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Research report

Acute neuregulin-1 signaling influences AMPA receptor mediated responses in cultured cerebellar granule neurons

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ABSTRACT

Neuregulin-1 (NRG1) is a trophic and differentiation factor that signals through ErbB receptor tyrosine kinases to regulate nervous system development. Previous studies have demonstrated that NRG1 affects plasticity at glutamatergic synapses in principal glutamatergic neurons of the hippocampus and frontal cortex; however, immunohistochemical and genetic analyses strongly suggest these effects are indirect and mediated via ErbB4 receptors on GABAergic interneurons. Here, we used cultured cerebellar granule cells (CGCs) that express ErbB4 to analyze the cell-autonomous effects of NRG1 stimulation on glutamatergic function. These cultures have the advantage that they are relatively homogenous and consist primarily of granule neurons that express ErbB4. We show that acute NRG1 treatment does not affect whole-cell AMPA or NMDA receptor (NMDAR) mediated currents in CGCs at 10–12 days *in vitro*. NRG1 also does not affect the frequency or amplitude of spontaneous AMPAR or NMDAR mediated miniature excitatory post-synaptic currents (mEPSCs). To further investigate the effects of NRG1 on activity-dependent plasticity of glutamatergic synapses in CGCs, we characterized the effects of high-glyine/0 Mg^{2+} (which activates synaptic NMDARs) on AMPAR-mEPSC frequency and amplitude. We show that high-glycine induces a form of chemical long-term potentiation (chemLTP) in CGCs characterized by an increase in AMPAR-mEPSC frequency but not amplitude. Moreover, NRG1 induces a decrease in AMPAR-mEPSC frequency following chemLTP, but does not affect AMPAR-mEPSC amplitude. CGCs in our cultures conditions express low levels of GluR1, in contrast to dissociated hippocampal cultures, but do express the long isoform of GluR4. This study provides first evidence that (1) high-glycine can induce plasticity at glutamatergic synapses in CGCs, and (2) that acute NRG1/ErbB-signaling can regulate glutamatergic plasticity in CGCs. Taken together with previous reports, our results suggest that, similar to Schaeffer collateral to CA1 synapses, NRG1 effects are activity dependent and mediated via modulation of synaptic AMPARs.

1. Introduction

Neuregulin-1 (NRG1) is a trophic and differentiation factor that signals through ErbB receptor tyrosine kinases to regulate nervous system development and function (reviewed in [1,2,10]). In the developed nervous system, neuregulin signals through ErbB4 to influence transmission and plasticity at glutamatergic synapses [3,15,17,34]. Specifically, acute activation of NRG1/ErbB4-signaling pathways inhibits both the induction and maintenance of LTP at CA3 → CA1 synapses [15,17,34]. Given the well-established requirement of CA3 → CA1 LTP in hippocampal-dependent learning (reviewed in [41]), understanding the mechanisms through which NRG1 regulates plasticity at these synapses has important implications. ErbB4 is abundantly expressed in GABAergic interneurons but expression is undetectable in principal glutamatergic neurons of the hippocampus and frontal cortex [9,30,31,45]; moreover, ablation of the ErbB4 gene in GABAergic interneurons, but not pyramidal neurons, blocks the inhibition of LTP induction by NRG1 [3]. Therefore, NRG1-dependent effects on LTP are indirect in that they do not require ErbB4 signaling in CA1 pyramidal cells. Several lines of evidence support the idea that NRG1/ErbB4-signaling directly regulates postsynaptic function of glutamatergic synapses on ErbB4-expressing neurons. First, ErbB4 colocalizes with post-synaptic AMPA and NMDARs [13,25,32] and the carboxyl-terminal

Abbreviations: AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; CGCs, cerebellar granule cells; chemLTP, high-glycine/0 Mg^{2+}-induced LTP; KA, kainate; LTP, long-term potentiation; mEPSC, miniature excitatory post-synaptic currents; NMDA, N-methyl-D-aspartic acid; NRG1, neuregulin-1; PSD-95, post-synaptic density 95; PDZ, post-synaptic density 95-Drosophila disc large tumor suppressor-zonula occludens-1.

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PDZ-ligand domain of ErbB4 interacts directly with members of the family of MAGUK-type synaptic scaffolding proteins including PSD-95/SAP-90 [13,14], indicating that ErbB4 receptors are positioned to directly modulate AMPA and NMDARs and/or associated regulatory proteins. Second, deletion of ErbB4 in PV-positive interneurons inhibits formation of functional glutamatergic synapses on these neurons [43], demonstrating that ErbB4 controls glutamatergic synapse development. Although central to understanding NRG1-dependent regulation of glutamatergic function and plasticity, the direct and acute effects of NRG1/ErbB4-signaling on the function of glutamatergic synapses and/or glutamate receptors in ErbB4-expressing neurons have been difficult to establish in hippocampal and/or cortical preparations.

In this study, we used cultured cerebellar granule cells (CGC) to test the hypothesis that acute NRG1-signaling influences NMDAR and AMPAR-mediated responses in ErbB4-expressing neurons. In the context of the current study, CGCs provide the following experimental advantages: (1) CGCs express ErbB4 and are capable of responding to NRG1: for example, chronic NRG1/ErbB4-signaling effects have been characterized, and include enhanced neurite outgrowth and enhanced expression of NR2C and $GABA_A\beta2$ subunits, as well as activation of PI_3-kinase and cdk5 [29,36,37,47,48]; (2) CGCs are well-characterized with regard to developmental and subcellular expression of AMPAR and NMDAR subtypes [5,8,11], (3) CGCs are relatively homogenous, consisting of >95% granule neurons which form glutamatergic connections in culture, and (4) CGCs are small with few dendrites, allowing for high resolution recordings of AMPAR and NMDAR medicated responses using whole-cell voltage-clamp techniques [11,24,42].

To determine whether acute ErbB4 stimulation affects glutamatergic transmission in CGCs, we first characterized the effects of NRG-1β on AMPA and NMDAR-mediated whole-cell responses and mEPSCs in CGCs using whole-cell voltage clamp techniques. Because NRG1 effects may be activity-dependent, we also investigated the acute effects of NRG-1β and ErbB-inhibition on AMPAR-mediated responses following chemical LTP induction via high-glycine/0 Mg^{2+}.

2. Experimental procedures

2.1. CGC cultures

A key strength and unique aspect of the research presented in our manuscript using dissociated cerebellar granule cells is that the culture is very homogenous because the ratio of granule cells to other cell types is high. In addition, the small and round morphology of the CGCs are easily differentiated from GABAergic neurons. We detected GAD-67 expression by immunofluorescence in only approximately 5% of cells; Purkinje cells and glia are mostly not viable in the culture conditions used. We also took advantage of the fact that granule neurons are interconnected in these cultures. Following synapse maturation, about 7–8 days in our CGC cultures, functional excitatory synapses can be observed as NMDA and AMPA-mEPSCs in response to spontaneous vesicular release [21,22].

CGCs were prepared as previously described [21] with minor modifications. Cerebella were harvested from 4 to 5 day old C57BL/6J mice. All procedures were approved by the IACUC at the NIH. Following induction of hypothermia, the cerebella from 2 to 3 mice were placed in cold Solution I (HBSS minus Ca^{2+}, Mg^{2+}, HCO_3^- and supplemented with 0.3% BSA and 14 mM glucose, 15 mM HEPES, 4.2 mM $NaHCO_3$, and 1.5 mM $MgSO_4{\cdot}H_2O$). After removing meninges, cerebella were incubated in Solution I containing 0.05% trypsin (Sigma, St. Louis, MO) for 5 min at 37 °C. The solution was replaced with cold Stop Solution (Solution I containing 0.25% soybean trypsin inhibitor [Cat. No. 65035, Calbiochem, LaJolla, CA], and 0.04% DNAse I). Neurons were dissociated in Solution I by trituration on ice. Cells were pelleted and resuspended in CGC media [basal Eagle's medium supplemented with 10% bovine calf serum, 2 mM glutamine (all from Invitrogen, Carlsbad, CA), Primocin (AmaxaBioSystems, Gaithersburg, MD) and 25 mM KCl] and maintained at 37 °C, 5% CO_2. Neurons were plated in 24 well dishes at density of 1×10^6 cell/ml directly onto acid-washed coverslips coated with 50 μg/ml poly-D-lysine. After 5 days *in vitro*, the medium was changed to low (5 mM) KCl to enhance functional synapse formation [minimal essential medium with 5 mg/ml glucose (Invitrogen) supplemented with 10 μM cytosine-arabanofuanoside (Sigma), 2 mM glutamine, insulin transferrin supplement (Sigma), and Primocin (AmaxaBioSystems)].

2.2. Western blot analysis

Hippocampal cultures (prepared as described in [17,18]) and CGC cultures were rinsed with cold PBS and lysed in buffer (50 mM EDTA, 150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100) containing Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN). Lysates were cleared by centrifugation at 12,000 rpm for 5 min at 4 °C using a 5415C microcentrifuge (Eppendorf, Hamburg, Germany). Proteins were quantified using the BioRad Protein Assay Reagent (BioRad, Hercules, CA). Proteins (20 μg/lane) were electrophoretically separated using Novex Tris–glycine 4–12% gradient gels and electroblotted onto nitrocellulose membranes. Immunoblots were blocked (5% nonfat milk in 0.1% Tween-20 in TBS) and separately probed with the following primary antibodies: rabbit polyclonal against the C-terminus of the long isoform of GluR4 (1:1000, Cat No. 06-308, Millipore, Billerica, MA); rabbit polyclonal anti-GluR1 (1:1000, Cat No. AB1504, Millipore); rabbit monoclonal anti-ErbB4 mAB-10 (1 μg/ml [45]); polyclonal anti-NR2B (1:1000, Cat No. 06-600, Millipore); rabbit polyclonal anti-NRG1 (1:500, Cat No. 348, Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-clathrin heavy chain (1:2000, clone TD.1, Cat No. sc-12734, Santa Cruz). Blots were extensively washed in TBS-T and then incubated with either donkey-anti-rabbit or sheep-anti-mouse IgG conjugated to HRP. Signals were visualized with the ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ). As an additional loading control, blots were stripped and re-probed with antibodies for GAPDH (0.5 μg/ml, Millipore), α-tubulin (Cell Signaling Technology, Beverly, MA) or clathrin heavy chain (1:2000, Cat No. 12734, Santa Cruz).

2.3. Electrophysiology

We used standard whole-cell voltage clamp techniques to assess AMPA and NMDAR-mediated responses. All recordings were performed at room temperature from CGCs at 9–12 days *in vitro*. We used CGCs > 9 day *in vitro* because AMPA and NMADAR-mediated mEPSCs were rarely observed in younger cultures. Electrodes were made from thin-wall borosilicate glass capillaries (Wiretrol II; Drummond) with a two-stage vertical puller. Recording electrodes were filled with intracellular solution containing (in mM): 0.6 EDTA, 5 ATP Mg Cl_2, 0.2 GTP, 145 potassium gluconate, 10 HEPES, pH 7.2 with KOH and adjusted to 300 mOsM with sucrose. Typical pipette resistances were 3–5 MΩs. Whole-cell voltage-clamp recordings were performed using an Axopatch 200B capacitor-feedback patch clamp amplifier (Axon CNS Molecular Devices, Sunnyvale, CA) at a holding potential of −60 mV. Currents were filtered at 1 kHz and digitized at 10 kHz using a Dell computer equipped with Digidata 1322 analogue-to-digital board and pClamp10 software (Axon CNS Molecular Devices). Access resistance was monitored throughout the recording using transient current responses to hyperpolarizing 5 mV pulses. Off-line data analysis and fitting were performed with Clampfit 10.2 (Axon CNS Molecular Devices). CGCs with capacitance >10 pF (unlikely to represent CGCs) or exhibiting a change in access resistance >10% were excluded from the analysis.

NMDAR and AMPAR-mEPSCs were identified from continuous recordings using Clampfit 10 (Axon CNS Molecular Devices) and the software's event detection template matching functions. If AMPAR-mEPSC frequencies were <0.04 Hz at baseline recordings were terminated and/or omitted from the analysis. NMDAR-mEPSC and AMPAR-mEPSC decays were fit using Clampfit 10 (Axon CNS Molecular Devices) from averages of 1 to 20 consecutive events selected using the software's event detection template matching functions. Current decays were fit using a single-exponential equation [$I(t) = I \times \exp(-t/\tau)$].

2.4. Recording solutions

Glass cover slips with CGCs or hippocampal neurons were placed in a recording chamber (total volume ~0.5 ml) with a solution exchange rate of 5 ml/min. Control bath extracellular solution (ECF) contained (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, 5 glucose, as well as 10 μM D-serine and 500 nM TTX (all from Sigma) and adjusted to 325 mOsM with sucrose and pH 7.4. Recording solutions used to isolate AMPAR and NMDAR-mediated whole-cell currents and mEPSCs were delivered using a gravity-fed Y-tubing system as described [28]. The outflow of the Y-tubing system was placed approximately 50 μm from the recorded cell. Throughout the experiment, cells were continually perfused through the Y-tubing system with either control ECF or specified recording ECF. For recording of whole-cell NMDAR-currents, NMDA (200 μM, Tocris, Ellisville, MO) was applied in Mg^{2+}-free ECF also containing 500 nM TTX (Calbiochem), 50 μM bicuculline (to block $GABA_A$ receptors, from Tocris). NMDAR-mediated mEPSCs were recorded in Mg^{2+}-free ECF also containing 500 nM TTX, 50 μM bicuculline, 20 μM D-serine, and 5 μM NBQX (to block AMPARs) as described [35]. To record whole-cell AMPAR-mediated currents, kainate (KA) was applied in ECF also containing 1 mM $MgCl_2$, 500 nM TTX, and 50 μM bicuculline. To verify that kainate-evoked responses were mediated by AMPARs, in a subset of cells, GYKI 52466 (50 μM), an AMPAR specific receptor antagonist at this concentration, was applied prior to and during kainate application. AMPAR-mediated mEPSCs were recorded in ECF containing 1 mM $MgCl_2$, 500 nM TTX and 50 μM bicuculline (as described in [21]). For treatment with high-glycine/0 Mg^{2+}, cells were exposed to high-glycine solution (ECF containing 200 μM glycine, 0 $MgCl_2$, 2 mM $CaCl_2$, and 0.1 μM strychnine) for 5 min at room temperature. NRG-1β (recombinant EGF-like domain, corresponding to NRG1-β1, amino