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Sp4-dependent repression of neurotrophin-3 limits dendritic branching

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ABSTRACT

Regulation of neuronal gene expression is critical to establish functional connections in the mammalian nervous system. The transcription factor Sp4 regulates dendritic patterning during cerebellar granule neuron development by limiting branching and promoting activity-dependent pruning. Here, we investigate neurotrophin-3 (NT3) as a target gene important for Sp4-dependent dendritic morphogenesis. We found that Sp4 overexpression reduced NT3 promoter activity whereas knockdown of Sp4 increased NT3 promoter activity and mRNA. Moreover, Sp4 bound to the NT3 promoter in vivo, supporting a direct role for Sp4 as a repressor of NT3 expression. Addition of exogenous NT3 promoted dendritic branching in cerebellar granule neurons. Furthermore, sequestering NT3 blocked the continued addition of dendritic branches observed upon Sp4 knockdown, but had no effect on dendrite pruning. These findings demonstrate that, during cerebellar granule neuron development, Sp4-dependent repression of neurotrophin-3 is required to limit dendritic branching and thereby promote acquisition of the mature dendritic pattern.

Introduction

The pattern of dendrites elaborated by a neuron determines integration of inputs. Defects in dendritic patterning are characteristic of many neurodevelopmental and mental retardation disorders (Dierssen and Ramakers, 2006; Galvez et al., 2005; Lee et al., 2003; Purpura, 1975). Dendritic development is a highly dynamic process that includes stages of addition, growth, branching, and stabilization or elimination of dendrites. Dendritic arborization patterns are regulated during development by the coordinated action of extracellular signals and gene expression programs (McAllister, 2000; Redmond and Ghosh, 2005). In addition to well-described roles in neuronal survival and differentiation, secreted polypeptide growth factors known as neurotrophins regulate growth and patterning of axons and dendrites in the mammalian nervous system (Friedman and Greene, 1999; Kaplan and Miller, 2000; Lewin and Barde, 1996; McAllister et al., 1999; Reichardt, 2006). The functions and regulated expression of neurotrophins and their receptors during dendritic development remains incompletely understood.

Neurotrophin 3 (NT3) has unique and sometimes overlapping or even opposing functions compared to the other neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 4/5 (NT4/5). Among the functions described for NT3, NT3 regulates survival and differentiation of neurons in the cerebellum (Bates et al., 1999; Katoh-Semba et al., 1996; Neveu and Arenas, 1996). NT3 expression in the cerebellum is highest in the embryo and NT3 expression markedly declines with postnatal cerebellar maturation (Ernfors et al., 1990; Katoh-Semba et al., 1996; Maisonpierre et al., 1990a; Neveu and Arenas, 1996; Rocamora et al., 1993). The high affinity NT3 receptor, TrkC, is expressed in the cerebellum from the third week after birth (Neveu and Arenas, 1996; Segal et al., 1995). Thus, there is a switch in NT3 levels from high to low and in TrkC levels from low to high during postnatal development of the cerebellum. The transcriptional programs that control the balance between NT3 and TrKC gene expression during cerebellar maturation have not been fully described. The effects of NT3 on growth and branching of dendrites depend on neuronal cell type and brain region. For example, NT3 has been shown to either promote or limit dendritic growth and branching in distinct layers of pyramidal neurons (Baker et al., 1998; McAllister et al., 1995, 1997). In early cerebellar granule neurons, addition of exogenous NT3 was shown to alter neurite morphology (Segal et al., 1995), however, little is known about the effects of NT3 on dendritic morphogenesis in these neurons.

We have identified an essential role for the transcription factor Sp4 during dendritic development in cerebellar granule neurons (Ramos et al., 2007). Knockdown of Sp4 in organotypic slices and dissociated cultures led to an increased number of highly branched dendrites. Total dendrite number is the summation of dendrites added less those that are removed. Time course analysis revealed that upon Sp4 knockdown, removal of dendrites was blocked and new dendritic

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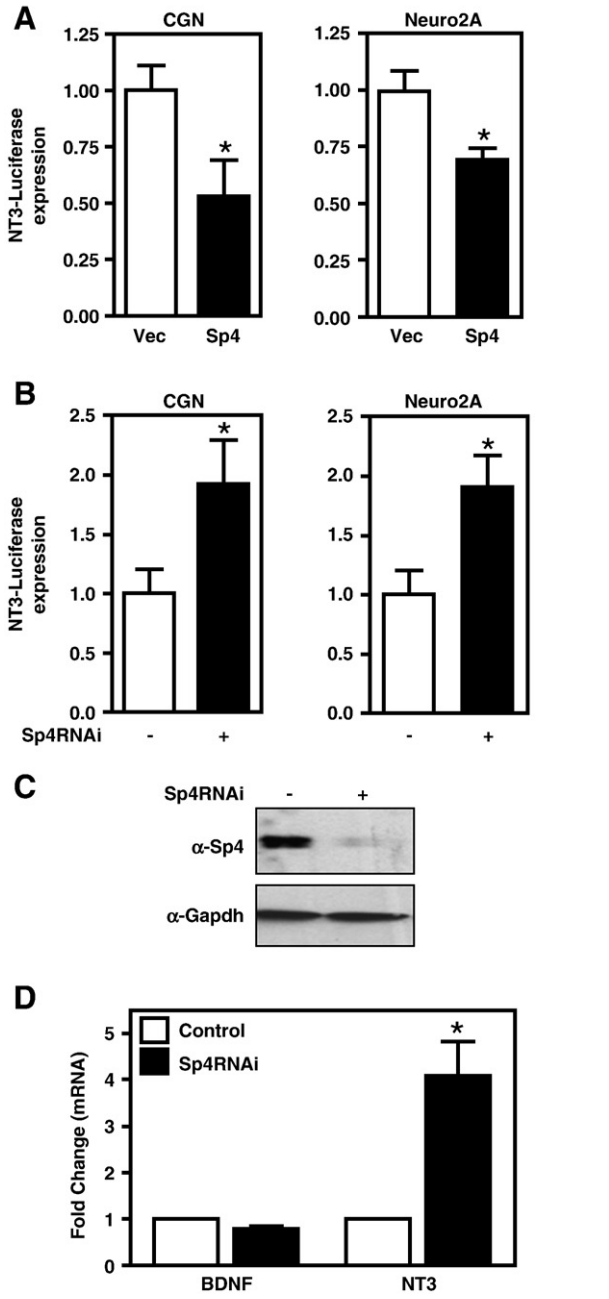

Fig. 1. Sp4 transcription factor represses neurotrophin-3 expression. Cerebellar granule neurons (CGN) or Neuro2A cells were co-transfected with NT3 (−3275/+91)-luciferase reporter and either (A) FlagSp4 (shaded bar) or empty vector (Vec) (open bar), or (B) Sp4RNAi (shaded bar) or control RNAi (open bar). Data represent the resulting luciferase activity normalized to each control condition. (Two-tailed *t*-test, $p<0.05$ (*)) (C and D) Neuro 2A cells were transfected with pMSCVpuro U6/GFP RNAi or U6/Sp4 RNAi plasmid. (C) After selection, cell lysates were immunoblotted for Sp4 and Gapdh. (D) Total RNA was subject to RT-qPCR with primers specific for NT3, BDNF and Gapdh. Values represents mean ± SEM generated from experiments performed in triplicate and normalized to Gapdh expression levels. Student's *t*-test was used to determine the significance between groups. Asterisk denotes statistically significant value relative to control RNAi (*$p<0.05$).

branches continued to be added at later times. Furthermore, overexpression of Sp4 was sufficient to promote dendritic pruning in non-depolarizing conditions. These studies revealed that Sp4 is required to limit addition of new branches and promote activity-dependent pruning of dendrites. Like the related Sp1 and Sp3 proteins, Sp4 binds to specific promoter elements to regulate transcription (Black et al., 2001; Lerner et al., 2002; Philipsen and Suske, 1999; Ross et al., 2002), but the number and nature of Sp4 target genes that mediate dendritic patterning have not been described. Analysis of mutant mice expressing low levels of Sp4 revealed an age-dependent decrease of NT3 mRNA in the hippocampus (Zhou et al., 2005). When Sp4 was knocked out specifically in neural crest, NT3 levels were not affected, although a reduction in levels of TrkC mRNA was observed (St Amand et al., 2006). These studies suggest context-dependent regulation of NT3 and TrkC by Sp4.

In this study, we have investigated the hypothesis that the transcription factor Sp4 regulates NT3 expression to control dendritic development during postnatal maturation of cerebellar granule neurons. We show here that Sp4-dependent down-regulation of NT3 expression is required to suppress continued addition of dendritic branches during cerebellar granule neuron maturation. Inhibition of NT3 signaling was not observed to alter dendrite pruning, suggesting that Sp4 regulates distinct target genes to promote dendritic pruning and limit branch addition. Furthermore, we show that Sp4 activated expression of TrkC, suggesting that opposing regulation by Sp4 may contribute to the dramatic change in NT3/TrKC ratios that occurs during cerebellar maturation. Our findings indicate that Sp4-dependent transcriptional regulation of neurotrophin-3 contributes to proper dendritic morphogenesis during cerebellar development.

Results

Sp4 represses NT3 expression in developing cerebellar granule neurons

Many stages of postnatal cerebellar granule neuron development, including dendritic growth, branching and pruning, are faithfully recapitulated *in vitro* (Gaudilliere et al., 2004; Powell et al., 1997; Ramos et al., 2007). Sp4 protein levels are high in the postnatal

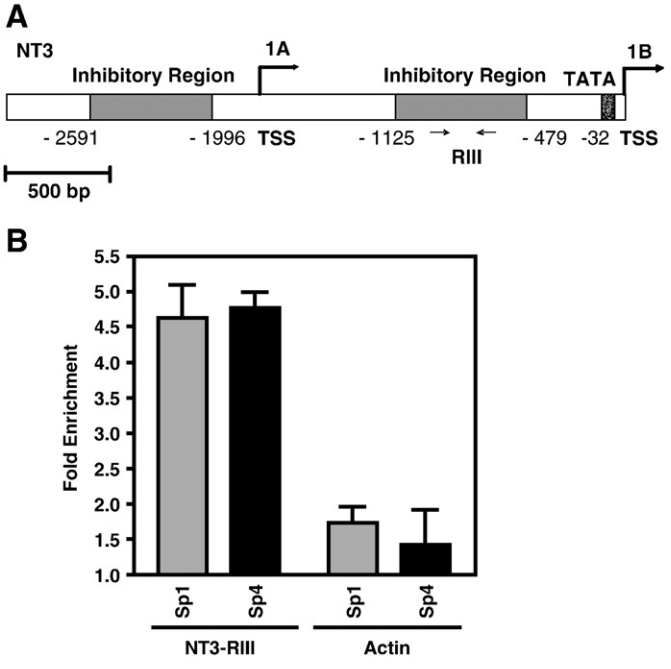

Fig. 2. Sp4 binds to the neurotrophin-3 promoter in cerebellar granule neurons. (A) Schematic of NT3 promoter in mouse. Positions of two transcription start sites (1A) and (1B), as well as the TATA box and two inhibitory regions (shaded grey) enriched in Sp4 binding sites are indicated. Arrows represent the locations of the primers used to amplify region RIII for chromatin immunoprecipitation (ChIP) assays. (B) Sp transcription factors bound to the inhibitory region of the endogenous NT3 promoter. ChIP assays were performed in cerebellar granular neurons, using antisera specific for Sp1 and Sp4 transcription factors, or Luciferase as control IgG. Immunoprecipitated DNA was analyzed by real-time PCR with oligonucleotides that amplified a fragment within the inhibitory region RIII of the NT3 promoter or a control fragment within the coding region of the beta-actin gene. Percent input normalized to control IgG is shown.