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

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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.

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 (Hippenmeyer 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).

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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 III *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_L) that normally innervate the dorsal limb bud (Fig. 2C, D). The weakly positive Islet-1/2 marker is used to label the LMC_L, 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_L 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).

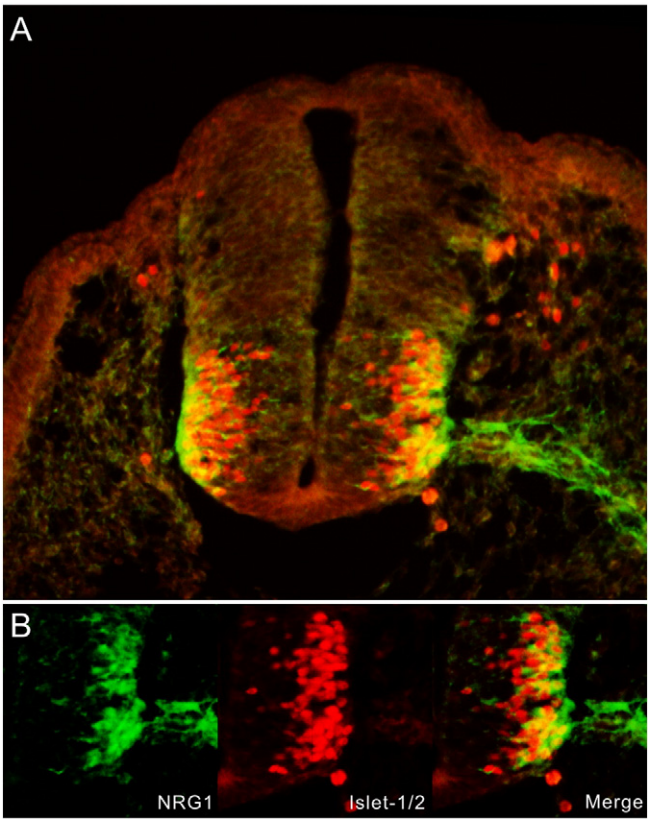

Fig. 1. NRG1 expression is maximal in motor neurons that have extended their axons. (A) Stage 18 embryonic chicken spinal cord was double-labeled with antibodies against NRG1 (green) and the motor neuron marker Islet-1/2 (red). Motor neurons that had extended their axons in the lateral spinal cord had the highest level of NRG1 protein expression. Higher power views are shown in (B).

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_L 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