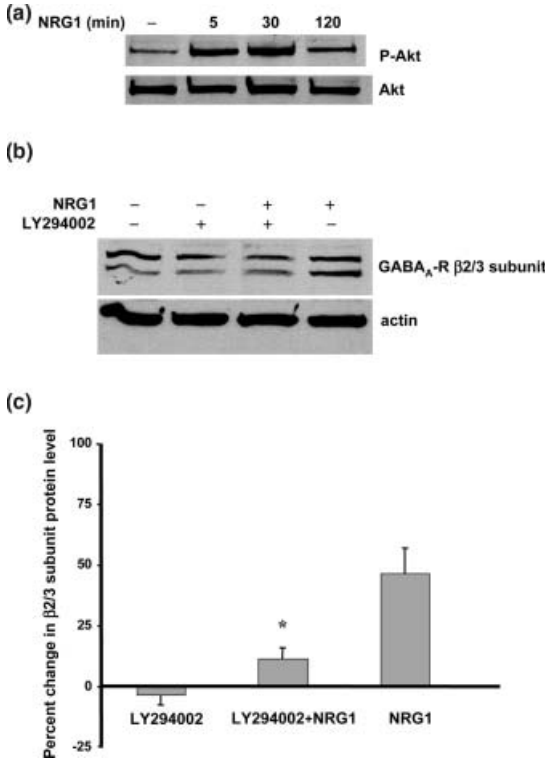

Fig. 5 PI3K pathway activation participates in NRG1-induced β2/3 subunit expression. (a) NRG1 triggers signaling through the PI3K pathway. Western blot of cultures treated with NRG (4 nM) for the indicated times were probed with anti-pAkt (top) and anti-Akt antibodies (bottom). (b) NRG1 signaling through the PI3K pathway induces β2/3 subunit expression. Western blot of β2/3 subunit protein (top panel) was reprobed with the anti-actin antibody (bottom). Similar results were obtained in 3 independent experiments. (c) Quantitative analysis of $GABA_A$ receptor β2/3 subunit expression in cells treated with or without LY294002 (50 μM) and NRG. Values are means ± SEM of 3 independent experiments; $^{*}p < 0.05$ in comparison with NRG-mediated increase in the absence of LY294002.

NRG1 on the induction of $GABA_A$ receptor β2/3 subunit polypeptide expression (Fig. 4c), demonstrating the importance of this pathway. Exposure to U0126 alone did not alter cell number and had no effect on cell morphology (not shown).

Similar studies demonstrated that the PI3K pathway is also important in mediating the NRG1-induced increase in $GABA_A$ receptor β2/3 subunit expression. First, western blots demonstrated that NRG1 activates targets of PI3K. Exposure of the neurons to NRG for 5 min resulted in an increase in Akt phosphorylation that was maximal at 30 min and declined after 2 h (Fig. 5a). NRG-induced Akt phosphorylation was blocked when ErbB4 receptor activation was inhibited with AG1478 (data not shown). To determine whether pathway activation was required to induce subunit expression, the cells were treated with NRG1 in the presence of LY294002, an inhibitor of PI3K (Vlahos *et al*. 1994). Western blots revealed that the inhibitor selectively blocked the effects of NRG1 on β2/3 subunit polypeptide expression (Fig. 5b), reducing it to near background levels (Fig. 5c).

Studies in myotubes have shown that association of cdk5 with ErbB3 is necessary to trigger the effects of NRG. This interaction is required for NRG-induced activation of the MAPK (Fu *et al*. 2001) or PI3K/Akt signaling pathways and induction of nAChR subunit expression (Tansey *et al*. 1996; Li *et al*. 2003). To test whether the cdk5 pathway participates in regulating $GABA_A$ receptor subunit expression in the CNS, the cultures were treated with roscovitine, a selective inhibitor of cdk5 (De Azevedo *et al*. 1997). Roscovitine reduced the effects of NRG1 on $GABA_A$ receptor β2/3

subunit expression in a concentration-dependent manner (Fig. 6a). At a concentration of 40 μM, the inhibitor completely blocked induction of subunit expression (Fig. 6b), To demonstrate that the effects of roscovitine were specific, additional studies were performed to demonstrate that NRG1-stimulated ErbB4 phosphorylation was not altered by this treatment (Fig. 6c). In addition, roscovitine had no effect on NRG1-stimulated phosphorylation of MAPK and Akt (Fig. 6d), findings indicating that signaling through neither pathway was altered.

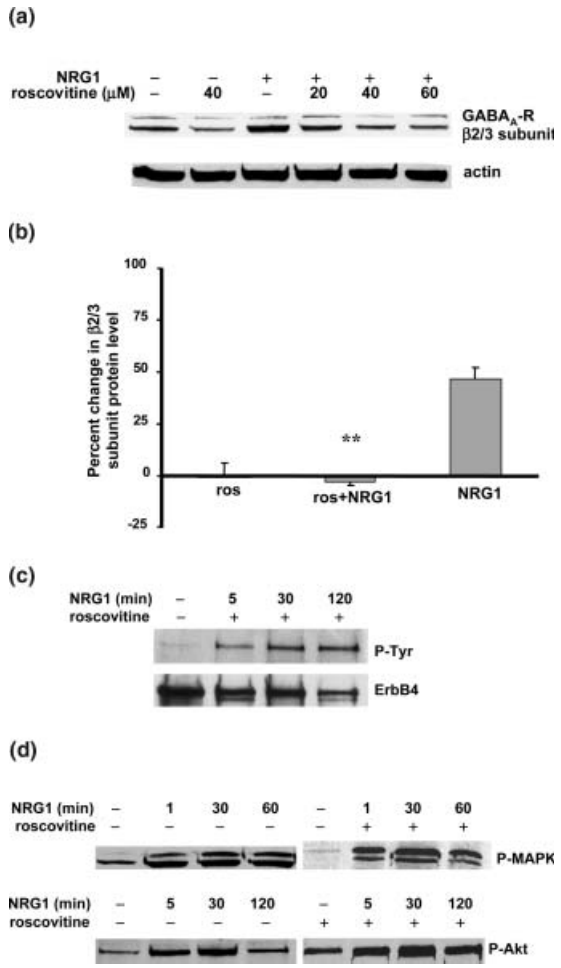

Fig. 6 Cdk5 is required for mediating NRG1-induced β2/3 subunit expression. (a) Representative western blot (top) of samples from cultures treated with the indicated concentrations of roscovitine and/or NRG. After β2/3 subunit protein was examined, the membrane was stripped and reprobed with the anti-actin antibody (bottom). (b) Quantitative analysis of $GABA_A$ receptor β2/3 subunit expression in cells treated with or without roscovitine (40 μM). Values are means ± SEM of 4 independent experiments; $^{**}p < 0.01$. (c) Roscovitine exerts no effect on NRG1-activated ErbB4 phosphorylation. Samples from cultures treated in the absence or presence of NRG1 (4 nM) and roscotivtine for the indicated times were analyzed on western blots probed with antibodies to phosphotyrosine and ErbB4. Similar results were obtained in 3 experiments. (d) Roscovitine fails to block the MAPK or Akt pathways. Samples from cultures treated in the absence or presence of NRG1 (4 nM) and roscovitine were analyzed on western blots probed for MAPK (top) or PI3K (bottom) activity. Similar results were obtained in 3 independent experiments.

Discussion

In this report we demonstrate that NRG1 induces a time- and concentration-dependent change in $GABA_A$ receptor β2/3 subunit expression in freshly dissociated P10 cerebellar granule neurons in culture. Its effects on subunit expression are initiated by activation of the ErbB4 receptor. NRG1 then triggers several intracellular signaling pathways to induce its effects; inhibitors selective for the MAPK, PI3K and cdk5 cascades all blocked the up-regulation of β2/3 subunit polypeptide expression.

Together with our earlier studies, the current findings demonstrate that the effects of NRG1 on $GABA_A$ receptor β2/3 subunit expression are dependent on cerebellar granule neuron maturity and differentiation in culture. Whereas 2 days are required for NRG1 to induce an increase in β2 subunit expression in cells prepared at P6 (Rieff *et al*. 1999), its effects are more rapid at P10. These findings suggest that NRG1, which is present in mossy fibers that innervate granule neurons *in vivo* (Ozaki *et al*. 2000), accelerates the differentiation of neurons already committed to express the $GABA_A$ receptor phenotype. Whether NRG1 activates the same signaling pathways to induce subunit expression in cells at P6 and P10 remains to be determined.

Our findings demonstrate that different NRG1 fragments can stimulate $GABA_A$ receptor β2/3 subunit expression. In this study, we used a 71-residue NRG1 peptide containing the β1 EGF-like domain. This peptide stimulates ErbB4 receptor signaling and induces expression of $GABA_A$ receptor β2/3 subunit expression in cerebellar granule neurons. This NRG1 peptide has also been shown to induce nAChR subunit expression in myocytes (Ozaki *et al*. 1997; Si *et al*. 1998; Fu *et al*. 2001). In our previous study, we found that a NRG1 peptide containing both the Ig- and EGF-like domains induced subunit expression in P6 cells (Rieff *et al*. 1999). Because these two NRG1 fragments are effective at similar concentrations (1–4 nM), it is likely that the EGF-like domain is sufficient to stimulate the ErbB4 receptor, in agreement with previous reports (Ozaki *et al*. 1997; Si *et al*. 1999).

Consistent with earlier findings (Rio *et al*. 1997; Garcia *et al*. 2000; Huang *et al*. 2000; Buonanno and Fischbach 2001), our studies demonstrate that the effects of NRG1 require ErbB receptor activation. Although other investigators reported that granule neurons express all three ErbB receptor subtypes (Ozaki *et al*. 1998), we found that P10 cerebellar granule neurons maintained for up to 6 days in

culture express only ErbB3 and ErbB4. ErbB3 is probably not important in the function of NRG1 in our cultures because ErbB3 can signal only as a heterodimer with ErbB2, a receptor not detected in our cells. Consequently, our studies focused on NRG1-induced activation of ErbB4, which can function as a homodimer. Since selective blockade of ErbB4 receptor activation or down-regulation of its expression inhibited the effects of NRG1, we conclude that this receptor is responsible for regulating $GABA_A$ receptor β2/3 subunit expression in P10 cultured cerebellar granule neurons.

NRG1-induced regulation of $GABA_A$ receptor β2/3 subunit expression in cerebellar granule neurons requires at least three signaling pathways, including MAPK, PI3K and cdk5. This suggests that signals from these three pathways converge, possibly at the level of $GABA_A$ receptor β2 subunit gene transcription. The details of how ErbB4 activates these pathways and how they converge to regulate $GABA_A$ receptor β2/3 subunit expression remain a subject for further study. Our data, however, allow us to rule out some possibilities. Since inhibiting the cdk5 pathway did not affect ErbB4 activation or MAPK and PI3K signaling, it is possible that all three pathways are activated in parallel. Alternatively, cdk5 signaling may lie downstream of the other two pathways. Both scenarios are different from the one reported for myocytes, where inhibition of cdk5 signaling interferes with ErbB activation by NRG (Fu *et al*. 2001). This difference could reflect the fact that these tissues express different ErbB receptors. Whereas the effects of NRG on the neuromuscular junction are mediated by ErbB2/ErbB3 heterodimers, and cdk5 regulates receptor activation by phosphorylating ErbB3, its effects on granule neurons occur through ErbB4, which may not be regulated by cdk5.

These studies provide novel information on the mechanisms underlying the effects of NRG1 on $GABA_A$ receptor β2/3 subunit expression in cerebellar granule neurons. Our understanding of this complex regulatory process, however, is far from complete. Whereas the increase in MAPK and PI3K activity following treatment with NRG1 is transient and peaks within 30 min of stimulation, a detectable change in subunit expression requires several hours. Whether this delay reflects the time required for new subunit synthesis, or the synthesis and activation of additional signaling proteins and transcription factors, remains to be determined. Studies currently underway should further unravel this process and identify some of the downstream signaling events.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (NS34317).

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