Rapid transient isoform-specific neuregulin1 transcription in motor neurons is regulated by neurotrophic factors and axon–target interactions

Jiajing Wang a, Abdelkrim Hmadcha a,b, Vaagn Zakarian a, Fei Song a,c,*, Jeffrey A. Loeb a,c,*

a The Center for Molecular Medicine & Genetics, Wayne State University School of Medicine, Detroit, MI 48201, USA
b Department of Stem Cells, CABIMER–Fundación Progreso y Salud, Sevilla 41092, Spain
c Department of Neurology and Rehabilitation, The University of Illinois at Chicago, Chicago, IL 60612, USA

ARTICLE INFO

Article history:
Received 26 August 2013
Revised 16 March 2015
Accepted 21 April 2015
Available online 22 April 2015

Keywords:
Neuregulin1
Neurotrophic factor
Brain-derived neurotrophic factor
Transcriptional regulation
Motor neuron

Abstract

The neuregulins (NRGs) are a family of alternatively spliced factors that play important roles in nervous system development and disease. In motor neurons, NRG1 expression is regulated by activity and neurotrophic factors, however, little is known about what controls isoform-specific transcription. Here we show that NRG1 expression in the chick embryo increases in motor neurons that have extended their axons and that limb bud ablation before motor axon outgrowth prevents this induction, suggesting a trophic role from the developing limb. Consistently, NRG1 induction after limb bud ablation can be rescued by adding back the neurotrophic factors BDNF and GDNF. Mechanistically, BDNF induces a rapid and transient increase in type I and type III NRG1 mRNAs that peak at 4 h in rat embryonic ventral spinal cord cultures. Blocking MAPK or PI3K signaling or blocking transcription with Actinomycin D blocks BDNF induced NRG1 gene induction. BDNF had no effect on mRNA degradation, suggesting that transcriptional activation rather than message stability is important. Furthermore, BDNF activates a reporter construct that includes 700 bp upstream of the type I NRG1 start site. Protein synthesis is also required for type I NRG1 mRNA transcription as cycloheximide produced a super-induction of type I, but not type III NRG1 mRNA, possibly through a mechanism involving sustained activation of MAPK and PI3K. These results reveal the existence of highly responsive, transient transcriptional regulatory mechanisms that differentially modulate NRG1 isoform expression as a function of extracellular and intracellular signaling cascades and mediated by neurotrophic factors and axon–target interactions.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Proper function of the nervous system requires orchestrated communication between neurons and many other cell types. Some of this communication occurs through the regulated release of growth and differentiation factors such as NRG1. Alternative splicing produces both membrane-bound and secreted forms of NRG1 (Falls, 2003; Mei and Xiong, 2008) that have been shown to be important in many aspects of nervous system and cardiac development and linked to peripheral nerve injury, heart failure, schizophrenia, multiple sclerosis, and cancer. All NRG1 splice forms share an EGF-like domain necessary and sufficient to activate hetero- and homo-dimeric combinations of ErbB2, ErbB3, and ErbB4 receptors (Esper et al., 2006). NRG1–ErbB signaling has been implicated in regulating Schwann cell survival, growth, differentiation, and myelination (Nave and Salzer, 2006; Ma et al., 2011), for modulating the expression of acetylcholine receptors at the neuromuscular junction (NMJ) (Li et al., 2004; Schmidt et al., 2011; Ngo et al., 2012), and inducing muscle spindle differentiation (Hippchenmeyer et al., 2002).

Much of how different NRG1 isoforms are spatially segregated is due to alternative splicing (Falls, 2003; Mei and Xiong, 2008). Most are produced as transmembrane precursors processed through proteolytic cleavage (Kalinowski et al., 2010; La Marca et al., 2011; Luo et al., 2011). Cleavage of type I and type II NRG1 isoforms sheds their extracellular domains producing biologically active soluble forms with an N-terminal, heparin-binding domain (HBD) used for selective cellular targeting to heparan sulfate proteoglycan (HSPG) rich cell surfaces (Loeb et al., 1999; Pankonin et al., 2005; Ma et al., 2009, 2011). Type III NRG1 isoforms have a hydrophobic cysteine-rich domain (CRD) keeping them membrane-tethered and enabling signaling through cell–cell contact (Wang et al., 2001).
The regulatory mechanisms that produce various NRG1 isoforms are not well understood, however, in schizophrenia transcriptional regulation of specific isoforms has been implicated (Stefansson et al., 2002). We have shown that NRG1 expression can be mediated by neurotrophic factors, providing a positive feedback loop with nearby cells (Esper et al., 2006; Ma et al., 2011). At the NMJ, muscle targets produce neurotrophic factors, including BDNF and GDNF (Henderson et al., 1993, 1994) that induce NRG1 mRNA and protein expression (Loeb and Fischbach, 1997) and promote the rapid release of soluble NRG1 from sensory and motor neuron axons in a dose- and time-dependent manner (Esper and Loeb, 2004, 2009). Here, we provide evidence that target-derived neurotrophic factors promote both type I and type II NRG1 expression in developing chick motor neurons in ovo and in mammalian cultured motor neurons. Mechanistically, the effects of BDNF on NRG1 transcription are rapid and transient and require both intracellular signaling cascades and ongoing protein synthesis. These studies are important for understanding the bidirectional communication between motor neurons and muscle targets during development and in pathological conditions.

2. Results

2.1. Axon–target interactions regulate NRG1 mRNA expression

We have previously observed that NRG1 protein and mRNA increases in spinal motor neurons following their birth and migration towards the lateral portion of the developing spinal cord in chicken embryos (Loeb et al., 1999). Using the homeodomain motor neuron marker Islet-1/2, NRG1 protein expression is seen to be highest in those motor neurons that have completed their migration and have extended their axons into the surrounding mesoderm (Fig. 1). These observations suggest that factors provided to outgrowing axons promote NRG1 expression. In order to test for this, unilateral hind limb bud ablation was performed in ovo at E2.5, prior to axon outgrowth into the limb bud (Tosney and Landmesser, 1985) (Fig. 2A, B). With this model, motor axons spiral into a ball in the absence of a target to innervate. After limb bud ablation, but before the period of programmed cell death at E6, NRG1 mRNA levels did not increase on the ablated side as they do on the control side in the lateral portion of the lateral motor column (LMC) that normally innervate the dorsal limb bud (Fig. 2C, D). The weakly positive Islet-1/2 marker is used to label the LMC, which shows no reduction of motor neuron numbers even after ablation (data not shown). This marker was used for double labeling radioactive in situ experiments in Fig. 2C showing decreased mRNA levels in the LMC on the side of the limb ablation (Fig. 2D). Consistently, quantitative RT-PCR (qPCR) using isoform-specific primers showed reduced expression of both type I/II (HBD) and type III (CRD) NRG1, suggesting that axon target interactions are important to induce both of these major NRG1 isoform classes (Fig. 2E).

2.2. Neurotrophic factors can restore NRG1 mRNA expression in motor neurons lacking targets

A lack of neurotrophic support is one possible explanation for the failure of NRG1 mRNA induction following limb bud ablation. Developing muscles provide a range of neurotrophic factors that support motor neuron survival and neuromuscular junction development (Levi-Montalcini and Calissano, 1979; Henderson et al., 1993, 1994). These factors have distinct expression profiles at different developmental stages. Therefore, we asked whether exogenous BDNF, GDNF, or NGF could rescue NRG1 mRNA expression after unilateral limb ablation. This was determined by measuring the ratio of NRG1 mRNA levels in the LMC, on the operated versus control sides of the spinal cord at E6 with or without addition of these factors at E4 (Fig. 3A). While both BDNF and GDNF maintained normal NRG1 mRNA levels in motor neurons that lack targets, NGF failed to rescue expression (Fig. 3A, B). This is consistent with their known presence during development and known actions, since both BDNF and GDNF receptors have been shown to be expressed in developing motor neurons (Henderson et al., 1993; Homma et al., 2003), and muscle- and Schwann cell-derived BDNF and GDNF have been shown to be potent survival factors for motor neurons (Yan et al., 1992; Henderson et al., 1994), whereas NGF and its receptors have little effect on motor system development (Funakoshi et al., 1993; Ip et al., 2001).

2.3. Type I and type III NRG1 mRNAs are rapidly and transiently upregulated by neurotrophic factors in mammalian motor neuron cultures

To address further the mechanism by which neurotrophic factors regulate NRG1 mRNA expression, we utilized an established, rat embryonic ventral spinal cord culture system in which we have previously shown rapid (within 4 h) effects of BDNF and GDNF on NRG1 mRNA levels (Loeb et al., 1999). Using isoform-specific qPCR, we found type I NRG1 mRNA was induced by both BDNF and GDNF, whereas type III NRG1 mRNA was induced to a smaller extent only by BDNF (Fig. 4A). No significant change was observed for type II NRG1 mRNA. Type I NRG1 mRNA peaked at 4 h, but declined rapidly by 6 h, and then returned to baseline 8 h after BDNF application, whereas type III NRG1 was also induced at 4 h, it did not return to baseline until 8 h (Fig. 4B). This difference in kinetics was seen consistently for both type I and type III NRG1 isoforms (n = 3–6). The demonstration that both type I and type III, but not type II, NRG1 mRNA levels are rapidly and transiently induced with BDNF
stimulation, but with distinct temporal profiles, suggest both common and unique regulatory mechanisms.

2.4. TrkB, MAPK and PI3K signaling are required for BDNF-induced NRG1 expression

To explore the signaling pathways involved in the regulation of type I and type III NRG1 mRNA by BDNF, we pretreated cultured cells with specific inhibitors prior to BDNF application. BDNF binds to either the TrkB tyrosine kinase or the low-affinity nerve growth factor receptor p75 and is known to lead to the activation of mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K) and phospholipase C-γ (PLC-γ) (Segal, 2003). Inhibition of TrkB activation by K-252a blocked type I and type III NRG1 mRNA induction by BDNF at 4 h (Fig. 5A, B). Furthermore, inhibition of either MAPK by the MEK inhibitor PD98059 or U0126, or PI3K by LY294002 or Wortmannin, blocked NRG1 mRNA induction by BDNF (Fig. 5C, D). These findings suggest that TrkB, MAPK, and PI3K signaling pathways are all involved in type I and type III NRG1 mRNA induction.

2.5. BDNF-induced NRG1 induction requires new transcription

The increase in mRNA levels could have resulted from either an increased rate of transcription, mRNA stabilization, or both. To distinguish between these, we measured mRNA levels after BDNF-treatment in the presence or absence of the transcription inhibitor Actinomycin D (ActD). Pretreatment with ActD for 30 min completely prevented NRG1 mRNA induction by BDNF, demonstrating that ongoing gene transcription is necessary for BDNF-evoked type I and type III NRG1 mRNA upregulation (Fig. 6A). To address whether the turnover rate of NRG1 mRNA is altered by BDNF, cultures were treated with ActD in the presence and absence of BDNF and mRNA levels were measured as a function of time. Interestingly, type I NRG1 mRNA was relatively short-lived, with half-life of about 1 h, whereas type III NRG1 mRNA was more stable, with twice the half-life of about 2 h (Fig. 6B). Since the presence or absence of BDNF had no clear effects on the turnover ratio of either type I or type III NRG1 mRNA, these findings suggest the most important effect of BDNF is on transcriptional activation rather than on mRNA stability.
Given the importance of transcription, we next investigated the effects of BDNF on the 5' cis-elements upstream to type I and type III NRG1 transcripts in rat embryonic ventral spinal cord cultures. Firefly luciferase constructs were prepared containing the 5' promoter regions flanking the transcription start sites for rat type I (−649 to +5) and type III (−1343 to +31) NRG1 (Fig. 7A). Each of these constructs was co-electroporated with the control vector pRL-TK in order to normalize the NRG1 induced luciferase activity with Renilla luciferase activity. After 24 h, BDNF induced the type I promoter-driven luciferase. While the type-III promoter luciferase activity was induced with the same trend, it did not reach statistical significance (Fig. 7B). These findings suggest that cis-acting elements contribute to type I NRG1 transcription induced by BDNF.

2.6. Protein synthesis is required to maintain type I NRG1 mRNA transcription

The transient nature of NRG1 mRNA induction by BDNF could have important biological implications. Exactly how NRG1 transcription is precisely shut off after 4–6 h after BDNF exposure was assessed using the protein synthesis inhibitor cycloheximide (CHX) in rat embryonic ventral spinal cord cultures with and without BDNF treatment.
3. Discussion

3.1. A reciprocal axon–target feedback loop mediated by NRG1 and neurotrophic factors

Both neuron-derived NRG1 and target-derived neurotrophic factors have been implicated to be important in neuromuscular system development. The functions of these mediators rely not only on the dynamic distribution of their receptors, but also on the temporal and spatial expression profile of the ligands. This diversity allows a multi-directional crosstalk between cells that can be both temporally and spatially restricted, depending on the need biologically. Here we have focused on the extracellular signaling mechanisms that underlie axon–target interactions in the developing spinal cord and limb bud. We show that axon–target interactions promote NRG1 expression in spinal motor neurons and that this effect can be replicated by target-derived neurotrophic factors, such as BDNF and GDNF. In vitro, we show that BDNF induces a rapid, yet transient upregulation of NRG1 mRNA that works through new transcription and is mediated by TrkB–MAPK and TrkB–PI3k signaling pathways as well as by distinct 5′ regulatory cis-elements. Both MAPK and PI3k signaling pathways have been shown to be activated by GDNF in different circumstances as well (Airaksinen and Saarma, 2002). The rapid (4 h) nature of the induction may be important to increase transcription in only those axons that make proper contact with their target. The transient nature of the induction (sharply falls after 4 h) requires protein synthesis and is associated with a rapid shut down of the TrkB signaling cascades. Biologically, this could be important to limit NRG1 expression in axons that do not have sustained contact with their destined targets. For secreted (type I) forms of NRG1, in addition to these transcriptional mechanisms, we have previously shown that BDNF can work posttranslationally to promote the local release of NRG1 from axons via a mechanism that requires protein kinase C-δ (Esper and Loeb, 2004, 2009).

In addition to survival, target-derived neurotrophic factors have been implicated in supporting motor neuron function by eliciting transcriptional and translational changes (Chowdary et al., 2012). In the present study, deprivation of target-derived neurotrophic factors by unilateral removal of developing limb buds prior to the period of programmed cell death did not lead to programmed cell death, but did lead to changes in two out of the three major spliced forms of NRG1. How neurotrophic factor signaling in axons is relayed to motor neuron nuclei is not entirely clear, but has been extensively studied (Segal, 2003; Zweifel et al., 2005). Neurotrophic factor signaling pathways can also differ depending on the cellular localization of their receptors (Chowdary et al., 2012). For example, retrograde NGF–TrkA signaling

![Fig. 5. Trk receptor, MEK, and PI3K inhibitors block the effects of BDNF on NRG1 mRNA. Rat embryonic ventral spinal cord cultures were exposed to K-252a (200 nM) (A, B) or PD98059 (10 μM), U0126 (10 μM), LY294002 (50 μM), or Wortmannin (200 nM) (C, D) for 30 min prior to adding BDNF (100 ng/ml) for 4 h. The inhibitor pretreatment resulted in the abrogation of BDNF induction on both type I and type III NRG1 compared to the diluents alone (no inhibitor). Data are reported as the mean ± SEM of at least three independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001, ns not-significant, two-way ANOVA followed by Bonferroni post hoc test by comparison to control.](image-url)
on axons results in Erk5 activation to mediate NGF pro-survival signaling in dorsal root ganglion cells, while activation of TrkA in the cell bodies of these neurons uses Erk1/2 activation (Watson et al., 2001). It has been reported that Akt can be phosphorylated by PDK1 at threonine 308 (Thr308) in the activation loop of the kinase and mTORC2 complex at serine 473 (Ser473) in the hydrophobic motif in different circumstances (Vanhaesebroeck and Alessi, 2000; Sarbassov et al., 2005). Thus, future studies could explore the activation dynamics of different phosphorylation sites within Akt signaling pathway upon BDNF. Another level of complexity is that there are a number of seemingly redundant neurotrophic factors that can converge on common pathways.

3.2. The complexity of NRG1 gene structure and isoform-specific expression

The human NRG1 gene is one of the longest and complex genes in the genome that is located on chromosome 8p12 and spans over 1 M bp. Recent studies from the ENCODE project have shown regions within this gene that have DNase hypersensitivity implicating the presence of cis-regulatory elements (Rosenbloom et al., 2013). One of the highest DNase-sensitive clusters is located —1–3 kb to the transcription start site of type I NRG1. Consistently, this enrichment region has high density of H3K27Ac and H3K4Me3 histones and transcriptional factor binding sites, determined by ChIP-seq analysis. All of these markers suggest that the —4–5 kb DNA fragment might be important regulatory regions for type I NRG1 expression (Rosenbloom et al., 2013). Consistently, we showed that BDNF can induce transcription of a portion of this cis-element. It would be worthwhile to investigate if this region, especially the region within the first intron that has not been analyzed before (Frensing et al., 2008; Liu et al., 2011), is responsible for the binding of regulatory factors induced by neurotrophic factors signaling.

Alternative splicing produces a daunting array of NRG1 proteins that have been actively investigated over the years (Falls, 2003). In this study we focused on 3 major protein forms that have been shown to have important differences in how and where they signal. For example, type I and type II forms both produce secreted proteins that have heparin-binding domains, but type II has an additional N-terminal Kringle domain of unclear function. Despite the similarity, we found that BDNF had no effect on type II NRG1 transcription, but affected both type I and type III. Type III NRG1 isoforms are unique in that they have a second membrane-spanning region thought to activate its receptors through direct cell–cell interactions (Wang et al., 2001). While BDNF induced both type I and type III NRG1 forms by 4 h, there were differences...
in both the magnitude of the effect and the kinetics. Type III NRG1 induction by BDNF was lower, but stayed turned on longer (over 6 h) and was more slowly degraded than type I NRG1. In addition, the BDNF effect on the 5′ cis-regulatory type III elements was less strong than for type I NRG1 at different time points (data not shown), suggesting the requirement of additional regulatory regions or additional mechanisms. A functional interaction between these two promoters also cannot be ruled out. Finally, the mechanism requiring protein synthesis that shuts off type I NRG1 expression does not occur for type III forms as cycloheximide had no effect on type III NRG1. Taken together, each splice form has transcriptional and posttranscriptional regulatory machinery that can fine-tune its expression leading to a dynamic, spatially and temporally unique pattern of NRG1 signaling in motor neurons. The distinct regulatory patterns of neurotrophic factors exert their differential effects on different isoforms of NRG1 provide a level of precision and complexity. This is because modes of function of the isoforms are complementary, with diffusion and binding to HSPGs for type I versus cell–cell contact for type III; and because their respective targets can be different.

3.3. Wider roles for NRG1–BDNF reciprocal signaling in the nervous system

NRG1, neurotrophic factors, and their respective receptor systems have all been shown to play critical roles in many aspects of nervous system development and in disease. For example, both signaling pathways are involved in long-term potentiation (LTP) in the hippocampus, however, studies have shown opposite effect with BDNF facilitating and NRG1 suppressing LTP (Patterson et al., 1996; Huang et al., 2000; Chen et al., 2010). The expression of BDNF and NRG1 are tightly regulated by neuronal activity both at the neuromuscular junction (Loeb et al., 2002) and in the CNS (Flavell and Greenberg, 2008; Liu et al., 2011). However, the link between these two signaling pathways in the CNS has not been investigated. Thus, similar regulatory machinery might exist in the CNS as we have found peripherally here. Models of epilepsy have also been used to show activity dependence. Activity-dependent transcription of BDNF has been shown to promote epileptogenesis (He et al., 2004) and NRG1 mRNA increases in the hippocampus after a single electrical stimulation-induced seizure (Tan et al., 2012). Finally, NRG1 has recently been implicated in models of chronic pain after nerve injury (Calvo et al., 2010) and in both human tissues and an animal model of ALS through activation of microglia (Song et al., 2012). Understanding extracellular signaling through neurotrophic factors as well as the intracellular signaling cascades that promote NRG1 expression could therefore be used to develop therapeutic targets for these disorders.

4. Materials and methods

4.1. Reagents

The following reagents were purchased from Sigma-Aldrich: K-252a (K1639, dissolved in DMSO), PD98059 (P215, dissolved in DMSO), LY294002 (L9908, dissolved in DMSO) and Wortmannin (W1628, dissolved in DMSO), Actinomycin D (A1410, dissolved in DMSO), Cycloheximide solution (C4859). U0126 (#9903, dissolved in DMSO) was obtained from Cell Signaling Technology.

4.2. Chick eggs, unilateral limb ablation and in ovo treatment

Fertilized chicken eggs were obtained from Michigan State University Poultry Farms and incubated in a Kuhl rocking incubator at 50% humidity. Limb buds were removed unilaterally on E2.5 and embryos...
were returned to the incubator until indicated times as previously described (Tosney and Landmesser, 1985). Any unsuccessful ablations, that did not yield an absent limb, were not used for the study. Recombinant BDNF, GDNF (Amgen), or NGF (Life Technologies) was added as described previously (Loeb et al., 2002; Ma et al., 2009). In brief, 1 μg/embryo/day of BDNF, GDNF or NGF were prepared in saline containing 0.2% BSA, respectively and added onto the choorioallantoic membrane for two consecutive days. Embryos were collected at indicated times and processed for total RNA, or in situ hybridization combined with immunofluorescence staining as described below. Staging of chick embryos was determined according to Hamburger–Hamilton (HH) stage series (Hamburger and Hamilton, 1951): E3 (stage 18–19); E4 (stage 23–24); E5 (stage 26–27); E6 (stage 28–29).

4.3. Immunofluorescence staining and in situ hybridization

Embryos were fixed in 4% paraformaldehyde in PBS at 4 °C overnight, washed in PBS, equilibrated in 30% sucrose, and sectioned into 20 μm sections. Neuregulin was labeled using 1310 antibodies against the proNGR1 precursor cytoplasmic tail (Loeb et al., 1999). RT-97 (1:50) and Islet-1/2 (1:50) were obtained from Developmental Studies Hybridoma Bank, University of Iowa, to label neurofilament and spinal cord motor neurons, respectively. Sections were incubated with antibodies in blocking solution (10% normal goat serum, 0.1% Triton X-100 in PBS) overnight at 4 °C, followed by incubation with the corresponding goat anti-mouse or anti-rabbit Alexa Fluor antibodies (1:500; Life Technologies) for visualization. Radioactive in situ hybridization was performed using 35S-labeled RNA probes as previously described (Beaumont et al., 2012). In brief, sense and antisense 35S-labeled RNA probes were generated from linearized full-length chicken proAβA cDNA clones by in vitro transcription. Probes were purified on NuClean R50 Sephadex columns (Shelton Scientific). Tissues were hybridized at 52 °C overnight, followed by washing, and dehydrated in ethanol. Slides were then dipped in photographic emulsion (Kodak NTB), dried, and exposed for 7 days or longer at 4 °C.

4.4. Imaging and quantitative analysis

Digital images were captured using a Nikon Eclipse E600 microscope with a Princeton Instruments Micromax cooled CCD digital camera. Signal intensities were quantified using Meta Morph image analysis software (Molecular Devices). Spinal cord lateral motor columns (lateral portions) were defined using a double labeling immunofluorescence and radioactive in situ hybridization protocol by first labeling the sections with Islet-1/2, showing a reduced signal in these regions (Jessell, 2000). In situ hybridizations were performed on the same sections. Regions of interest (ROIs) were selected based on weaker Islet-1/2 signal at the most lateral part of the ventral spinal cord. In situ signal intensity was measured for each ROI and the ratios of operated/control sides were calculated by dividing average gray value of operated side by the counterpart of control side within the same section.

4.5. Primary rat embryonic ventral spinal cord cultures

Primary rat embryonic ventral spinal cord cultures were prepared as described previously (Loeb and Fischbach, 1997). Briefly, ventral spinal cord vessels were dissected from embryos from timed pregnant Sprague Dawley rats (Harlan) using the ventral two-third of the spinal cords of embryonic day 15 (E15) rat embryos. Cells were plated on laminin and poly-D-lysine-coated 24-well plates at density of 200,000 cells/cm² in Leibovitz’s L-15 Glutamax Medium supplemented with N-2 supplement, MEM vitamin solution, penicillin/streptomycin (Life Technologies), 6 mM NaHCO3, 6 μg/ml chick E11 pectoral muscle extract, and 54 μg/ml imidazole. Cultures were maintained at 37 °C with 5% CO2. At day in vitro 3 (DIV3), cultures were treated with BDNF, GDNF, or NT-3 for indicated time periods. In some experiments, inhibitors were added 30 min before the neurotrophic factors. At the end of treatments, cells were washed with cold PBS and subjected to total RNA or protein extraction.

4.6. RNA isolation and qPCR

5- to 6-somite segments of lumbar spinal cords were harvested from unilateral limb ablated chick embryos at E6 and then separated for RNA extraction. Total RNA from both tissue and cell cultures were isolated using RNeasy mini kit (Qiagen). Equal amounts of total RNAs were reverse transcribed using oligo(dT) by Superscript First-Strand Synthesis System (Life Technologies). Chick Ig-NRG1 and CRD-NRG1 transcripts were detected as previously described (Ma et al., 2011). Rat type I and type II NRG1 were measured by Rn00580917_m1 and Rn01482172_m1, respectively; type III NRG1 were detected by: forward primer, 5'-TCTAACTTTCCACATCGACATC; reverse primer, 5'-TCTCTATAGATGGCAGG; and taqman probe, 6FAM-ACGACTGGGACCGAC. Rat GAPDH was detected by Rn99999916_s1 for normalization (Life Technologies). Message levels of NRG1 isoforms were normalized to GAPDH. qPCR data were collected from at least three biological replicates, and ΔΔCt was used for calculations.

4.7. Protein isolation and immunoblotting

Total protein from rat embryonic ventral spinal cord cultures was extracted using RIPA lysis and extraction buffer containing 25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 0.1% SDS, and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Equal amounts of protein samples were loaded for immunoblotting using antibodies from Cell Signaling Technology at the following dilutions: Phosho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000, #4695); Phospho-p38 MAPK (1:1000, #8690); p38 MAPK (1:1000, #8690); Phospho-Akt (Ser473) (1:1000, #4370), p44/42 MAPK (Erk1/2) (1:1000, #4895); Phospho-p38 MAPK (Thr180/Tyr182) (1:1000, #4511), p38 MAPK (1:1000, #8690); Akt (pan) (1:1000, #4685). SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for signal detection. Blots were first probed with antibodies against phospho-proteins and then stripped for reprobing.

4.8. Dual luciferase reporter assay

5'- flanking regions of type I and type III NRG1s were amplified by PCR from rat genomic DNA using the forward primer, 5’-TGCTTAGGAGAGCTTAGCTGAGT, and the reverse primer, 5’-TCAGCAGTTTCTCACCAGAAGAGTA for type I; forward primer, 5’-TAAGATGCTGTGAGTGAAC, and reverse primer, 5’-ATGTCGCGGGAAAAACTTCTC for type III. They were subcloned into pGL3B vector, upstream to the firefly luciferase gene, and verified by sequencing. Dissociated cells from rat embryonic ventral spinal cord were cotransfected by electroporation (Neon Transfection System, Life Technologies) with type I promoter–Luc or type III promoter–Luc together with thymidine kinase promoter–Renilla luciferase reporter plasmid (pRL-TK) at ratio of 50:1. 12 h after transfection, cells were treated with BDNF as indicated for 24 h before lysis. Luciferase activities were assayed using Dual-Luciferase Reporter Assay System (Promega) by a Fluoroskan Ascent microplate luminometer (Thermo Scientific). Firefly luciferase activity was normalized by Renilla luciferase activity to eliminate sample variation.

4.9. Statistical analysis

A paired two-tailed Student t-test was used to calculate differences between two groups. Statistical differences for multiple group comparisons were calculated using a one-way ANOVA followed by Dunnett post hoc test. For comparisons of multiple groups with different treatments, a two-way ANOVA followed by Bonferroni post hoc test by comparison to control was used. Normalized values were averaged and reported as the mean ± SEM of at least three independent experiments. Statistical
significance is presented as *p < 0.05, **p < 0.01, ***p < 0.001, ns not significant.

Acknowledgments

This work was supported by NIH Grant R01 NS059947 and the Miller Amyotrophic Lateral Sclerosis Center.

References


